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RESEARCH PAPER

Abiotic stress and self-destruction: *ZmATG8* and *ZmATG12* gene transcription and osmotic stress responses in maize

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Abstract Water deficit is one of the most important stresses affecting the maize crop. Whilst the development of osmotic stress tolerant maize (*Zea mays* L.) genotypes is an effective approach for reducing yield losses, understanding of the basic mechanisms of response and tolerance is limited. Under normal conditions, autophagy works at baseline levels to maintain cellular homeostasis. However, under abiotic stress conditions, this process intensifies to remove damaged or unwanted cytoplasmic materials or to recycle materials to provide anabolic substrates and metabolites to cells. This process is mediated by *ATG* genes (AuTophaGy-related genes). Autophagosome expansion and maturation is mediated by ATG8 and ATG12 ubiquitin-like conjugation systems. In this sense, the aim of this study was to characterize the regulation and transcriptional profile of the *ZmATG8* gene and isoforms, together with the *ZmATG12* gene, in maize landrace seedlings under osmotic stress conditions. A difference in transcript profile was observed between two studied landraces, with higher transcript accumulation in landrace Argentino Amarelo, which was more affected by osmotic stress. Under the stress conditions, all *ZmATG* genes studied showed an increase in transcript accumulation in shoot tissues in this landrace. In contrast, for landrace Taquarão a reduction in gene expression was detected, with the exception of *ZmATG8b*. For root tissues under stress, landrace Argentino Amarelo showed an increase in transcript accumulation for the *ZmATG* genes, with the exception of *ZmATG8b*, whilst for landrace Taquarão an increase in expression of *ZmATG8e* and *ZmATG12* was observed, with a reduced expression of *ZmATG8c*. The *ZmATG* genes presented *cis*-regulatory elements involved in osmotic stress response via abscisic acid (ABA)-dependent and ABA-independent signaling. As such, it is suggested that the known transcription factors involved in the osmotic stress signaling response may act in the regulation of *ZmATG* genes. This work provides evidence of autophagy transcriptional signaling in response to osmotic stress in maize.

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Introduction

The degradation and recycling of cellular components in plant cell vacuoles through autophagy (self-feeding) enables the maintenance of cellular homeostasis, development and stress tolerance. In this process, the products belonging from degradation of proteins and organelles are sent to the cytoplasm for reuse (Üstün, Hafrén, & Hofius, 2017; Bassham, 2018).

In plants, three types of autophagy have been described (Marshall & Vierstra, 2018). In microautophagy, the cytoplasmic content is trapped by invagination of the tonoplast (vacuole surface), while in macroautophagy, the cytoplasmic content is trapped in autophagosomes and then carried to the vacuole. Mega-autophagy is the most extreme form, where a rupture or permeabilization of the tonoplast and the release of hydrolases occurs, leading to cytoplasm degradation. This characterizes the last stage of programmed cell death. Macroautophagy (which will be called autophagy hereafter) has been shown to play a critical role in adapting plants to drastic environmental stresses, such as carbon and nitrogen deficiency, heat, drought, salinity, oxidative and osmotic stress, and also in adaptation to biotic stresses (Han, Yu, Wang, & Liu, 2011; Signorelli, Tarkowski, den Ende, & Bassham, 2019).

Autophagy is initiated by the formation of a double membrane from the endoplasmic reticulum and other membranes, generating a phagophore. The development of phagophores requires the coordinated action of proteins encoded by AuTophaGy-related genes (*ATG*). Increased accumulation of *ATG* gene transcripts in response to adverse environmental conditions has been demonstrated in different species such as tomato, pepper, rice and wheat, demonstrating that autophagy is transcriptionally regulated by abiotic stresses (Signorelli, Tarkowski, den Ende, & Bassham, 2019). However, the mechanism responsible for transcription induction is not yet known (Avin-Wittenberg, 2019).

Silencing of *AtATG18a* resulted in higher sensitivity of *Arabidopsis thaliana* plants when subjected to salinity, drought and other osmotic stress conditions (Liu, Xiong, & Bassham, 2009). On the other hand, overexpression of apple (*Malus domestica*) *MdATG3* in *A. thaliana* increased tolerance to salinity and osmotic stress (Wang, Sun, Jia, & Ma, 2017). Similarly, overexpression of apple *MdATG18a* in tomato and apple plants increased drought stress tolerance when compared to wild type plants (Sun et al., 2018). These findings highlight the role of autophagy in the tolerance mechanism of plants. Some authors suggest that stress tolerance is related to autophagy protecting cells from programmed cell death (Williams et al., 2015).

Maize (*Zea mays* L.) is the third most consumed cereal in the world and ensuring yields under osmotic stress is of great importance for global food security, especially in the context of climate change and increased frequency of drought episodes (Su et al., 2019). As the development of osmotic stress tolerant genotypes is an effective way to reduce yield losses due to drought, the identification of mechanisms and genes associated with osmotic stress tolerance is fundamental to assist the plant breeding process. The genetic variability required for plant breeding can be obtained either by assessing the genetic pool available in natural populations such as landraces or through artificial mutation using different tools.

Given the critical role of autophagy in development and stress responses, a better understanding of the initial activation of this process in cultivated plants is beneficial to numerous agricultural applications (Tang & Bassham, 2018). In this sense, this work aimed to study the transcriptional profile of both the *ZmATG8* gene and isoforms and the *ZmATG12* gene, in landrace maize seedlings under control and osmotic stress conditions.

Material and methods

Plant material

Maize landraces displaying different responses to osmotic stress were identified in a previous screening of a group of Southern Brazilian maize landraces (data not shown). Osmotic stress was simulated by applying a solution containing Polyethylene glycol 6000 (PEG 6000). The osmotic potential was previously determined from a screening assay using −0.2, −0.4, −0.6, −0.8 and −1.0 Mpa. Based on this assay (Fig. 1A,B), we identified the −0.2 MPa dose to negatively affect seed germination and seedling establishment, whilst maintaining shoot and root tissue development. Maize landraces Taquarão (T) and Argentino Amarelo (S) were selected based on contrasting responses to osmotic stress.

Seeds were germinated under osmotic potential of 0 MPa (control) and −0.2 MPa (osmotic stress). For germination, three sheets of Germitest-type paper soaked with water (0 MPa) and PEG 6000 solution (−0.2 MPa) were used, moisted with an equivalent of 2.5 times the paper weight (v/w). The experiment was conducted in a completely randomized design with three biological replicates, with 50 seeds per replicate. Seeds were maintained in a germination chamber at 25 °C for 7 days. Seedlings subjected to the 0 MPa condition received water after 4 days incubation. After 7 days, 20 seedlings were morphologically characterized per treatment, with the remaining seedlings frozen in liquid nitrogen for RNA extraction.

Morphological traits

The morphological traits of shoot and root length, fresh and dry shoot and root mass were measured in seedlings under control (0 MPa) and osmotic stress (−0.2 MPa) conditions. Dry and fresh mass was determined after shoot and root lengths were estimated. For fresh and dry mass, shoots were separated from the roots and both were packed in paper bags and dried in a forced air oven at 80 °C for 72 h. Samples were weighed on an analytical balance accurate to 0.001 g. Considering that the studied landraces present intrinsic developmental characteristics, the relative performance (RP) was calculated to evaluate the effect of the osmotic stress on the morphological traits. For the calculation we used the equation: RP variable = ($\bar{x}$ variable at −0.2 MPa / $\bar{x}$ variable at 0 MPa) * 100.

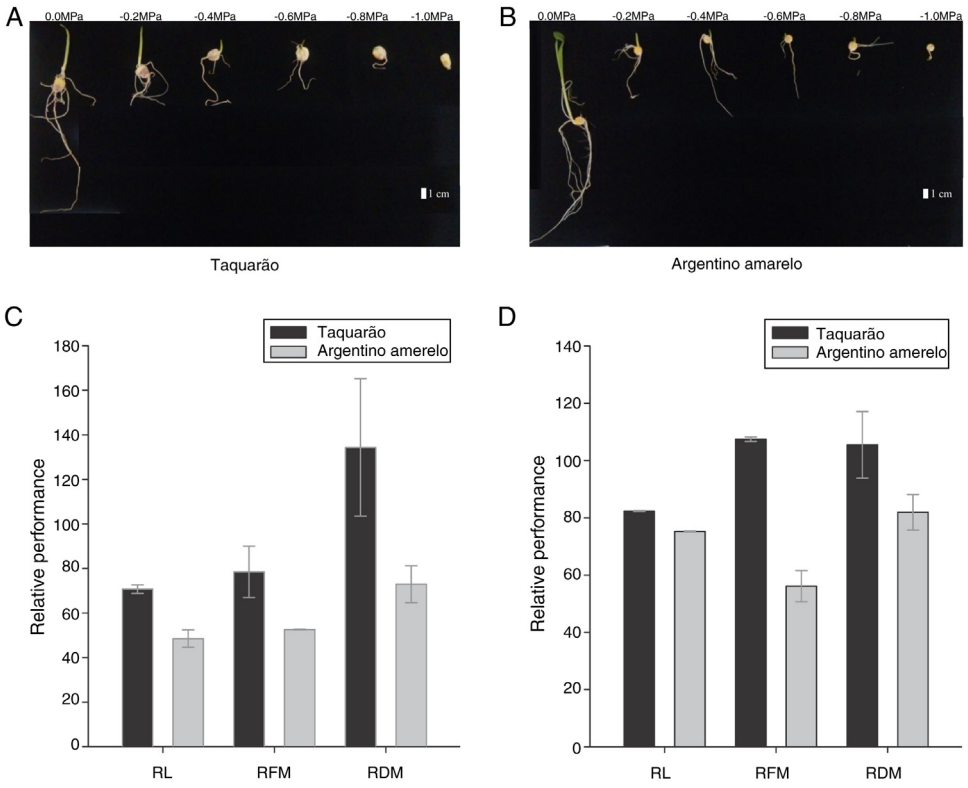

Figure 1 Polyethylene glycol 6000 dose screening and phenotypic characterization through relative performance of maize landraces Taquarão and Argentino Amarelo under osmotic stress conditions. A: Taquarão. B: Argentino Amarelo. C: Shoot morphological traits (shoot length - SL; shoot fresh mass - SFM; shoot dry mass - SDM). D: Root morphological traits (root length - RL; root fresh mass - RFM and root dry mass - RDM). Bars represent the standard deviation (n = 3, containing 20 seedlings each).

Real time qRT-PCR analyses

Total RNA was extracted from 2 g of shoot and root tissue using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) following the protocol described by the manufacturer. RNA quantity and purity were verified by spectrophotometry and integrity by agarose gel electrophoresis. Samples were treated with DNase I (Invitrogen), and PCR reactions using RNA not converted into cDNA were also performed to confirm the absence of genomic DNA. Each sample (1 μg) was converted into cDNA using oligo (dT) with the commercial SuperScript® III first-strand system kit (Invitrogen).

The qRT-PCR experiment was performed according to the MIQE guidelines (Bustin et al., 2009) using oligonucleotides for the *ZmATG8a*, *ZmATG8b*, *ZmATG8c*, *ZmATG8d*, *ZmATG8e* and *ZmATG12* genes (Li et al., 2015). Three reference genes, namely *ZmUBC* (Li et al., 2015), *ZmβACT* (Zhang, Lei, Lai, Zhao, & Song, 2018) and *ZmGAPDH* (Zhang et al., 2017) were employed (Supplementary file 1).

Validation experiments were performed using four cDNA dilutions to determine the amplification efficiency and specificity of each oligonucleotide. Oligonucleotides that were 90–110% efficient and with only one peak in the dissociation curve were used. Gene expression assays were conducted on an Applied Biosystems 7500 fast real-time PCR system thermal cycler using SYBR™ Green PCR Master Mix (Applied Biosystems). Three independent biological replicates from each sample and three technical replicates from each biological replicate were employed. Quantification was conducted according to the ΔΔCt method (Livak & Schmittgen, 2001). Expression data were subjected to stability analysis using DataAssist ™ v3.0 Software (Applied biosystems). After analysis, *ZmβACT* and *ZmGAPDH* showed score values below 1.0, and were used to normalize the expression data of the *ZmATG* target genes. The control condition of each landrace and in each tissue was used as the baseline for determining relative RNA levels. Expression results were presented in heat maps using the Multi Experiment Viewer (TIGR MeV) software (Saeed et al., 2003).

Transcriptional regulation

In order to understand the similarities and differences between the *ZmATG8* isoforms, sequence conservation analysis was performed. Conservation analysis of predicted promoter sequences and gene sequence of *ZmATG8* isoforms was performed using Motif-based sequence analysis tools (MEME) version 5.0.5 software (Bailey & Elkan, 1994). Also, the potential transcriptional regulation of the *ZmATG8* gene and isoforms, as well as the *ZmATG12* gene was assessed by a *cis*-regulatory element analysis (CREs). The sequences of the putative *ZmATG* promoters (1 Kb upstream of the transcription start site) were obtained from the Ensembl Plants - *Zea mays* platform (http://plants.ensembl.org/Zea_mays/Info/Index) in the B73 reference genome. The sequences were

subjected to CREs analysis on the New PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace) platform (Higo, Ugawa, Iwamoto, & Korenaga, 1999). To identify unique and common CREs between the genes studied, a Venn diagram was generated using the Orange v. 3.18 (Demsar et al., 2013).

Results and discussion

Phenotypic profile of landraces under osmotic stress

Different maize landraces represent part of the gene pool available for exploitation by plant breeders in the development of new genotypes. The Brazilian landraces Taquarão and Argentino Amarelo displayed differential responses to osmotic stress (Fig. 1). In Taquarão, osmotic stress had a minor effect on shoot and root length and on fresh and dry shoot and root mass (Fig. 1A, C, D). On the other hand, in Argentino Amarelo, osmotic stress had a stronger effect on shoot and root traits (Fig. 1B–D). Impairment of seedling growth and development was expected, since it is known that osmotic stress causes several effects on the plant. In particular, osmotic stress affects the roots, causing inhibition of elongation due to decreased cell wall extension and reduced turgor. On the other hand, root elongation can be preserved by protecting the apical elongation zone through abscisic acid (ABA) signaling. In shoots, ABA accumulation leads to stomatal closure and consequent reduced leaf photosynthesis leaf, causing reduction in turgor and assimilate consumption, respectively, resulting in reduction of leaf expansion (reviewed in Yang, Rao, & Horst, 2013).

The morphological profile obtained in this study suggests that at the seedling stage Taquarão was less affected than Argentino Amarelo under the stress, producing more roots to compensate for the osmotic stress, as can be seen for RFM and RDM (Fig. 1B–D). Given this observed contrasting response, the two landraces were used as a model to investigate the transcriptional profile of genes associated with autophagy and provide insights into the potential involvement of this degradation and recycling process in the osmotic stress response.

Expression profiles of *ATG* genes in maize landraces under osmotic stress

Under normal conditions, autophagy works at baseline levels to maintain cellular homeostasis. However, under abiotic stress conditions this process intensifies to remove damaged or unwanted cytoplasmic materials, or to recycle cytoplasmic materials to provide anabolic substrates and metabolites to cells (Wang, Xu, Wang, & Galili, 2017). This process is mediated by *ATG* genes. The proteins encoded by these genes can be divided into four functional groups: (i) the ATG1 kinase complex involved in initiating autophagosome formation; (ii) the ATG9 complex for membrane recruitment; (iii) the phosphatidylinositol 3-kinase (PI3K) complex for vesicle nucleation; and (iv) the ATG8 and ATG12 ubiquitin-like conjugation systems for vesicle expansion and closure (reviewed in Yang, Bu, Huang, & Chen, 2019). In the maize genome, five isoforms exist for the *ATG8* gene (*ZmATG8a*, *ZmATG8b*, *ZmATG8c*, *ZmATG8d* and *ZmATG8e*), in contrast to only one *ATG12* gene (*ZmATG12*) (Chung, Suttangkakul, & Vierstra, 2009). Here, we characterized the transcriptional profile of the genes associated with these ubiquitin-like conjugation systems, which act in the final step of autophagosome development, in two maize landraces under osmotic stress.

It is known that the plant shoot and root may show different responses to stresses. In this sense, the transcriptional profile of both the *ZmATG8* gene and isoforms and the *ZmATG12* gene was analyzed in both tissues. Under osmotic stress, an increase in transcript accumulation was observed for the *ZmATG8* gene (*ZmATG8a*, *ZmATG8b*, *ZmATG8c*, *ZmATG8d* and *ZmATG8e*) in shoot tissues for the landrace Argentino Amarelo, which was more affected by this condition. On the other hand, a reduction in transcript accumulation was observed in the less affected landrace Taquarão (Fig. 2A). Such transcriptional profiling indicates that in maize seedlings under osmotic stress genes that encode proteins associated with autophagy are activated.

In Argentino Amarelo roots under osmotic stress, a similar an increase in the expression of most of the *ZmATG* genes studied (except *ZmATG8c*) (Fig. 2B) was observed to that in shoot tissues, again suggesting activation of autophagy signaling. In Taquarão roots subjected to osmotic stress, by contrast, two genes showed increased transcription, namely *ZmATG8e* and *ZmATG12* (Fig. 2B). This expression profile suggests an initial signaling process of autophagy in roots of this landrace.

Since osmotic stress is initially perceived by roots, one would expect major changes in the expression of *ZmATG* genes in this tissue, different to what was observed in the present study (Fig. 2). A reasonable explanation for this could be the influence of ABA signaling, which undoubtedly performs a central role in the osmotic stress response. It is currently known that during drought, ABA produced in leaves can have a major role in signaling of the stress when compared to the ABA produced in the roots (McAdam, Manzi, Ross, Brodribb, & Gomez-Cadenas, 2016).

The involvement of autophagy in response to osmotic stress was first elucidated in *A. thaliana* by detecting increased *AtATG18a* gene expression and induction of autophagosome formation in response to this condition (Liu, Xiong, & Bassham, 2009; reviewed in Tang & Bassham, 2018). More recently, several studies have shown induction of *ATG* genes in response to osmotic stress in cultivated plants such as apple, foxtail millet, barley, pepper, rice, tomato and wheat (reviewed in Tang & Bassham, 2018). The fundamental role of autophagy for plant survival under osmotic stress has been demonstrated from studies on Arabidopsis *atg5*, *atg7* and *RNAi-AtATG18a* mutants, which fail to activate autophagy and are hypersensitive in this condition (Liu et al., 2009; Zhou et al., 2013). Similar responses have also been observed in autophagy-defective wheat and tomato plants (Kuzuoglu-Ozturk et al., 2012; Zhu et al., 2018).

Our results provide evidence for the activation of genes associated with autophagy in sensitive maize seedlings under osmotic stress. The question raised is: what is the role of autophagy in this stress condition? Is it a cause or a consequence of the sensitivity expressed by the genotype? Cellular damage caused by osmotic stress results from reactive oxy-

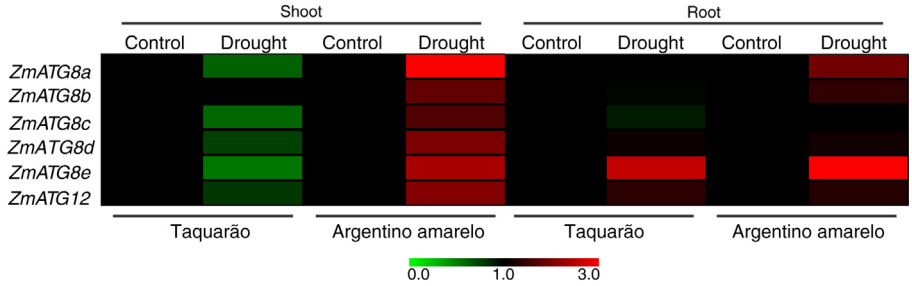

Figure 2 Relative quantification of *ZmATG8* and their isoforms and *ZmATG12* gene expression in shoot (A) and root (B) tissues of Taquarão and Argentino Amarelo landraces subjected to osmotic stress (−0.2 MPa) for 7 days. Control samples were used as standard calibrators.

gen species (ROS), which are produced in greater quantities under such conditions. At low levels, ROS act as cellular signaling components, but when at high levels they become extremely deleterious, damaging the membrane and other cellular components, resulting in oxidative stress and eventually cell death (Cruz de Carvalho, 2008). Plant root tissues sense osmotic stress and send a signal to the shoot, leading to stomatal closure. This mechanism, whilst promoting a reduction of water loss, also limits the entry of CO_2, directly and indirectly influencing the reduction of liquid photosynthesis and increasing ROS production. CO_2 limitation causes a reduction in $NAPD^+$ regeneration through the Calvin Cycle, leading to a reduction in the photosynthetic electron transport chain, and to greater electron leakage to O_2 by the Mehler reaction. Also, under osmotic stress, the production of hydroxyl radicals occurs in the thylakoid through iron-catalyzed reduction of hydrogen peroxide by both superoxide dismutase and ascorbate (Cruz de Carvalho, 2008). Oxidative damage can be prevented by the action of the cell antioxidant system (enzymes and other antioxidant molecules), but if this mechanism is not efficient and sufficient, the cellular damage caused by oxidative stress will be inevitable and autophagy will be required to prevent cell death.

Transcriptional regulation of *ZmATG8* and *ZmATG12* genes

In order to understand the transcriptional regulation of *ZmATG8* isoforms, an analysis of the conservation of the gene sequence and the predicted promoter sequence was performed. Based on *in silico* analysis, the gene sequence was conserved between the isoforms, which explains the maintenance of the same function between copies (Fig. 3A). This conservation was expected, given the mechanisms of control that prevent the occurrence of mutations in genic regions. On the other hand, the predicted promoter region presented differences related to motifs (Fig. 3B), which is interesting, given that isoforms can be regulated in response to different conditions. The predicted promoters of the *ZmATG8b* and *ZmATG8c* isoforms appear to have higher motif conservation, suggesting a similar regulation.

From the observation that the predicted promoters seem to present structural differences, an analysis of occurrence of *cis*-regulatory elements (CREs) was performed. CREs are involved in the control of development and physiology through the regulation of gene expression, and their divergence is a common cause of evolutionary changes (Wittkopp & Kalay, 2012). In this study, a total of 137 different CREs were identified. Among them, while some CREs are common in promoters of all genes analyzed, other CREs are unique to a particular promoter (Supplementary file 2). The identified CREs are responsive to different environmental conditions, tissues, in response to phytohormones and involved in plant growth and development processes, as well as in response to biotic and abiotic stresses. Most CREs are binding sites for transcription factors (TFs) known to regulate abiotic stress-responsive genes (Fig. 4A). Interestingly, CREs were identified that are binding sites for TFs known to be involved in osmotic stress response signaling (Fig. 4B).

With regard to the regulation of *ZmATG8* gene isoforms, the occurrence of CREs differed, with the lowest number present in the *ZmATG8c* isoform. Although each isoform harbours unique CREs, the majority of CREs are shared (Fig. 4C and Supplementary file 3). While on one hand a TF can activate all isoforms due to the presence of common CREs, isoforms can also show a particular regulation arising from unique CREs in their promoters. This finding may explain the variation in the expression profile of *ZmATG8* isoforms, as observed in the root tissues of the Taquarão landrace under osmotic stress (Fig. 2B).

Considering that the proteins *ZmATG8* and *ZmATG12* act in the same stage of the autophagy process, we investigated evidence for common regulation of these genes In fact, a large number of shared CREs were observed between the promoters of these two genes (Fig. 4D), suggesting regulation under similar conditions. However, it should be noted that among the promoters of the *ZmATG8* isoforms there are a large number of CREs that are not present in the *ZmATG12* promoter (Fig. 4D and Supplementary file 3), suggesting differential regulation.

ABA and *ZmATGs*

The response to osmotic stress occurs through the activation of two categories of genes, regulatory genes that comprise TFs and functional genes that encode proteins involved in tolerance to the stress caused in the cell. The latter can include LEA proteins, molecular chaperones, antioxidant system enzymes and sugar and proline amino acid biosynthesis enzymes (as reviewed in Singh & Laxmi, 2015). In this sense, the *ATG* genes fall into the functional gene cat-

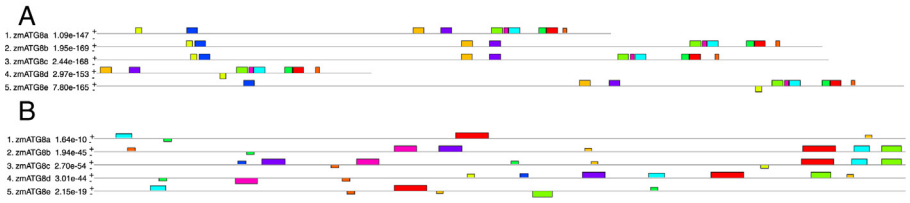

Figure 3 Conservation of motifs in promoter (A) and gene (B) sequences from *ZmATG8* and their isoforms. Boxes with the same color represent the conserved sequences among *ZmATG8* isoforms.

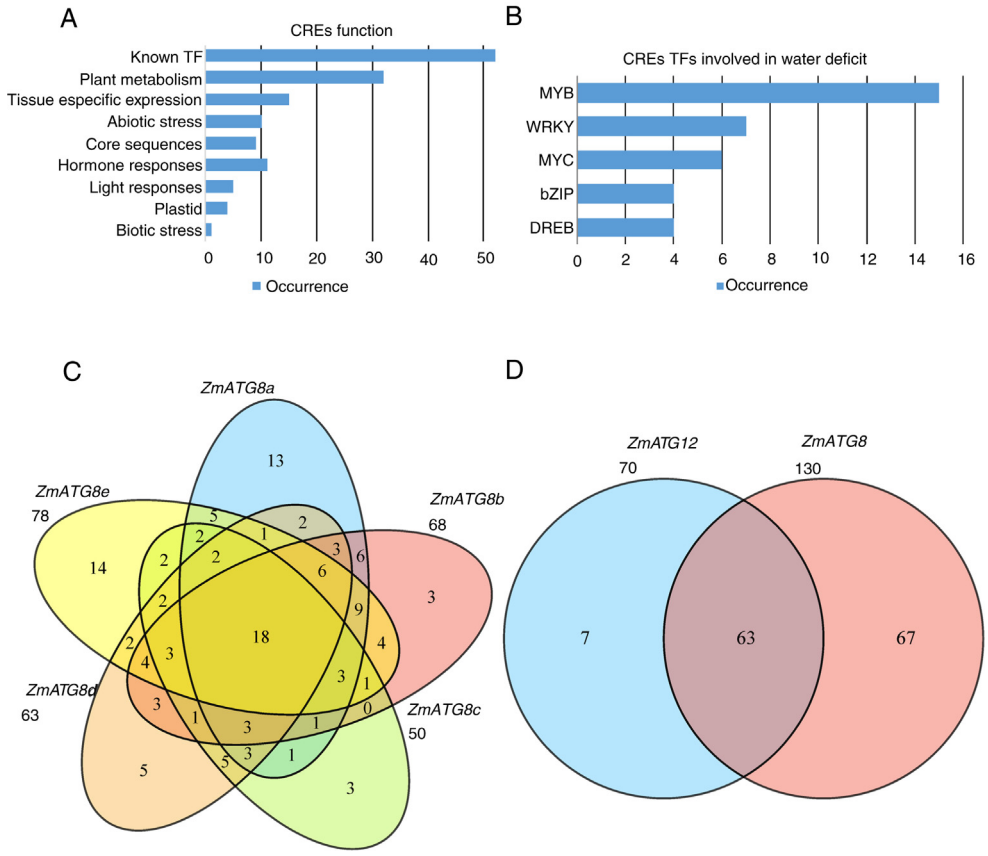

Figure 4 Occurrence of *cis* regulatory elements (CREs). A: Occurrence of CREs in promoters of *ZmATG8* and their isoforms and in the *ZmATG12* gene, according to function; B: Occurrence of CREs in promoters of *ZmATG8* and their isoforms and in the *ZmATG12* gene which are targets of transcription factors involved in water deficit responses. C: Venn diagram showing CREs in common among *ZmATG8* gene isoforms; D: Venn diagram showing the CREs in common between the *ZmATG8* and *ZmATG12* genes.

egory and act when the abovementioned mechanisms are insufficient to prevent cell damage.

Under osmotic stress conditions, the phytohormone ABA plays an important role in plant survival through the induction of physiological and biochemical changes. Many studies have already shown that signaling in response to osmotic stress may be ABA-dependent by the acting of AREB/ABF (Abscisic acid-responsive element binding protein/ABRE binding factor) TFs. These are members of the bZIPs (basic leucine zipper) subfamily that recognize and bind on ABRE (ABA responsive element) CREs present in specific gene promoter regions. In addition, other TFs also act on ABA-dependent signaling, such as MYBs (myeloblastosis) and MYCs (myelocytomatosis) (reviewed in Nakashima, Yamaguchi-Shinozaki, & Shinozaki, 2014; reviewed in Singh & Laxmi, 2015). In addition to these, it has been reported that WRKY TFs also act on ABA-dependent pathways through recognition and binding to the CRE W-box (revised in Singh & Laxmi, 2015). ABA-independent signaling can also occur through the action of the transcription factor DREB/CBF (Dehydration-responsive element binding protein/C-repeat binding factor) that recognizes and binds in CREs DRE/CTR (dehydration-responsive element/C-repeat) (reviewed in Nakashima et al., 2014; reviewed by Singh & Laxmi, 2015). Participation of the NAC TF is also involved, acting dependently and independently of ABA (Singh & Laxmi, 2015). Considering this complex regulation in osmotic stress, we identified CREs present in the promoters of the studied *ATG* genes that act in this signaling cascade, suggesting that *ATG* genes are involved in the response to osmotic stress.

Quantity of a same CRE

0.0 12.0

ABA independent signalling							
		ZmATG8a	*ZmATG8b*	*ZmATG8c*	*ZmATG8d*	*ZmATG8e*	*ZmATG12*
DREB TFs	DRE2COREZMRAB17	0	0	0	1	1	0
	DRECRTCOREAT	0	0	2	1	1	0
	CBFHV	2	0	3	2	2	0
	CRTDREHVCBF2	2	0	0	0	0	0

ABA dependent signalling							
		ZmATG8a	*ZmATG8b*	*ZmATG8c*	*ZmATG8d*	*ZmATG8e*	*ZmATG12*
AREB/ABF TFs	ABRELATERD1	0	0	0	1	2	0
	ABRERATCAL	0	1	1	0	4	0
	CE3OSOSEM	0	0	0	0	1	0
	RYREPEATBNNAPA	1	0	0	2	0	1
MYC TFs	MYBST1	1	4	0	1	1	0
	MYCATERD1	1	1	0	0	0	1
	MYCATRD22	1	1	0	0	0	1
	MYCCONSENSUSAT	10	12	2	8	12	12
MYB TFs	MYB1AT	0	3	0	2	1	2
	MYB1LEPR	0	0	0	0	1	0
	MYB26PS	0	1	0	0	0	0
	MYB2AT	1	0	0	0	0	0
	MYB2CONSENSUSAT	0	0	1	0	2	0
	MYBATRD22	0	0	0	0	1	0
	MYBCORE	6	3	2	1	4	1
	MYBCOREATCYCB1	0	0	0	1	0	1
	MYBGAHV	1	0	1	0	1	1
	MYBPLANT	1	1	0	0	2	0
	MYBPZM	2	1	0	1	2	0
	IBOX	2	0	0	0	1	1
	L1BOXATPDF1	0	0	0	0	0	1
	TATCCAOSAMY	0	1	0	1	1	0
WRKY TFs	WBBOXPCWRKY1	1	0	0	0	0	1
	WBOXATNPR1	2	1	1	0	2	2
	WBOXHVISO1	4	3	2	0	2	1
	WBOXNTCHN48	1	0	2	1	0	0
	WBOXNTERF3	5	3	5	1	5	2
	WRKY71OS	11	6	6	1	9	5
	ELRECOREPCRP1	0	0	1	0	0	0

Figure 5 Occurrence of *cis* regulatory elements (CRE) involved in osmotic stress signaling by the ABA-independent and ABA-dependent pathways in the predicted promoters of *ZmATG8* and their isoforms and in *ZmATG12*.

Taking into account the signaling pathways under osmotic stress, i.e., ABA-dependent and ABA-independent, it was verified that the *ZmATG8b* isoform and the *ZmATG12* gene could not be activated by the ABA-independent pathway, since they do not present DRE/CRT CREs. This result suggests that they are not downstream of DREB regulation (Fig. 5).

Promoters of all genes presented CREs involved in ABA-dependent signaling (Fig. 5), suggesting that ABA plays an important role in regulating *ZmATG8* and *ZmATG12* genes under osmotic stress. However, a range of different types of CREs were identified in each promoter, with, for example, the *ZmATG8e* promoter showing the highest occurrence of ABRE (Fig. 2A).

MYCCONSENSUSAT, MYBCORE and WBOXNTERF3/WRKY71OS CREs, as binding sites of MYC, MYB and WRKY TFs, respectively, occur in all promoters, suggesting an effective participation of these TFs in the activation of the studied genes. Some reports have shown increased accumulation of *WRKY71* gene transcripts under osmotic stress conditions (Fei et al., 2019). Interestingly, this TF specifically binds in CRE WRKY71OS, suggesting that *ZmATG* genes may be activated via WRKY71 during osmotic stress.

Overall, our results provide evidence for involvement of DREB/CBF, AREB/ABF, MYB, MYC and WRKY TFs in osmotic stress signaling responses, acting on *ZmATG8a, ZmATG8c, ZmATG8d and ZmATG8e*, but not on *ZmATG8b* and *ZmATG12* transcriptional regulation. These findings suggest that the *ZmATG*s are functional genes involved in the plant response to this stress.

Conclusions and future prospects

This study provides insights into the transcriptional activation of *ZmATG8* and *ZmATG12* genes during osmotic stress responses in maize seedlings. These genes appear as potential candidates for functional characterization under

osmotic stress conditions. Our results pave the way for further studies on ATG proteins and the monitoring of autophagy in stress conditions. If postulates are validated, these genes could be monitored and included in molecular breeding strategies for the development of osmotic tolerant genotypes.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biori.2019.12.001.

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