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Research article

# Spatial identification of transcripts and biological processes in laser micro-dissected sub-regions of waterlogged corn roots with altered expression of phytoglobin

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ABSTRACT

Over-expression of the corn phytoglobin *ZmPgb1.2* increases tolerance to waterlogging, while suppression of *ZmPgb1.2* compromises plant growth. To unravel compartment-specific transcriptional changes evoked by ZmPgb1.2 during hypoxia, laser micro-dissected sub-regions from waterlogged roots of WT and *ZmPgb1.2* overexpressing [ZmPgb1.2(S)] plants were probed for global transcriptional analysis using next generation RNA sequencing. These sub-regions included compartments within the meristematic, elongation, and maturation zone. Of the 149 genes differentially expressed by the up-regulation of *ZmPgb1.2*, 78 occurred within the meristematic region and included genes involved in jasmonic acid synthesis and response, ascorbic acid metabolism, and ethylene signalling. The *ZmPgb1.2* regulation of these genes, discussed in the context of known functions of Pgbs, was further validated by monitoring their expression in meristematic cells of waterlogged roots suppressing *ZmPgb1.2*. Of the 27 genes differentially expressed by the over-expression of *ZmPgb1.2* in the elongation zone, pyruvate kinase and alcohol dehydrogenase showed an expression pattern correlated to the level of *ZmPgb1.2* in the tissue. The transcriptional induction of these two enzymes in hypoxic domains of the elongation zone over-expressing *ZmPgb1.2* suggests the activation of the fermentation pathway which might be required to sustain metabolic flux and production of ATP in support of cell elongation.

## 1. Introduction

Flooding and excessive moisture are conditions frequently experienced by plants which reduce agricultural yields by limiting growth and development. Exposure to these conditions, which can be continuous or ephemeral, triggers tolerance and adaptive responses reflected by the diverse anatomical, physiological and molecular responses exhibited by flooding-prone plants (Voesenek and Bailey-Serres, 2013). One of the most severe perturbations ascribed to flooding is hypoxia, the reduced availability to atmospheric oxygen, due to its reduced diffusion in solutions. Oxygen-requiring metabolic pathways, some of which are crucial for energy production, are directly affected by hypoxia. Reduction in mitochondrial ATP synthesis in favor of substrate-level phosphorylation occurs under low oxygen conditions and physiological mechanisms regulating photosynthesis, gas exchange, nutrient assimilation and reallocation, and hormonal responses are compromised in plants (Vartapetian and Jackson, 1997). Many of these disturbances often occur above the water level in tissues not directly experiencing oxygen depletion and are attributable to metabolic perturbations in the roots. This concept is best exemplified by the reduction in photosynthetic capacity and transpiration rate occurring in flooded plants which are due to a direct effect on stomata density of ABA originated by hypoxic roots (Bai et al., 2013). Root cells are the first to sense and respond to flooding-induced hypoxia, and prolonged exposure to low oxygen can compromise their function and alter the behaviour of the overall root system.

The iterative generation of all cell types in the roots is regulated by the activity of the root apical meristem (RAM), comprising stem cells

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surrounding mitotically inactive quiescent cells (QC) which act as the "organizing center". With the QC suppressing their differentiation, the stem cells generate derivatives contributing to the formation of apical (columella and lateral root cap) and basal (epidermis, cortex, endodermis and stele) tissues (Dolan et al., 1993). Derivatives of the stem cells undergo a differentiation path progressing through an elongation (E) zone and a maturation (M) zone along the root profile. The precise and highly regulated regenerative nature of the RAM is very susceptible to perturbation in environmental conditions, including hypoxia. Accumulation of ethylene-induced reactive oxygen species (ROS) followed by premature cell differentiation and programmed cell death (PCD) occurred in hypoxic root meristematic cells (Mira et al., 2016b). The hypoxic roots exhibited growth retardation, and in some instances growth cessation, with negative repercussions on the overall plant performance, including reduced photosynthetic rate and leaf damage (Youssef et al., 2016).

Among factors mediating flooding tolerance are phytoglobins (Pgbs), heme-containing proteins identified in all nucleated organisms with the major identified function of scavenging nitric oxide (NO) especially under condition of stress (Hill, 2012). Nitric oxide, generated during hypoxia possibly via nitrate reductase, influences root behaviour and structure, as observed during the formation of aerenchyma (Wany et al., 2017). The close link between Pgb and flooding responses is apparent by changes in *Pgb* expression level induced during hypoxia (Silva-Cardenas et al., 2003) that correlated with flooding tolerance (Campbell et al., 2015). When over-expressed in several systems, including Arabidopsis, corn and alfalfa, *Pgbs* ameliorate flooding tolerance by possibly keeping a high energy status required to sustain growth (Hunt et al., 2002; Dordas et al., 2003; Igamberdiev and Hill, 2004). Pgbs have also been characterized as "survival factors" where they have been shown to influence plant morphogenesis (Huang et al., 2014). This concept was further extended in hypoxic corn roots where the presence of ZmPgbs in the root tip scavenged NO, protected cells from dying, and contributed to the retention of a functional RAM by suppressing ethylene and ROS production, executors of the death program (Mira et al., 2016b). The ZmPgb protective role was more prominent in the center of the root tip, harboring the stem cells, and resulted in enhanced root growth under conditions of hypoxia compromising growth in WT roots. Hypoxic-root growth inhibition was further aggravated by the suppression of *ZmPgbs* triggering over-production of ethylene, ROS and massive PCD (Mira et al., 2016b). Of note, the effects of *ZmPgb* expression on root growth were specific to hypoxia, with no visible deviations in root morphology and plant behaviour under normoxic conditions.

Examination of gene expression in maize root cortical cells during lysigenous aerenchyma formation identified major changes in genes associated with the generation and scavenging of ROS (Rajhi et al., 2011; Yamauchi et al., 2011). There was no indication of major changes in *Pgb* gene expression in these cells. Since the suppression of genes required for the production of ROS is associated with *Pgb* expression (Stasolla and Hill, 2017; Mira et al., 2016b), one would anticipate a lack of involvement of Pgb in ROS-associated programmed cell death in cortical cells during lysogenous aerenchyma formation. Considering the evidence that Pgbs have a positive influence on tolerance to hypoxia and that their expression is cell-specific (Mira et al., 2016b, 2017), comparative transcriptional analyses were conducted using laser microdissection of waterlogged WT plants and plants over-expressing *ZmPgb1.2* to assess how Pgb influenced cell-specific gene expression during hypoxia in root tip sub-regions. The transcript profiles of these sub-regions, including meristematic cells (Me), as well as cells within the elongation (E) and maturation (M) zone, identified potential candidate genes that were further probed in the respective sub-regions of flooded roots in which the level of *ZmPgb1.2* was repressed. Collectively, this study provides an unprecedented high-resolution analysis of molecular responses triggered by hypoxia and influenced by ZmPgbs.

## 2. Materials and methods

### 2.1. Plant material and waterlogging treatment

Characterization of the transgenic plant material, and details of the waterlogging treatment have been described previously (Youssef et al., 2016). Briefly, three-leaf stage WT plants, as well as plants over-expressing or down-regulating *ZmPgb1.2* [ZmPgb1.2 (S) or ZmPgb.1.2(A)] were grown in 12 inch pots containing Metro-Mix 900 (composed of sphagnum peat moss, bark, perlite, and vermiculite) under a 16 h photoperiod of 22 °C light/20 °C dark. Waterlogging was imposed by maintain the water 2 cm above the soil surface for three consecutive days. Pots wits a bottom opening of 1 cm where placed in a large plexiglass container as described in Youssef et al. (2016).

### 2.2. Tissue processing and embedding

Waterlogged roots were gently removed from the soil and 1 cm apex segments were immediately fixed in 33% glacial acetic acid and 66% ethanol overnight at 4 °C. For each biological replicate, about 30 root apexes were collected from 10 different plants of the same genotype. The samples were washed three times in 70% ethanol and dehydrated with a graded (85, 95 and 100%) ethanol series and gradually infiltrated with xylenes and subsequently embedded in Paraplast Plus paraffin (McCormick Scientific, St. Louis, MO, USA) (Rao et al., 1989; Chan et al., 2016). Serial longitudinal sections (7 μm thickness) were mounted on Membrane Slides (Leica Microsystems) and then dewaxed in xylene for 1 min.

### 2.3. Laser microdissection (LMD) of root sub-regions

The different root sub-regions were dissected from longitudinal sections. They included two compartments within the meristematic domain: the proximal meristem (PMe) and distal-lateral meristem (DLMe), as well the elongation zone of the central (EC), and lateral (EL) region, and the maturation zone of the central (MC), and lateral (ML) region (Fig. 1). Dissection was carried out using a Carl Zeiss PALM MicroBeam system (Carl Zeiss, Oberkochen, Germany). The tissue fragment was traced using the Freehand Tool in the PALMRobo software version 4.3. Laser values were set to 10 for speed, 1 for aperture, and 40 for power level, in order to limit the amount of energy utilized and tissue damage. The microdissected tissue (surface area per compartment > 1,000,000 $\mu m^2$) was immediately placed in 0.5 ml microcentrifuge tube caps (Fisher Scientific, Ottawa,ON, Canada) containing 30 μl of lysis buffer [from the Ambion® RNAqueous®-Micro Kit (Life Technologies, Carlsbad, CA, USA)]. Collection was performed within 1 h to minimize RNA degradation and in the event the lysis buffer evaporated during this time, more buffer was added and the volume recorded. Samples were either used immediately for RNA extractions or stored at −80 °C.

### 2.4. RNA extraction and amplification

Two biological replicates were used, each consisting of a minimum of 20 different roots, collected from 10 different corn plants. Extraction of RNA was performed using the Ambion® RNAqueous®-Micro Kit (Life Technologies).

The cDNA libraries were generated from the RNA samples using the Ovation® RNA-Seq System V2 kit (NuGEN, San Carlos, CA, USA) and fragmented using the NEBNext® dsDNA Fragmentase® kit (New England Biolabs, Ipswich, UK). The cDNA libraries were then prepared with the Illumina TruSeq™ RNA Sample Preparation v2 kit (Illumina, San Diego, CA, USA) using the low throughput protocol according to the manufacturer's instructions.

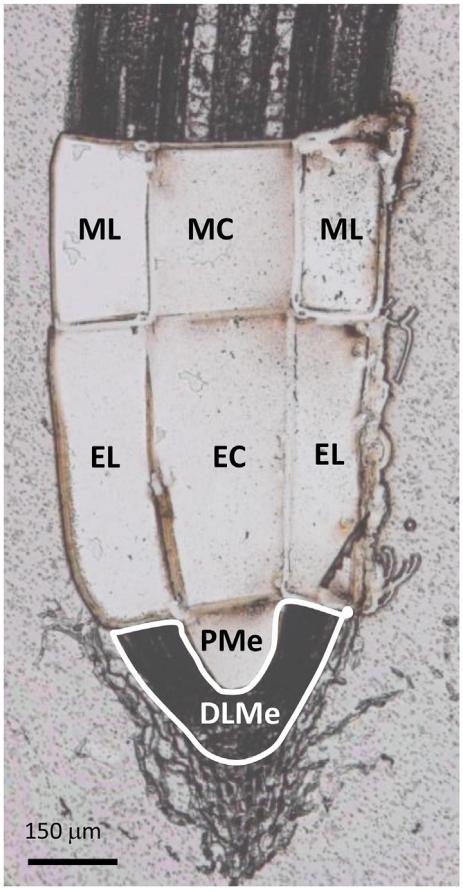


**Fig. 1.** The different sub-regions utilized for transcriptome studies of laser-microdissected hypoxic corn roots. DLMe, distal-lateral meristem; PMe, proximal meristem; EC, elongation zone of the central root region; EL, elongation zone of the lateral root region; MC, maturation zone of the central root region; ML, maturation zone of the lateral root region.

### 2.5. Validating sample quality

The quantity and quality of the extracted RNA samples and the cDNA libraries were assessed using Agilent RNA 6000 Pico Chips (Agilent Technologies, Santa Clara, CA, USA) and Agilent High Sensitivity DNA Chips (Agilent Technologies), respectively, following the manufacturer's instructions. Representative Electropherogram tracings from the Agilent 2100 Bioanalyzer for isolated RNA and cDNA library are shown in Supplemental Fig. 1.

### 2.6. RNA-sequencing and data analyses

The multiplexed samples were sequenced using The Illumina HiSeq 2500 platform at the Génome Quebec Innovation Center, obtaining 50 bp single-end reads. Reads were processed as described in Becker et al. (2017b). In brief, low quality reads were removed and adapters were clipped using the Trimmomatic tool. Reads passing quality filters were aligned to the Maize B73 v4 reference genome (Jiao et al., 2017) using TopHat2 of the Tuxedo pipeline (Kim et al., 2013). Cufflinks, cuffquant, cuffnorm, and cuffdiff (Trapnell et al., 2012) were used to generate normalized counts in FPKM and identify differentially expressed genes (pooled dispersion method, $q < 0.05$) between treatments. FPKMs were visualized using count and graph functions in Microsoft Excel. Patterns of gene expression were identified from FPKMs using a fuzzy k-means algorithm and visualized in R as barplots as described in Belmonte et al. (2013). To aid in data interpretation, all predicted protein sequences in the maize genome were aligned to both rice and Arabidopsis to identify putative homologs. Alignments were performed using NCBI-BLASTp with an e-value cutoff of $10^{-10}$. From aligned read files, raw counts were extracted using the HTSeq Python Framework with the following parameters: m union -f bam -stranded = no. Principle component analysis (PCA) was performed using raw counts input to the DESeq package (Anders and Huber, 2010).

### 2.7. Gene ontology (GO) term enrichment

For each pattern, gene ontology (GO) term enrichment was performed (Orlando et al., 2009). A GO term database was constructed for analyses using data from an existing GO term annotation (available at AgriGO: http://bioinfo.cau.edu.cn/) along with annotation information from putative rice and Arabidopsis homologs. A hypergeometric distribution test was performed using our GO database and the program SeqEnrich (Becker et al., 2017a) to identify statistically enriched ($p < 0.001$) GO terms overrepresented in each pattern and to assign a p-value. Statistical enrichment of GO terms was visualized using R and the gplots package.

### 2.8. Transcript abundance validation

The transcript abundance of selected genes was further validated using quantitative (q)RT-PCR analysis, as previously described (Mira et al., 2016a). The relative gene expression level was analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using actin as the reference gene.

## 3. Results

### 3.1. Global comparison of transcript levels in sub-regions of hypoxic maize roots

Imposition of hypoxic conditions compromises plant fitness and performance in corn (Youssef et al., 2016) by suppressing growth of roots and inducing death of the meristematic cells (Mira et al., 2016b). These effects were alleviated by over-expressing *ZmPgbs* and exaggerated in plants where the levels of *ZmPgbs* were reduced. To provide spatial resolution of transcriptional changes occurring in hypoxic corn roots, and to further investigate the protective role exerted by Pgbs, wild type (WT) plants and plants over-expressing *ZmPgb1.2* [ZmPgb1.2(S) line] were waterlogged for 3 days, and subsequently laser micro-dissected to isolate different root sub-regions and perform global transcriptional analysis using next generation RNA sequencing. The expression of selected genes obtained by comparing the transcriptome of WT and ZmPgb1.2(S) lines was subsequently tested in the respective root sub-regions of plants suppressing *ZmPgb1.2* [ZmPgb1.2(A)]. The choice to dissect the roots at day 3 was dictated by the peak in expression profile of *ZmPb1.2* measured in the root tips (1 mm) of waterlogged WT plants (Fig. 2A).

Transcript abundance was estimated as Fragments Per Kilobase of genes per Million mapped reads (FPKM) in which an expressed gene had a FPKM $\geq 1$ (Bhardwaj et al., 2015). The abundance level, categorized as low (FPKM 1–5), moderate (FPKM 5–25), or high (FPKM > 25), revealed that the largest percentage of detected transcripts had a moderate expression in all root compartments analyzed (Supplemental Fig. 2A). Principal component analysis, used to compare the transcript populations in the sub-regions of WT and ZmPgb1.2(S) roots, revealed a high degree of gene expression similarities shared by the same sub-regions regardless of the genotype (Supplemental Fig. 2B).

The expression level of *ZmPgb1.2* was measured by (q)RT-PCR in the different root sub-regions to confirm a low expression of the gene in the ZmPgb1.2(A) line (Fig. 2B). Relative to WT, the expression of *ZmPgb1.2* in the ZmPgb1.2(S) line was always higher in all root domains, validating the expression pattern generated from RNA sequencing (compare Fig. 2B with Supplemental Fig. 3). Note, however, that in the

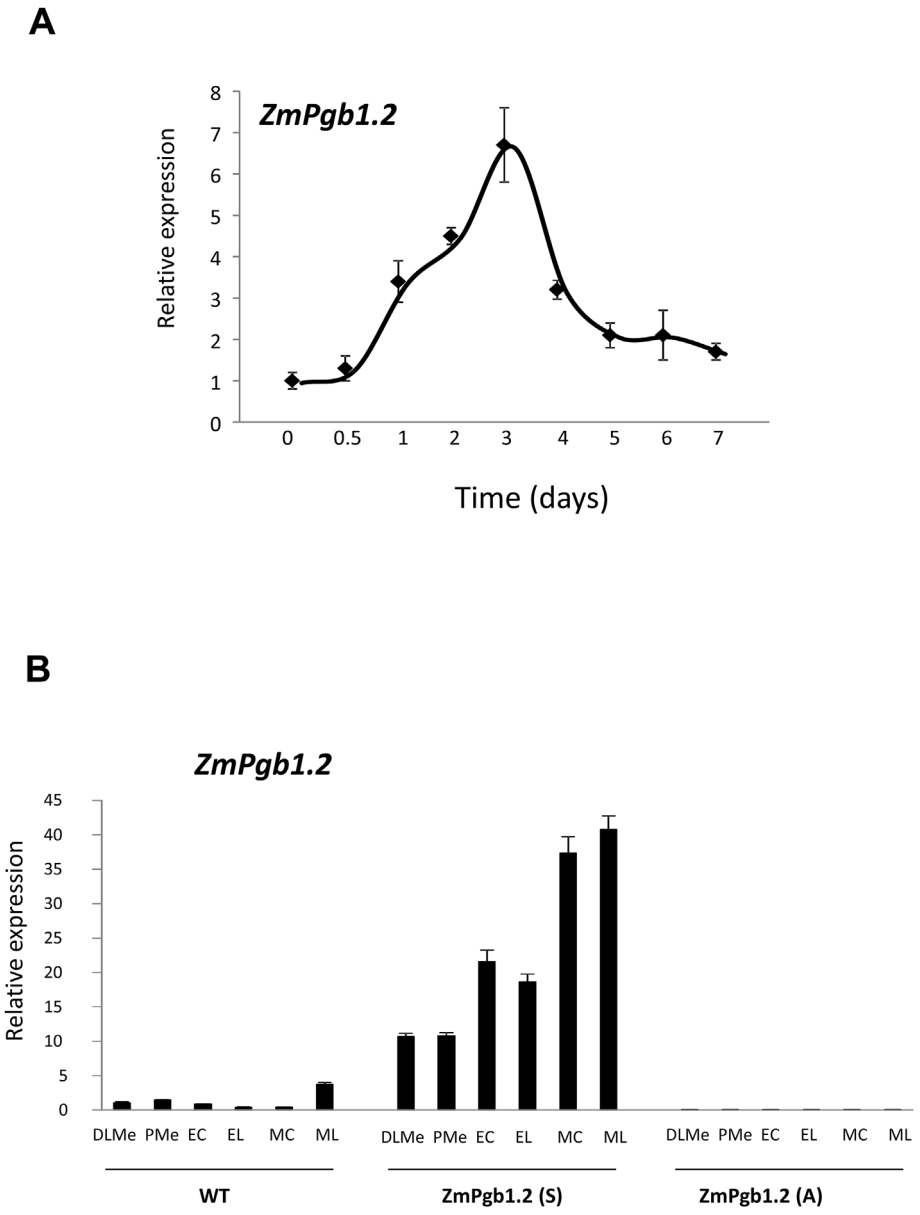


**Fig. 2.** Expression level of *ZmPgb1.2* measured by (q)RT-PCR in **(A)** root tips (1 mm) of waterlogged WT plants, and **(B)** in the dissected root sub-regions (shown in Fig. 1) of WT and transgenic plants after 3 days of waterlogging. Values ± SE are means three biological replicates.

ZmPgb1.2(S) line the relative proportions of *ZmPgb1.2* in the elongation zones (EC and EL) and the central maturation zone (MC), as a fraction of the total *ZmPgb1*.2 relative expression in the regions measured, are higher than what they are in the WT after 3 days of waterlogging (Fig. 2B).

### 3.2. Transcription gradient along the profile of hypoxic WT and ZmPgb1.2 over-expressing roots

To estimate the distribution of biological processes and molecular functions occurring along the root profile of hypoxic roots, transcripts were clustered into 21 patterns, based on their sub-region specific expression (Supplemental Figs. 4A and B). Genes included in pattern 1 (599 genes) and 3 (132 genes) followed an apical-basal gradient, with the highest expression in the apical meristematic tip (PMe and DLMe sub-regions), an intermediate expression in EC and EL, and the lowest expression in the mature basal domains (MC and ML). This was in contrast to genes of pattern 2 (1146 genes) and 8 (119) exhibiting a basal-apical gradient with a preferential expression in mature domains (ML and MC) and a limited expression in the apical sub-regions PMe and DLMe.

Heatmaps of enriched Gene Ontology (GO) terms were generated to identify categories of transcripts associated to specific functions. Transcripts with an apical-basal gradient, enriched in the meristematic regions relative to mature cells (patterns 1 and 3), included those associated with nucleic acid and nucleotide binding, DNA repair and replication mechanisms, cell proliferation, root morphogenesis, and transcription factors (Fig. 3). Specific genes included cyclins (B2:4, D3; 1, and D4; 1), topoisomerases (1 and 2), MUTS homologs (2, 6, and 7), and those participating in auxin flow (auxin import carrier1), and brassinosteroid perception (brassinosteroid insensitive1) (Table 1). Of interest, several transcription factors, such as those modulating ethylene responses (ERF-containing pathogenesis factor and ethylene-responsive factor 5) were preferentially abundant in hypoxic meristems (Table 1).

With the exception of the elongation zone of the Pattern 3 genes, there were no major shifts in pattern profiles as a result of over-expressing *ZmPgb1.2* (Fig. 3). In the EC zone, there was a more

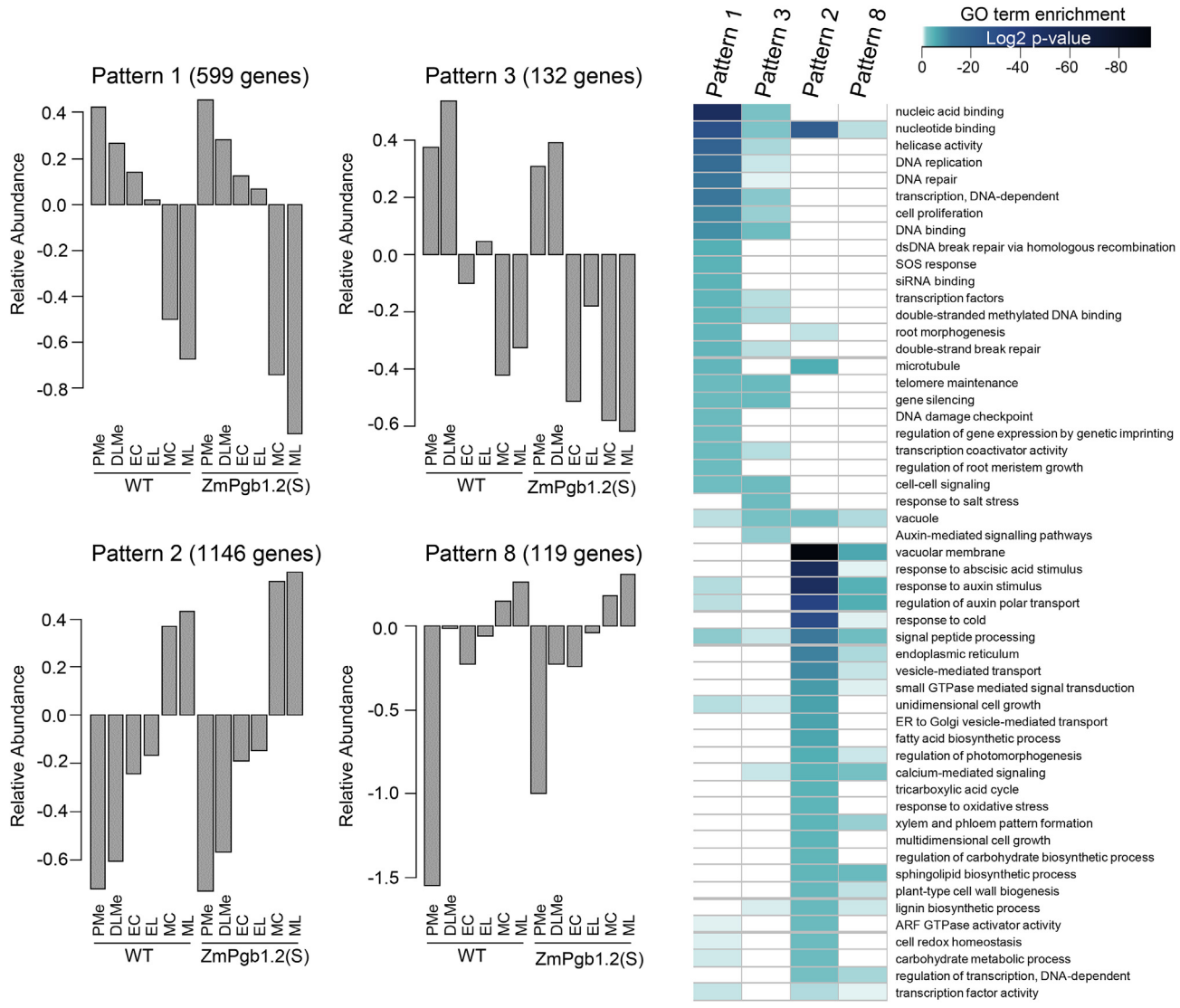


**Fig. 3.** Clusters of transcripts exhibiting an apical-basal (pattern 1 and 3) or basal-apical (pattern 2 and 8) gradient along hypoxic corn roots. Heatmaps of enriched Gene Ontology terms for the different patterns are also shown. Terms are considered enriched at $P < 0.001$ and greater statistical enrichment is represented by a darker color. PMe, proximal meristem; DLMe, distal-lateral meristem; EC, elongation zone of the central root region; EL, elongation zone of the lateral root region; MC, maturation zone of the central root region; ML, maturation zone of the lateral root region. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

pronounced down-regulation of Pattern 3 gene abundance compared to WT, while in the EL zone, the pattern shifted to one of over-abundance in the WT to under-abundance in ZmPgb1.2(S) line.

Genes exhibiting a basal-apical gradient and mostly present in mature domains of hypoxic roots (pattern 2 and 8) were mainly associated with vacuolar-, ER-, and Golgi vesicle-mediated transport, and differentiation processes (xylem and phloem pattern formation and lignin biosynthetic process) (Fig. 3). Both MC and ML sub-regions were enriched with transcripts contributing to responses to cold and oxidative stress. Mediators of cold responses, such as dehydrin COR410, cold acclimation WRCOR413, several heat shock proteins, and low temperature responsive proteins accumulated preferentially in mature subregions (Table 2). This was also the case of transcripts associated with oxidative stress, which included several coding for wound-responsive and disease resistant proteins, as well as peroxidases. Activation of stress responses was accompanied by the accumulation of transcripts contributing to cellular redox homesostasis, such as those participating in ascorbate recycling processes (dehydroascorbate reductase and monodehydroascorbate reductase1) and glutathione metabolism (glutathione peroxidases and transferases). Several executors of the endoplasmic reticulum (ER)-mediated apoptotic program were also preferentially transcribed in mature root tissue, these included Bax inhibitor-1, metacaspase5, autophagy3, and several apoptosis regulators (Table 2).

### 3.3. Radial transcription gradient in hypoxic WT and ZmPgb1.2 overexpressing roots

Within the meristematic (Me) region 412 genes were differentially expressed between the proximal meristem (PMe), and the distal-lateral meristem (DLMe), and the majority of these genes (245) were induced in the DLMe sub-region (Supplemental Fig. 5). A similar number of genes (391) were differentially expressed between the EC and EL subregions, with almost half of them being induced in EC. The expression of only 31 genes was found to differ between the MC and ML (Supplemental Fig. 5).

Categories of transcripts abundant mainly in the peripheral root domains (DLMe, EL, and ML), and clustered in patterns 7 (157 genes) and 5 (138 genes) (Fig. 4 and Supplemental Figs. 4A and B), comprised responses to growth hormone stimuli, including jasmonic acid and

**Table 1**
List of representative transcripts following an apical-basal gradient selected from patterns 1 and/or 3 (Fig. 3).

| Maize ID | Putative annotation | Associated GO term | pattern(s) |
|---|---|---|---|
| GRMZM2G138886 | Cyclin B2; 4 | cell proliferation | 1 |
| GRMZM2G161382 | Cyclin D3; 1 | cell proliferation | 1 |
| GRMZM2G178229 | Cyclin D4; 1 | cell proliferation | 1 |
| GRMZM2G064613 | NPK1-related protein kinase 1 | cell proliferation | 1 |
| GRMZM2G098828 | NPK1-related protein kinase 3 | cell proliferation | 1 |
| GRMZM2G111014 | DNA gyrase B2 | helicase activity | 1 |
| GRMZM2G574858 | SNF2 domain-containing protein | helicase activity | 3 |
| GRMZM2G021270 | Topoisomerase 1 | DNA binding | 1 |
| GRMZM2G121210 | Topoisomerase 2 | DNA binding | 1 |
| GRMZM2G076329 | Replication factor-A protein 1 | DNA replication | 1 |
| GRMZM2G056075 | MUTS homolog 2 | DNA repair | 1 |
| GRMZM2G421541 | MUTS homolog 6 | DNA repair | 1 |
| GRMZM2G110212 | MUTS homolog 7 | DNA repair | 1 |
| GRMZM2G030128 | DNA repair- protein (RAD50) | DNA repair | 1 |
| GRMZM2G071304 | DNA ligase 1 | DNA repair | 1 |
| GRMZM2G089743 | Argonaute 4-like | siRNA binding | 1 |
| GRMZM2G047143 | DNA-binding HORMA protein | nucleic acid binding | 1 |
| GRMZM2G149802 | DNA-directed DNA polymerases | nucleic acid binding | 1 |
| GRMZM2G129413 | Auxin import carrier1 | root morphogenesis | 1 |
| GRMZM2G146794 | Brassinosteroid insensitive 1 | root morphogenesis | 3 |
| GRMZM2G114775 | GATA transcription factor 16 | transcription factor | 1 |
| GRMZM2G366434 | Baby Boom | transcription factor | 1,3 |
| GRMZM2G027309 | NAC (No Apical Meristem) factor | transcription factor | 1 |
| GRMZM2G133331 | bZIP transcription factor | transcription factor | 1 |
| GRMZM2G173321 | AP2/B3-like transcriptional factor | transcription factor | 1 |
| GRMZM2G109480 | AP2/B3-like transcriptional factor | transcription factor | 1 |
| GRMZM2G065496 | AP2/B3-like transcriptional factor | transcription factor | 3 |
| GRMZM2G126566 | MYB domain containing protein | transcription factor | 1,3 |
| GRMZM2G012262 | WHIRLY 2 | transcription factor | 1 |
| GRMZM2G070295 | PLATZ transcription factor | transcription factor | 1 |
| GRMZM2G086573 | Aintegumenta-like 5 | transcription factor | 1,3 |
| GRMZM2G104074 | GRAB2 protein | transcription factor | 3 |
| GRMZM5G804893 | Nuclear factor Y, subunit B8 | transcription factor | 3 |
| GRMZM2G370715 | KOW-domain protein | transcription factor | 1 |
| GRMZM2G003466 | ERF-containing pathogenesis factor | transcription factor | 3 |
| GRMZM2G151542 Ethylene-responsive factor 5 | transcription factor | 1 | |

abscisic acid, as well responses to adverse conditions, such as oxidative stress, salt, and cold (Fig. 4). Within the meristematic region many of the stress responses appeared to be more active in the DLMe sub-region relative to the PMe sub-region.

Relative to the proximal meristem (PMe), the DLMe region was enriched during the hypoxic treatment with transcripts encoding the dehydration responsive element (DREB2B), the drought induced protein 21, alcohol dehedrogenase 2, several peroxidases and glutathione transferases (Fig. 5). This was associated with increased transcription of genes engaged in defense responses and the respiratory burst oxidase homolog B (RBOHB). Transcripts regulating the synthesis and signalling of jasmonic (lipoxygenase1, allene oxide synthase), and synthesis of ethylene (ACC oxidase1), also accumulated preferentially in DLMe (Fig. 5). This was in contrast to components of auxin transport (auxin efflux carrier) and response (target of Monopeteros 6, and SAUR auxin responsive) which were most abundant in the PMe sub-region (Fig. 5).

An enrichment of stress-related transcripts was also detected in the lateral domains of the elongation zone (EL), compared to the EC sub-region. Cells in the EL-sub-region accumulated higher levels of mRNAs encoding defensin precursors, glutathione transferases, glutathione peroxidase 6, monodehydroascorbate reductase1, and several hypoxia-related factors such as alcohol dehydrogenase, alternative oxidase1 and AAA-ATPase 1 (Fig. 6). Factors regulating the synthesis and signalling of jasmonic acid (lypoxygenase1 and 2, jasmonic acid induced protein, and allene oxide synthase), ABA (ABI-1) and brassinosteroids (brassinosteroid insensitive1) were also preferentially expressed in EL (Fig. 6). Relative to EC, EL was depleted in transcripts involved in glucan synthesis and metabolism, auxin synthesis (flavin monoxygenase), transport (PIN1), and response (target of Monopteros 6), as well as several transcription factors (Fig. 6).

In the maturation zone differences in transcription between the central (MC) and lateral (ML) sub-regions were limited, and included transcripts with diverse biological functions (Supplemental Fig. 6).

The transcription data generated from RNA sequencing were validated by q(RT)—PCR analyses on representative genes. Despite the expected differences in sensitivity of the two techniques, similar expression patterns, i.e. up-regulation or down-regulation, were obtained (Supplemental Figs. 7A and B).

### 3.4. Alteration of *ZmPgb1.2* evokes sub-region specific transcriptional changes in hypoxic roots

A total of 150 genes were differentially expressed by the up-regulation of *ZmPgb1.2*, with about half of the changes (78 genes) occurring in the meristematic (Me) sub-region (57 in DLMe and 21 in PMe) (Table 3).

In the PMe subregion, over-expression of *ZmPgb1.2* activated genes associated with jasmonic acid synthesis (lipoxygenase1) and response (jasmonate-induced protein), along with a gene associated with the oxidation of ascorbic acid (ascorbate peroxidase1), while reducing ethylene synthesis (ACC oxidase1) and response (ERF4) (Fig. 7A). These changes were specific to the expression level of *ZmPgb1.2*, as an opposite expression pattern was measured by qRT-PCR in the PMe sub-region of roots suppressing *ZmPgb1.2* (Fig. 7B). Interestingly, differential expression of two genes that have been associated with cell cycle regulation and auxin responses (F-Box/RNI-like) undergo opposite changes as a result of *ZmPgb1.2* expression during hypoxia, one increasing substantially, the other decreasing (Fig. 7A).

A similar preferential accumulation of lipoxygenase1 and ascorbate peroxidase1 also occurred in DLMe sub-regions of *ZmPgb1.2* over-

**Table 2**
List of representative transcripts following basal-apical gradient selected from patterns 2 and/or 8 (Fig. 3).

| Maize ID | Putative annotation | Associated GO term | pattern(s) |
|---|---|---|---|
| GRMZM2G147014 | Dehydrin COR410 | response to cold | 2 |
| GRMZM2G040030 | Cold acclimation WCOR413 | response to cold | 2 |
| GRMZM2G010000 | Heat shock protein DnaJ | response to cold | 2 |
| GRMZM2G415007 | Heat shock protein 70 (Hsp 70) | response to cold | 2 |
| GRMZM2G114793 | Heat shock protein 70 (Hsp 70) | response to cold | 2 |
| GRMZM2G11972 | DNAJ heat shock N-terminal domain- protein | response to cold | 2 |
| GRMZM2G137495 | DNAJ heat shock family protein | response to cold | 2 |
| GRMZM2G015605 | Low temperature and salt responsive protein | response to cold | 2 |
| GRMZM2G153369 | Low temperature and salt responsive protein | response to cold | 2 |
| GRMZM2G477325 | Hydrophobic TI6A (Low temperature-induced) | response to cold | 2 |
| GRMZM2G416308 | Proline extension-like receptor kinase 1 | oxidative stress | 2 |
| GRMZM2G106393 | Wound-responsive family protein | oxidative stress | 2 |
| GRMZM2G050730 | Stress responsive A/B Barrel Domain | oxidative stress | 2 |
| GRMZM2G180244 | Disease resistance protein (CC-NBS-LRR class) | oxidative stress | 2 |
| GRMZM2G180254 | Disease resistance protein (CC-NBS-LRR class) | oxidative stress | 2 |
| GRMZM2G174644 | Oxysterol-binding family protein | oxidative stress | 2 |
| GRMZM2G410175 | Peroxidase 2 | oxidative stress | 2 |
| GRMZM2G088765 | Peroxidase 2 | oxidative stress | 2 |
| GRMZM2G085967 | Peroxidase 39 | oxidative stress | 2 |
| GRMZM2G048474 | Peroxidase 1 | oxidative stress | 2 |
| GRMZM2G144648 | Peroxidase superfamily protein | oxidative stress | 2 |
| GRMZM2G085198 | Peroxidase superfamily protein | oxidative stress | 2 |
| GRMZM2G341934 | Peroxidase superfamily protein | oxidative stress | 2 |
| GRMZM2G136534 | Peroxidase superfamily protein | oxidative stress | 2 |
| GRMZM2G035502 | Dehydroascorbate reductase | redox homeostasis | 2,8 |
| GRMZM2G084881 | Monodehydroascorbate reductase 1 | redox homeostasis | 2 |
| GRMZM2G135893 | Glutathione peroxidase 6 | redox homeostasis | 2 |
| GRMZM2G329144 | Glutathione peroxidase 4 | redox homeostasis | 2 |
| GRMZM2G434541 | Glutathione S-transferase TAU 15 | redox homeostasis | 2 |
| GRMZM2G146246 | Glutathione S- transferase | redox homeostasis | 2 |
| GRMZM2G300965 | Respiratory burst oxidase homolog D | redox homeostasis | 2 |
| GRMZM2G479608 | Bax inhibitor-1 family protein | endoplasmic reticulum | 2 |
| GRMZM2G029087 | BAX inhibitor 1 | endoplasmic reticulum | 2 |
| GRMZM2G074404 | BAX inhibitor-1 | endoplasmic reticulum | 2 |
| GRMZM2G047274 | Metacaspase 5 | membrane | 2 |
| GRMZM5G847530 | Defender against death (DAD family) protein | membrane | 2 |
| GRMZM5G818887 | Autophagy 3 (APG3) | autophagy | 2 |
| GRMZM2G017013 | Apoptosis regulator BCL-2- athanogene1 | apoptosis | 2 |
| GRMZM2G029863 | Apoptosis regulator BCL-2- athanogene 3 | apoptosis | 2 |
| GRMZM5G860590 | DCD (Development and Cell Death) | apoptosis | 2 |
| GRMZM2G013448 | ACC oxidase 1 | ethylene pathway | 2,8 |
| GRMZM2G174347 | Ethylene response factor 2 | Transcription factor | 2,8 |
| GRMZM2G137413 | Auxin response factor 1 | auxin response | 2 |
| GRMZM2G050089 | Auxin efflux carrier family protein | auxin transport | 2 |

expressing roots, relative to their WT counterparts. This was concomitant with an increased expression of several genes encoding for cell wall modifying enzymes, including cinnamyl alcohol dehydrogenase, polyphenol oxidase, and xyloglucan endotransglucosylate/hydrolases 5 (Sanz et al., 2014) (Fig. 8A). The transcript levels of these genes were reduced in DLMe sub-regions where *ZmPgb1.2* was suppressed (Fig. 8B).

Compared to WT, DLMe over-expressing *ZmPgb1.2* was depleted in stress-related transcripts, including the drought-induced protein 21, a detoxification protein, and several late embryogenic abundant (LEA) proteins (Fig. 8A).

Of the 27 genes differentially expressed between the central elongation (EC) sub-regions of WT and ZmPgb1(S) lines, 13 accumulated preferentially in cells over-expressing *ZmPgb1.2*. (Fig. 9A). These included two key enzymes of ethanolic fermentation: pyruvate decarboxylase APK1B and alcohol dehydrogenase 1, both of which were suppressed in the respective compartment of roots with lower levels of *ZmPgb1.2* (Fig. 9B). Several transcripts of unknown function, together with others encoding proteins with diverse roles, ranging from defence (defensin precursor) to lipid transport/modification (lipid transferase1) accumulated at lower levels in EC over-expressing *ZmPgb1.2* relative to the EC subregion of WT roots (Fig. 9A).

Relative to WT, ML sub-regions over-expressing *ZmPb1*.2 had lower expression of a peroxidase 2 and a xyloglucan endotransglucosylase. Several stress-related transcripts, including a heat shock protein 21, thioredoxin 2, oxidative stress 3, and serine endopeptidase were detected at higher levels in the ML region of WT roots (Supplemental Fig. 8). A total of 28 genes were differentially expressed in the MC sub-regions. Only two of these: a protein of unknown function and a Kelch-domain protein were more abundant under those conditions where *ZmPgb1.2* was induced (Supplemental Fig. 8).

## 4. Discussion

This study provides a comprehensive and high resolution transcriptional analysis of hypoxic corn roots and documents changes in gene expression elicited by altered expression of *ZmPgb1.2*, which have been implicated in protecting root cells exposed to low oxygen levels (Mira et al., 2016b). Specifically, the hypoxic inhibition of root growth and overall plant performance were alleviated by the over-expression of *ZmPgb1.2* and aggravated by those conditions suppressing *ZmPgb1.2* (Youssef et al., 2016; Mira et al., 2016b). These effects, not observed under normoxic conditions where expression of *ZmPgb* had no effects on root morphology and behaviour, were ascribed to the protective role exercised by the gene in retaining the integrity of root tips under low oxygen levels. In agreement with previous studies (Zhao et al., 2008; Mira et al., 2016b), waterlogging resulted in a rapid rise in *Pgb* level within the root tip (Fig. 2A). The expression of *ZmPgb1.2* was observed in all meristematic sub-regions (Fig. 2B).

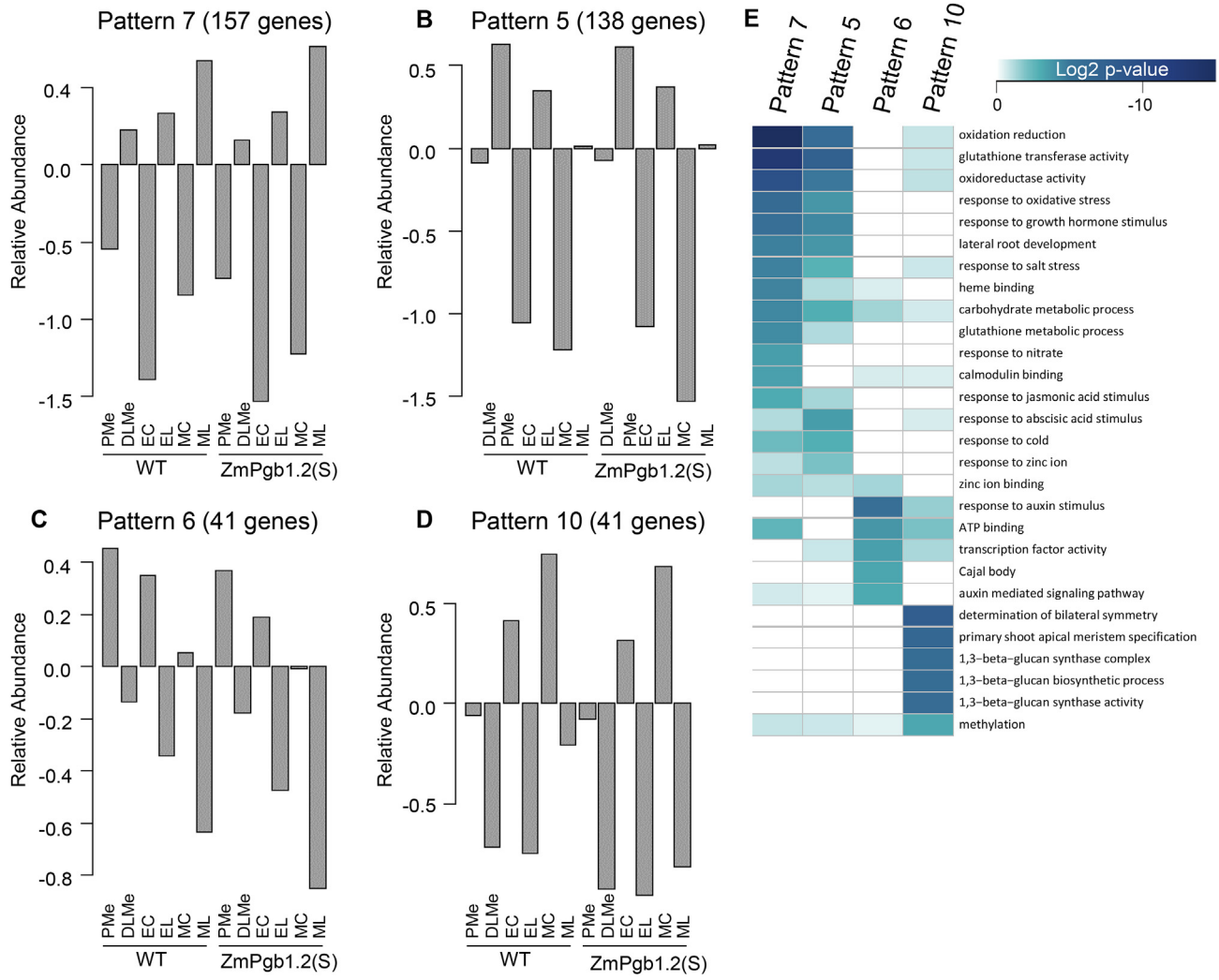


**Fig. 4.** Clusters of transcripts exhibiting a radial gradient in hypoxic corn roots. Heatmaps of enriched Gene Ontology terms for the different patterns are also shown. Terms are considered enriched at $P < 0.001$ and greater statistical enrichment is represented by a darker color. PMe, proximal meristem; DLMe, distal-lateral meristem; EC, elongation zone of the central root region; EL, elongation zone of the lateral root region; MC, maturation zone of the central root region; ML, maturation zone of the lateral root region. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In the apical-basal transcriptome pattern 599 genes (pattern1) and 132 genes (pattern 3) were preferentially induced in the root apex and down-regulated in the maturation zone of waterlogged roots (Fig. 3). The meristematic regions of hypoxic corn roots were enriched with biological processes contributing to cell proliferation, such as helicase activity DNA replication, and DNA repair. Specific cyclins and DNA binding and repair factors identified in this study (Table 1) are known modulators of the cell cycle (Gutierrez, 2009). The list also includes the transcription factors, Aintegumenta-like 5, Baby Boom, and several AP2/B3-like factors which govern the maintenance and function of the root meristem (reviewed in Drisch and Stahl, 2015). Consistent with the activation of ethylene responses under low oxygen conditions (Drew, 1997), and the susceptibility of hypoxic meristematic cells to the over-production of ethylene (Mira et al., 2016b), two ethylene responsive factors (ERF), ERF-containing pathogenesis factor and ERF5, were preferentially expressed in the meristematic domains of hypoxic roots (Table 1).

Several processes activated in response to stress (oxidative stress, cold, and ABA) were preferentially down-regulated in the apical domains of the root relative to the basal domains (Fig. 3, Patterns 2 and 8). Among the genes categorized in patterns 2 and 8 are several coding for heat shock proteins, as well as wound and disease resistance proteins (Table 2). Oxidative stress and damage resulting from the over-production of ROS is a typical consequence of many types of stress including hypoxia (Blokhina et al., 2003). Besides being formed as by-products during electron transport mechanisms in chloroplasts (Asada and Takahashi, 1987), ROS can be generated by the NADPH oxidase multicomplex enzyme (Torres and Dangl, 2005). The expression of the respiratory burst oxidase homolog D (RBOHD) (Table 2), a component of NADPH oxidase and reliable indicator of ROS production (Mira et al., 2016a), was preferentially down-regulated in the apical, metabolically more active hypoxic root cells while was induced in the mature root cells. Pronounced oxidative stress experienced by the basal domains of the roots is also confirmed by the elevated accumulation of transcripts coding enzymes scavenging ROS and suppressors of oxidative damage. These include several peroxidases such as glutathione peroxidase 4 and 6, as well as enzymes needed for the regeneration of ascorbic acid, dehydroascorbate reductase and monodehydroascrobate reductase 1 (Table 2). Ascorbic acid is one of the most effective antioxidants detoxifying ROS through its oxidation to monodehydroascorbate and its regeneration, mediated by dehydroascorbate reductase and monodehydroascrobate reductase, ensures survival under condition of prolonged stress (Smirnoff and Pallanca, 1996).

In the mature domains, the over-representation of several transcripts related to death mechanisms, including metacaspase 5, autophagy 3, and development of cell death (DCD), are most likely associated

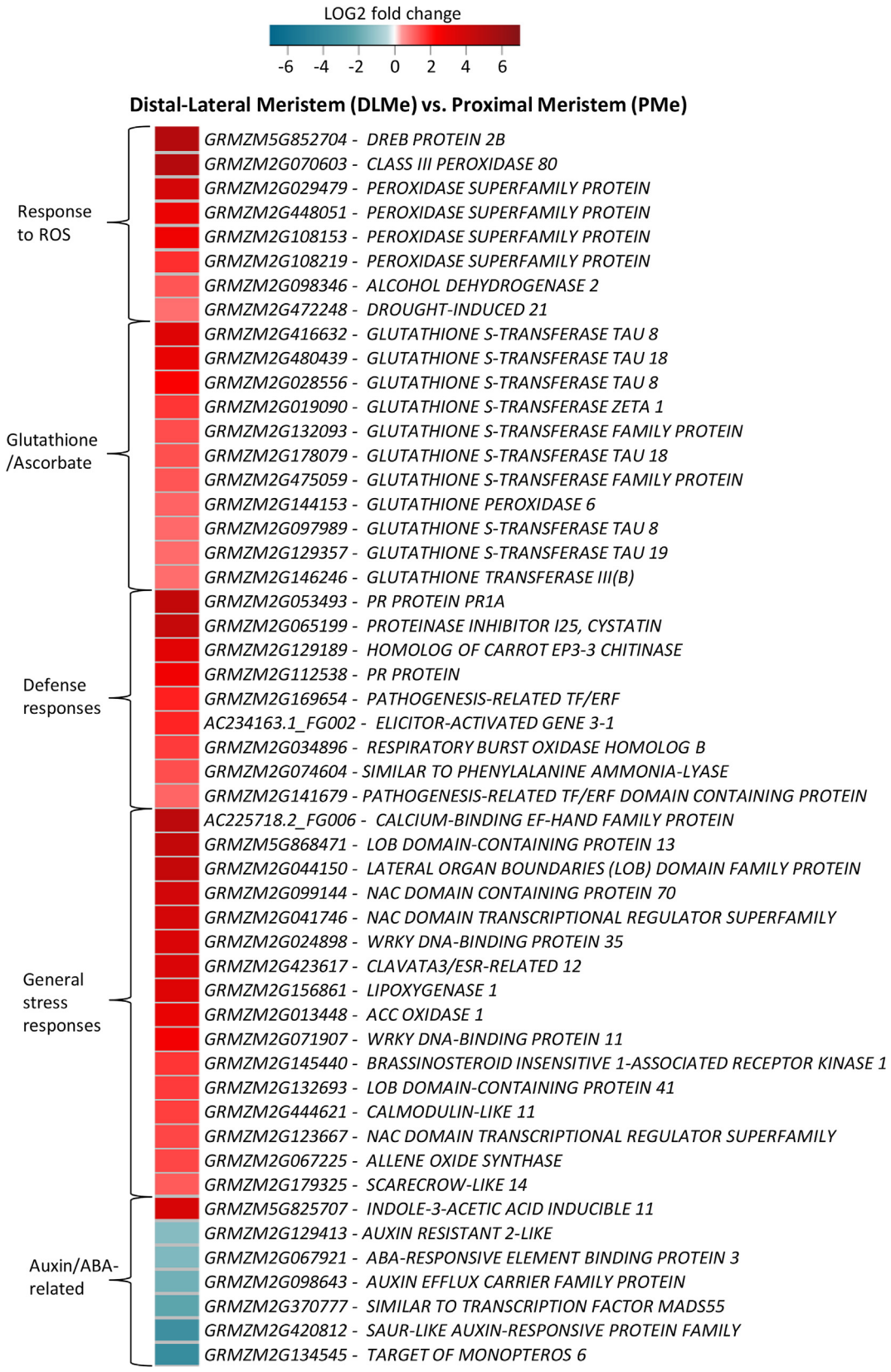


**Fig. 5.** Heat map of selected transcripts accumulating differentially between the DLMe and PMe sub-regions of hypoxic WT and ZmPgb1.2(S) roots.

to the induction of PCD during the formation of aerenchyma, an avoidance mechanism to oxygen deprivation (reviewed in Bailey-Serres et al., 2012). Among the diverse stimuli inducting PCD, the endoplasmic reticulum (ER) plays a key role in relaying apoptotic signals by sensing the balance between folded and unfolded proteins, and triggering the death process following the over-accumulation of unfolded or misfolded proteins (Boyce and Yuan, 2006). The observation that the ER-stress attenuator Bax inhibitor1 (Watanabe and Lam, 2008),

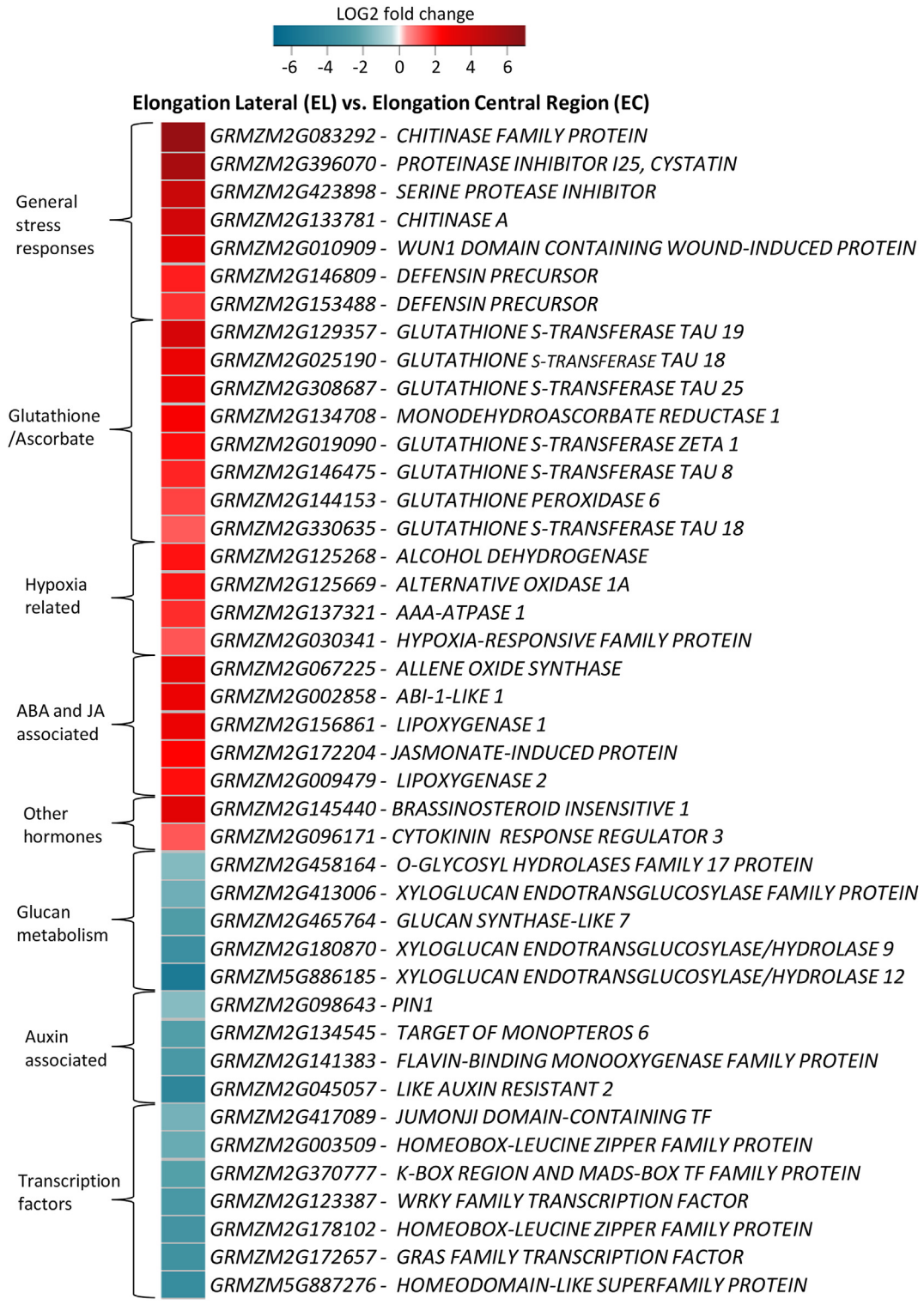


**Fig. 6.** Heat map of selected transcripts accumulating differentially between the EL and EC sub-regions of hypoxic WT and ZmPgb1.2(S) roots.

highly induced in death programs elicited by ER stress (Duan et al., 2010), is among the genes preferentially expressed in the basal zone of the root suggests the involvement of the ER in triggering death leading to the formation of aerenchyma.

Analyses of the radial expression gradient indicate that many stress responses are predominantly active in the lateral root domains (DLMe, EL, ML) (Figs. 3–7). Within the meristematic area, gene induction is preferential in the DLMe sub-region (Supplemental Fig. 5) and includes an array of stress-related genes some of which related to hypoxia such as the fermentation enzyme alcohol dehydrogenase 2 and others participating in glutathione/ascorbate metabolism, as well as general stress and defence responses (Fig. 5). This transcriptional diversification suggests that either hypoxic responses utilize intermediates common to other stress responses, or that hypoxic roots also experience other forms of abiotic and biotic stress. This notion was also supported by the activation of hormone-related genes such as brassinosteroid insensitive 1, conferring higher tolerance to cold when over-expressed (Kim et al., 2010a), and lipoxygenase 1 and allene oxide synthase, jasmonic acid

**Table 3**
Number of genes differentially expressed within the same sub-regions of WT and *ZmPgb1.2* over-expressing roots exposed to hypoxia. DLMe, distal-lateral meristem; PMe, proximal meristem; EC, elongation zone of the central root region, EL, elongation zone of the lateral root region; MC, maturation zone of the central root region, ML, maturation zone of the lateral root region.

| Sub-region | Differentially expressed genes |
|---|---|
| DLMe | 57 |
| PMe | 21 |
| EC | 27 |
| EL | 0 |
| MC | 29 |
| ML | 16 |

biosynthetic enzymes induced in response to pathogen attack (Savatin et al., 2014; Yang et al., 2015). A similar preferential activation of stress responses in the lateral root domain, relative to the central root domain, was also observed in the elongation (E) zone (Fig. 6), but less in the maturation (M) zone (Supplemental Fig. 6), an observation to be considered when performing studies using whole root tissue.

Our previous studies revealed that Pgbs influence the response of corn plants to oxygen deprivation, with the ectopic expression of *ZmPgb1.2* alleviating hypoxic-root growth inhibition while the suppression of *ZmPgb1.2* aggravating the inhibition (Mira et al., 2016b). The Pgb effects were more pronounced within the meristematic region and were mediated by NO and ethylene. Here we documented 78 genes differentially expressed in the root tip domains (DLMe and PMe) of hypoxic corn over-expressing *ZmPgb1.2* (Figs. 7 and 8), and identified potential candidates regulated by the levels of *ZmPgb1.2* in the tissue, i.e. showing an opposite expression behaviour between the *ZmPgb1.2* over-expressing [ZmPgb1.2(S)] and suppressing [ZmPgb1.2(A)] lines. Within the PMe sub-region these included genes participating in jasmonic acid synthesis (lipoxygenase 1) and response (jasmonate induced protein), as well as ascorbate metabolism (ascorbate peroxidase 1), which were over-represented in the ZmPgb1.2(S) line, while two involved in ethylene synthesis (ACC oxidase 1) and response [Ethylene Responsive Factor (ERF)4] were under-represented (Fig. 7A and B). The ZmPgb-regulation of these five candidate genes was also supported by localization analyses (Supplemental Fig. 9).

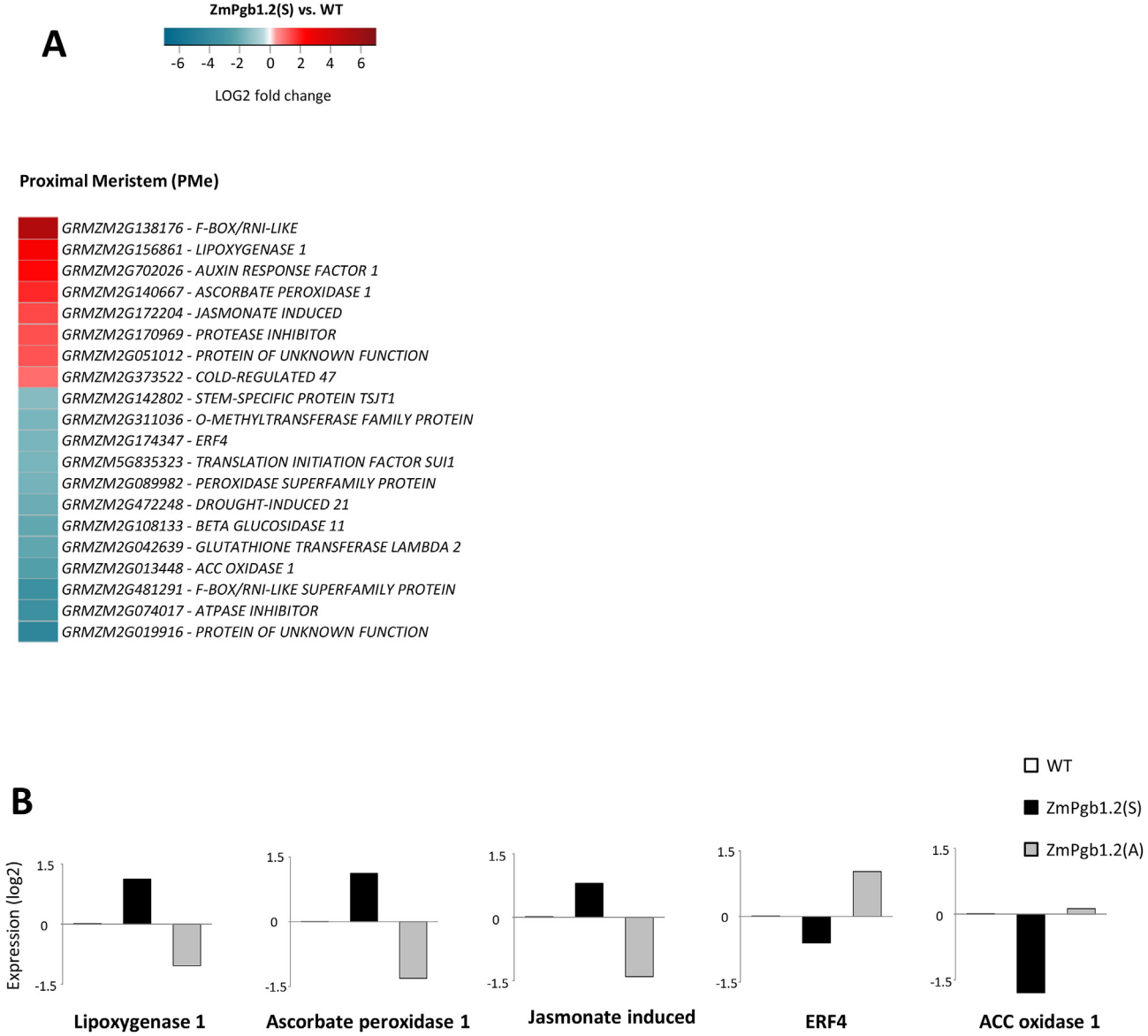


**Fig. 7.** (A) Genes differentially expressed by the over-expression of *ZmPgb1.2* in the proximal meristem PMe. **(B)** Expression levels of selected genes measured by (q) RT-PCR in the PMe sub-region of roots up-regulating [ZmPgb1.2(S)] or down-regulating [ZmPgb1.2(A)] *ZmPgb1.2*. Values are normalized to the value of WT set at 0.

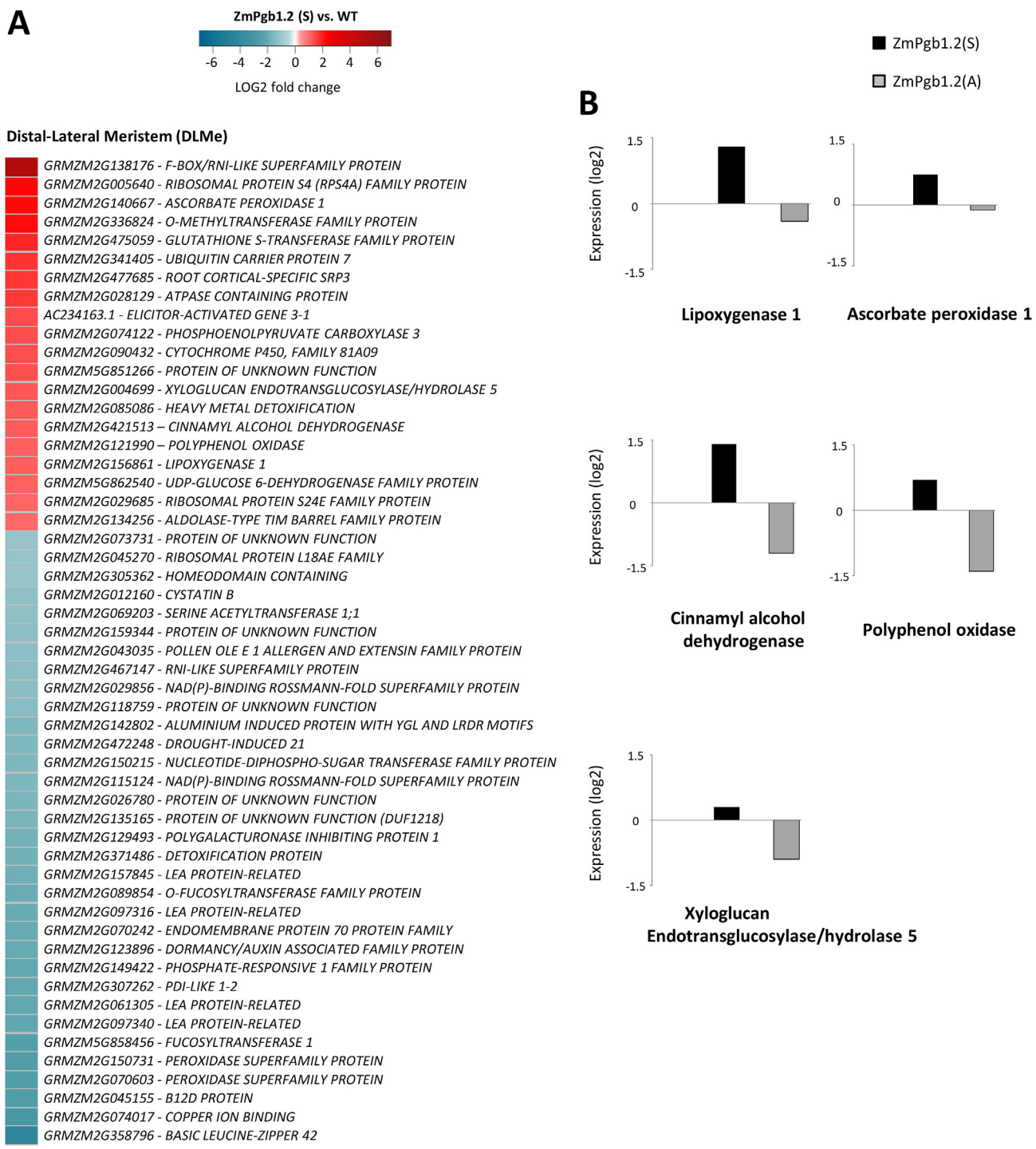


**Fig. 8. (A)** Genes differentially expressed by the over-expression of *ZmPgb1.2* in the distal-lateral meristem DLMe. **(B)** Expression levels of selected genes measured by (q)RT-PCR in the DLMe sub-region of roots up-regulating [ZmPgb1.2(S)] or down-regulating [ZmPgb1.2(A)] *ZmPgb1.2*. Values are normalized to the value of WT set at 0.

The transcriptional activation of jasmonic acid synthesis and response in the meristematic cells of the PMe sub-region over-expressing *ZmPgb1.2* and the repression in those suppressing *ZmPgb1.2* is intriguing, as the Arabidopsis Pgb suppresses accumulation of JA (Mira et al., 2016c). This discrepancy suggests the diverse, and in this case opposite, function of Pgbs between monocots and dicots and agrees with our previous studies revealing different Pgb mechanisms directing the same morphogenic event in corn, a monocot (Huang et al., 2014), and Arabidopsis, a dicot (Elhiti et al., 2013). Despite the well-recognized role of JA in defence responses to necrotrophic pathogens and insects (Wasternack and Hause, 2013) the participation of this hormone in hypoxic mechanisms has only been shown to be involved in the re-oxygenation process following submergence of Arabidopsis, where JA levels decreased during submergence and increased rapidly during the return to normoxic conditions (Yuan et al., 2017). The authors implicated MYC2 in the process, by which the factor was involved in increasing antioxidants. Jasmonic acid has been associated with reversible protein phosphorylation, particularly with respect to translation control through phosphorylation/dephosphorylation of transcription factors (Rojo et al., 1998). Major changes in the

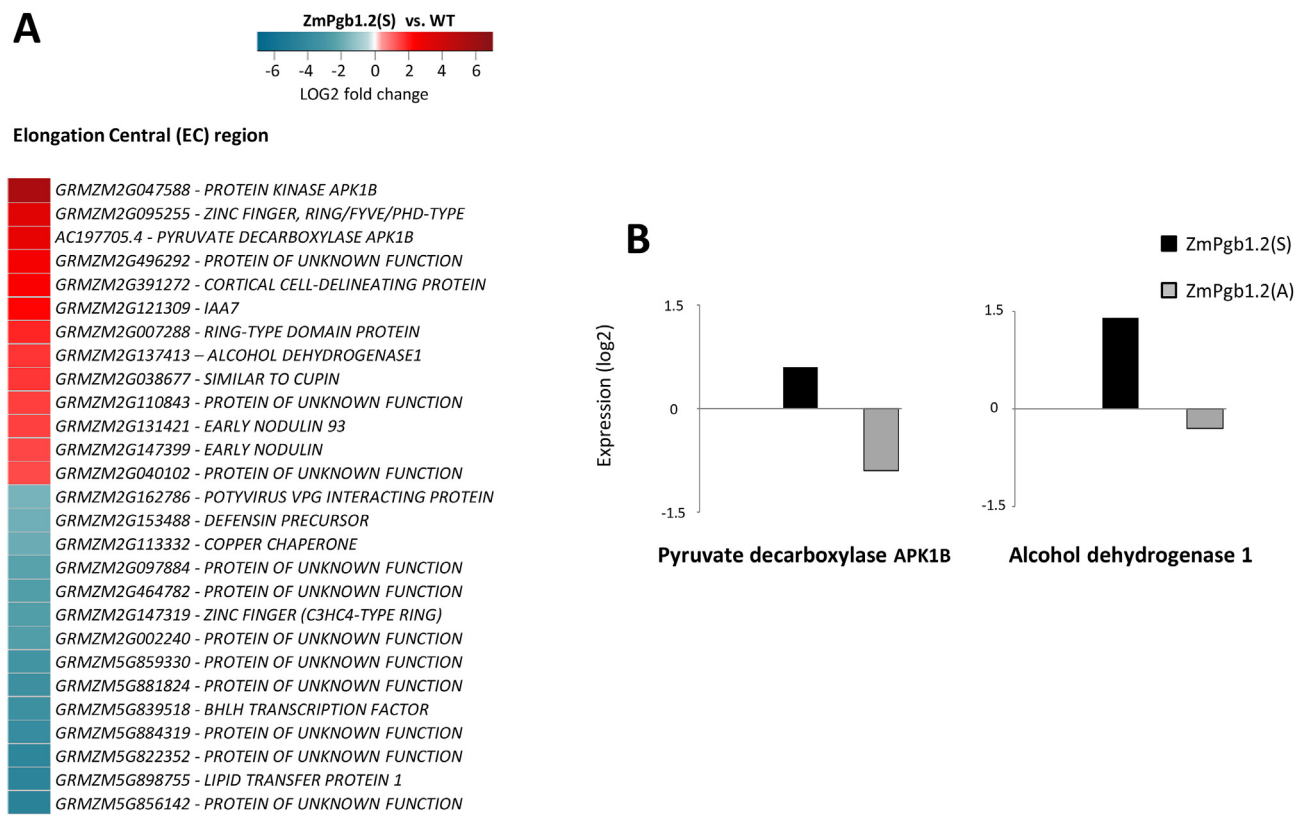


**Fig. 9. (A)** Genes differentially expressed by the over-expression of *ZmPgb1.2* in the central elongation sub-region EC. **(B)** Expression levels of selected genes measured by (q)RT-PCR in the EC sub-region of roots up-regulating [ZmPgb1.2(S)] or down-regulating [ZmPgb1.2(A)] *ZmPgb1.2*. Values are normalized to the value of WT set at 0.

phosphorylation of maize root ribosomal proteins has been observed as a result of hypoxic stress (Bailey-Serres and Freeling, 1990).

Evidence also suggests that the JA can enhance oxidative defences and the cellular detoxification system by favoring the formation of ascorbic acid, one of the most effective antioxidants detoxifying ROS. In the context of previous findings showing that the ROS-induced death of hypoxic root cells is mediated by Pgbs (Mira et al., 2016a) and the higher expression of ascorbate peroxiadase 1 in meristematic cells over-expressing *ZmPgb1.2* (Fig. 7), it is postulated that Pgb activation of JA synthesis and responses represents a potential protective strategy to limit ROS-induced oxidative stress and ensure survival of the RAM under low oxygen levels.

Accumulation of ROS in the meristematic regions of hypoxic roots is induced by the activation of ethylene synthesis and response which are promoted by the suppression of *ZmPgbs* and inhibited under conditions where *ZmPgbs* are over-expressed (Mira et al., 2016b). Consistent with this observation, the ethylene biosynthetic (ACC oxidase 1) and responsive (ERF4) genes are reduced in the PMe sub-region of the ZmPgb1.2(S) line and induced in the same sub-region of ZmPgb1.2(A) line (Fig. 7B). This regulation, however, was not observed in the DLMe sub-region where no differences in the expression of both ACC oxidase 1 and ERF4 were measured between lines. Besides confirming the cell-specific action of Pgbs, this observation suggests that the central domains of the meristem might be more susceptible to fluctuations in Pgbs levels, and ethylene synthesis and response, during period of oxygen deprivation.

The enrichment of ascorbate peroxidase 1 in the PMe and DLMe sub-regions of roots over-expressing *ZmPgb1.2*, and its depletion in the same sub-regions where the level of the gene is suppressed might also contribute to the specification of the quiescent cells (QC), the organizing center or the RAM (Petricka et al., 2012) during hypoxic conditions. Retention of quiescence in the QC requires a shift of the total ascorbate pool towards monodehydroascorbate, the oxidized form generated by ascorbate peroxidase. Perturbations of this redox balance depleting monodehydroascorbate, induce division of the QC, leading to the premature differentiation of the stem cells and inhibition of root growth (Jiang et al., 2003). Thus, the alleviation of root growth retardation in hypoxic roots over-expressing *ZmPg1*.2 might be due, at least in part, to the presence of an ascorbate peroxidase-mediated oxidized environment in the QC which is absent in roots down-regulating *ZmPgb1.2* and susceptible to hypoxia.

As well as affecting ascorbate peroxidase and lipoxigenase 1, ZmPgb1.2 also regulates the expression of several genes in the DLMe sub-region, including polyphenol oxidase, cinnamyl alcohol dehydrogenase, and xyloglucan endotransglucosylase (Fig. 8). Polyphenol oxidases are copper-containing enzymes which, by catalyzing the oxidation of diphenols to quinones, are implicated mainly in defence mechanisms to pathogens and herbivores (Yoruk and Marshall, 2007). Recent evidence suggests they possess antioxidant activity, mediated by JA, enhancing abiotic stress tolerance (Mayer, 2006); a function that might also contribute to relieve oxidative stress in the meristems, and account for the different growth performance of corn roots with altered expression of *ZmPgb1.2*. Cinnamyl alcohol dehydrogenase is a major enzyme involved in suberin and lignin biosynthesis and it is induced in tobacco roots in response to biotic and abiotic stress (Kim et al., 2010b). While meristematic regions of WT and *ZmPgb1.2* transgenic lines did not stain for lignin and suberin, both compounds preferentially accumulated in the endodermal and exodermal layers of the mature zone of the ZmPgb1.2 (S) line, and to a lower extent in the ZmPgb1.2(A) line (Supplemental Fig. 10). Based on this observation it is speculated that the activation of lignin and suberin production is initiated at a transcriptional levels in the meristematic zone, and completed in the maturation zone as the meristematic cells are rapidly displaced along apical-basal profile of the root. In the ZmPgb1.2(S) line the heavier

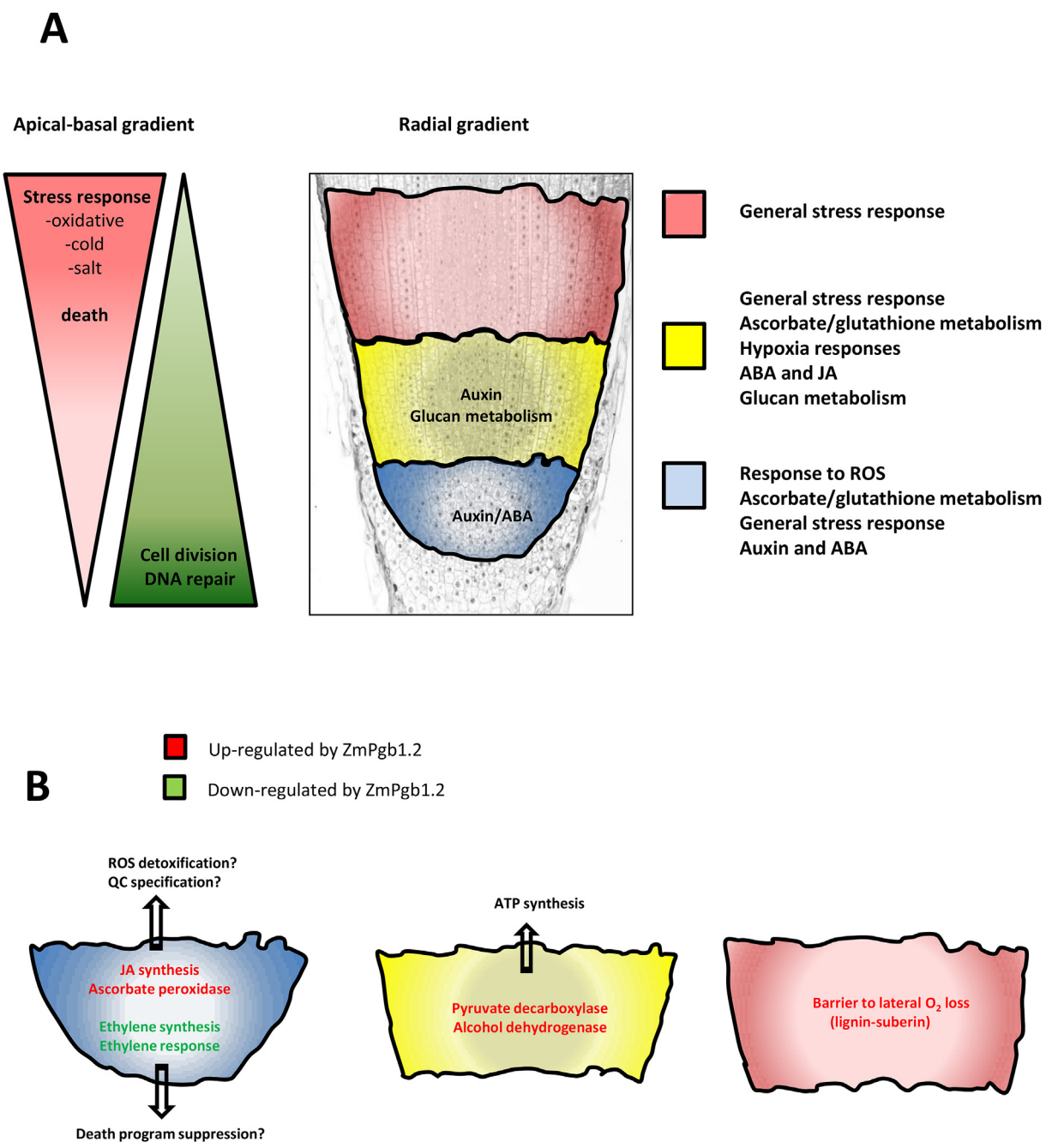


**Fig. 10.** Summary of transcriptional changes occurring within sub-regions of WT roots **(A)**, and between WT and *ZmPgb1.2* lines. In **(A)** the color gradation reflects transcript abundance. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

accumulation of both suberin and lignin in the endodermal and exodermal layers might provide a barrier inhibiting radial $O_2$ loss, a well documented strategy to cope with hypoxic conditions.

The regulation of xyloglucan endotransglucosylase by *ZmPgb1.2* (Fig. 8) also suggest modifications in cell wall components, as documented under diverse forms of abiotic and biotic stress (Le Gall et al., 2015). This enzyme was up-regulated in maize seedling shoots during hypoxic stress (Peschke and Sachs, 1994).

Hypoxic plants need to re-configure some of their metabolic pathways to compensate for reduced oxygen levels limiting or halting oxidative phosphorylation. Under these circumstances the glycolytic pathway is the only mean to produce ATP, and carbon flow through glycolysis requires $NAD^+$ regenerated by alcoholic fermentation (Roberts et al., 1984a, 1984b). Thus activation of the fermentative enzymes pyruvate decarboxylase and alcohol dehydrogenase represents a strategy to cope with hypoxia, as also revealed by the reduced tolerance to oxygen deficiency in plants lacking alcohol dehydrogenase function (Jacobs et al., 1988; Matsumura et al., 1995) and enhanced tolerance in those overexpressing pyruvate decarboxylase (Ismond et al., 2003). The regulation of alcohol dehydrogenase and pyruvate carboxylase by *ZmPgb1.2* (Fig. 9) suggests a pattern where the metabolic flux in the fermentative pathway, and hence production of ATP during hypoxia, is encouraged by the over-expression of *ZmPgb1.2* and inhibited by its suppression. The Pgb transcriptional regulation of the two fermentative enzymes was only observed in the EC sub-region where cells are actively elongating and most likely require ATP to sustain the auxin-induced elongation processes.

Besides providing an unprecedented high resolution overview of domain-specific transcriptional changes occurring in hypoxic corn roots, this study reveals that hypoxic stress triggers unique biological

responses in diverse root compartments (summarized in Fig. 10), which cannot be differentiated by conventional analyses using whole roots. Some of these responses are also modulated by ZmPgb1.2 which contributes to relieve hypoxic stress by inducing the expression of genes with diverse functions, ranging from hormonal signalling and detoxification processes to modified cell division. The expression pattern of these genes further attests that Pgbs operate in a cell and tissue-specific manner and suggests that understanding why Pgbs triggers some responses in some cells but not others is pivotal in revealing mechanisms of stress tolerance.

## Author contribution

MY performed the waterlogging experiments and assisted in the laser-microdissection and RNA library preparation. JM contributed to the tissue processing and the development of the RNA library. MM performed the expression studies by qRT-PCR and the RNA *in situ* hybridization analyses. MB and MB analyzed the RNA library. RH and CS designed the experiment, supervised the work and wrote the manuscript.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.036.

## References

Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome Biol. 11, R106.
Asada, K., Takahashi, M., 1987. Production and scavenging of active oxygen in chloroplasts. In: Kyle, D.J., Osmond, C.B., Arntzen, C.J. (Eds.), Photoinhibition. Elsevier, Amsterdam, pp. 227–287.
Bai, T., Li, C., Li, C., Liang, D., Ma, F., 2013. Contrasting hypoxia tolerance and adaptation in Malus species is linked to differences in stomatal behavior and photosynthesis. Physiol. Plantarum 147, 514–523.
Bailey-Serres, J., Freeling, M., 1990. Hypoxic stress-induced changes in ribosomes of maize seedling roots. Plant Physiol. 94, 1237–1243.
Bailey-Serres, J., Lee, S.C., Brinton, E., 2012. Waterproofing crops: effective flooding survival strategies. Plant Physiol. 160, 1698–1709.
Becker, M.G., Walker, P.L., Pulgar-Vidal, N.C., Belmonte, M.F., 2017a. SeqEnrich: a tool to predict transcription factor networks from co-expressed Arabidopsis and Brassica napus gene sets. PLoS One 12, e0178256.
Becker, M.G., Zhang, X., Walker, P.L., Wan, J.C., Millar, J.L., Khan, D., Granger, M.J., Cavers, J.D., Chan, A.C., Fernando, D.W.G., Belmonte, M.F., 2017b. Transcriptome analysis of the Brassica napus-Leptosphaeria maculans pathosystem identifies receptor, signaling and structural genes underlying plant resistance. Plant J. 90, 573–586.
Belmonte, M.F., Kirkbride, R.C., Stone, S.L., Pelletier, J.M., Bui, A.Q., Yeung, E.C., Hashimoto, M., Fei, J., Harada, C.M., Munoz, M.D., Le, B.H., Drews, G.N., Brady, S.M., Goldberg, R.B., Harada, J.J., 2013. Comprehensive developmental profiles of gene activity in regions and subregions of the Arabidopsis seed. Proc. Natl. Acad. Sci. U. S. A 110, E435–E444.
Bhardwaj, A.R., Joshi, G., Kukreja, B., Malik, V., Arora, P., Pandey, R., Shukla, R.N., Bankar, K.G., Katiyar-Agarwal, S., Goel, S., Jagannath, A., Kumar, A., Agarwal, M., 2015. Global insights into high temperature and drought stress regulated genes by RNA-Seq in economically important oilseed crop Brassica juncea. BMC Plant Biol. 15, 9.
Blokhina, O., Virolainen, E., Fagerstedt, K.V., 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann. Bot. 91, 179–194.
Boyce, M., Yuan, J., 2006. Cellular response to endoplasmic reticulum stress: a matter of life or death. Cell Death Differ. 13, 363–373.
Campbell, M.T., Proctor, C.A., Dou, Y., Schmitz, A.J., Phansak, P., Kruger, G.R., Zhang, C., Walia, H., 2015. Genetic and molecular characterization of submergence response identifies subtol6 as a major submergence tolerance locus in maize. PLoS One 10, e0120385.
Chan, A.C., Khan, D., Girard, I.J., Becker, M.G., Millar, J.L., Sytnik, D., Belmonte, M.F., 2016. Tissue-specific laser microdissection of the Brassica napus funiculus improves gene discovery and spatial identification of biological processes. J. Exp. Bot. 67, 3561–3571.
Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., Scheres, B., 1993. Cellular organisation of the Arabidopsis thaliana root. Development 119, 71–84.
Dordas, C., Hasinoff, B.B., Igamberdiev, A.U., Manac'h, N., Rivoal, J., Hill, R.D., 2003. Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. Plant J. 35, 763–770.
Drew, M.C., 1997. Oxygen deficiency and root metabolism: injury and acclimation under hypoxia. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 223–250.
Drisch, R.C., Stahl, Y., 2015. Function and regulation of transcription factors involved in root apical meristem and stem cell maintenance. Front. Plant Sci. 6, 505.
!Lost DataDuan, Y., Li, B., Wang, Y., Li, K., Sodmergen, Han, C., Zhang, Y., Li, X., 2010. An endoplasmic reticulum response pathway mediates programmed cell death of root tip induced by water stress in Arabidopsis. New Phytol. 186, 681–695.
Elhiti, M., Hebelstrup, K.H., Wang, A., Li, C., Cui, Y., Hill, R.D., Stasolla, C., 2013. Function of the type-2 Arabidopsis hemoglobin in the auxin-mediated formation of embryogenic cells during morphogenesis. Plant J. 74, 946–958.
Gutierrez, C., 2009. The Arabidopsis cell division cycle. Arabidopsis Book 7, e0120.
Hill, R.D., 2012. Non-symbiotic haemoglobins – What's happening beyond nitric oxide scavenging? AoB Plants 2012. https://doi.org/10.1093/aobpla/pls004.
Huang, S., Hill, R.D., Wally, O.S., Dionisio, G., Ayele, B.T., Jami, S.K., Stasolla, C., 2014. Hemoglobin control of cell survival/death decision regulates in vitro plant embryogenesis. Plant Physiol. 165, 810–825.
Hunt, P.W., Klok, E.J., Trevaskis, B., Watts, R.A., Ellis, M.H., Peacock, W.J., Dennis, E.S., 2002. Increased level of hemoglobin 1 enhances survival of hypoxic stress and promotes early growth in Arabidopsis thaliana. Proc. Natl. Acad. Sci. U. S. A. 99, 17197–17202.
Igamberdiev, A.U., Hill, R.D., 2004. Nitrate, NO and haemoglobin in plant adaptation to hypoxia: an alternative to classic fermentation pathways. J. Exp. Bot. 55, 2473–2483.
Ismond, K.P., Dolferus, R., de Pauw, M., Dennis, E.S., Good, A.G., 2003. Enhanced low oxygen survival in Arabidopsis through increased metabolic flux in the fermentative pathway. Plant Physiol. 132, 1292–1302.
Jacobs, M., Dolferus, R., Van den Bossche, D., 1988. Isolation and biochemical analysis of ethyl methanesulfonate-induced alcohol dehydrogenase null mutants of arabidopsis thaliana (L.) Heynh. Biochem. Genet. 26, 105–122.
Jiang, K., Meng, Y.L., Feldman, L.J., 2003. Quiescent center formation in maize roots is associated with an auxin-regulated oxidizing environment. Development 130, 1429–1438.
Jiao, Y., Peluso, P., Shi, J., Liang, T., Stitzer, M.C., Wang, B., Campbell, M.S., Stein, J.C., Wei, X., Chin, C.S., Guill, K., Regulski, M., Kumari, S., Olson, A., Gent, J., Schneider, K.L., Wolfgruber, T.K., May, M.R., Springer, N.M., Antoniou, E., McCombie, W.R., Presting, G.G., McMullen, M., Ross-Ibarra, J., Dawe, R.K., Hastie, A., Rank, D.R., Ware, D., 2017. Improved maize reference genome with single-molecule technologies. Nature 546, 524–527.
Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., Salzberg, S.L., 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36.
Kim, S.Y., Kim, B.H., Lim, C.J., Lim, C.O., Nam, K.H., 2010a. Constitutive activation of stress-inducible genes in a brassinosteroid-insensitive 1 (bri1) mutant results in higher tolerance to cold. Physiol. Plantarum 138, 191–204.
Kim, Y.H., Bae, J.M., Huh, G.H., 2010b. Transcriptional regulation of the cinnamyl alcohol dehydrogenase gene from sweet potato in response to plant developmental stage and environmental stress. Plant Cell Rep. 29, 779–791.
Le Gall, H., Philippe, F., Domon, J.M., Gillet, F., Pelloux, J., Rayon, C., 2015. Cell wall metabolism in response to abiotic stress. Plants 4, 112–166.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.
Matsumura, H., Takano, T., Yoshida, K.T., Takeda, G., 1995. A rice mutant lacking alcohol dehydrogenase. Breed Sci. 45, 365–367.
Mayer, A.M., 2006. Polyphenol oxidases in plants and fungi: going places? Phytochemistry 67, 2318–2331.
Mira, M., Hill, R.D., Stasolla, C., 2016a. Regulation of programmed cell death by phytoglobins. J. Exp. Bot. 67, 5901–5908.
Mira, M.M., El-Khateeb, E.A., SayedAhmed, H.I., Hill, R.D., Stasolla, C., 2017. Are avoidance and acclimation responses during hypoxic stress modulated by distinct cell-specific mechanisms? Plant Signal. Behav. 12, e1273304.
Mira, M.M., Hill, R.D., Stasolla, C., 2016b. Phytoglobins improve hypoxic root growth by alleviating apical meristem cell death. Plant Physiol. 172, 2044–2056.
Mira, M.M., Wally, O.S.D., Elhiti, M., El-Shanshory, A., Reddy, D.S., Hill, R.D., Stasolla, C., 2016c. Jasmonic acid is a downstream component in the modulation of somatic embryogenesis by Arabidopsis Class 2 phytoglobin. J. Exp. Bot. 67, 2231–2246.
Orlando, D.A., Brady, S.M., Koch, J.D., Dinneny, J.R., Benfey, P.N., 2009. Manipulating large-scale Arabidopsis microarray expression data: identifying dominant expression patterns and biological process enrichment. Methods Mol. Biol. 553, 57–77.
Peschke, V.M., Sachs, M.M., 1994. Characterization and expression of transcripts induced by oxygen deprivation in maize (*Zea mays* L.). Plant Physiol. 104, 387–394.
Petricka, J.J., Winter, C.M., Benfey, P.N., 2012. Control of Arabidopsis root development. Annu. Rev. Plant Biol. 63, 563–590.
Rajhi, I., Yamauchi, T., Takahashi, H., Nishiuchi, S., Shiono, K., Watanabe, R., Mliki, A., Nagamura, Y., Tsutsumi, N., Nishizawa, N.K., Nakazono, M., 2011. Identification of genes expressed in maize root cortical cells during lysigenous aerenchyma formation using laser microdissection and microarray analyses. New Phytol. 190, 351–368.
Rao, I.M., Arulanantham, A.R., Terry, N., 1989. Leaf phosphate status, photosynthesis and carbon partitioning in sugar beet. II. Diurnal changes in sugar phosphates, adenylates, and nicotinamide nucleotides. Plant Physiol. 90, 820–826.
Roberts, J.K.M., Callis, J., Jardetsky, O., Walbot, V., Freeling, M., 1984a. Cytoplasmic

acidosis as a determinant of flooding intolerance in plants. Proc. Natl. Acad. Sci. U. S. A. 81, 6029–6033.
Roberts, J.K.M., Callis, J., Jardetsky, O., Walbot, V., Freeling, M., 1984b. Mechanisms of cytoplasmic pH regulation in hypoxic maize root tips and its role in survival under hypoxia. Proc. Natl. Acad. Sci. U. S. A. 81, 3379–3383.
Rojo, E., Titarenko, E., Leon, J., Berger, S., Vancanneyt, G., Sanchez-Serrano, J.J., 1998. Reversible protein phosphorylation regulates jasmonic acid-dependent and -independent wound signal transduction pathways in Arabidopsis thaliana. Plant J. 13, 153–165.
Sanz, L., Fernandez-Marcos, M., Modrego, A., Lewis, D.R., Muday, G.K., Pollmann, S., Duenas, M., Santos-Buelga, C., Lorenzo, O., 2014. Nitric oxide plays a role in stem cell niche homeostasis through its interaction with auxin. Plant Physiol. 166, 1972–1984.
Savatin, D.V., Gramegna, G., Modesti, V., Cervone, F., 2014. Wounding in the plant tissue: the defense of a dangerous passage. Front. Plant Sci. 5, 470.
Silva-Cardenas, R.I., Ricard, B., Saglio, P., Hill, R.D., 2003. Hemoglobin and hypoxic acclimation in maize root tips. Russ. J. Plant Physiol. 50, 821–826.
Smirnoff, N., Pallanca, J.E., 1996. Ascorbate metabolism in relation to oxidative stress. Biochem. Soc. Trans. 24, 472–478.
Stasolla, C., Hill, R.D., 2017. Determining cellular responses: phytoglobins may direct the traffic. Trends Plant Sci. 22, 820–822.
Torres, M.A., Dangl, J.L., 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr. Opin. Plant Biol. 8, 397–403.
Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., Pachter, L., 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7, 562–578.
Vartapetian, B.B., Jackson, M.B., 1997. Plant adaptations to anaerobic stress. Ann. Bot. 79 (Suppl. ment), 3–20.
Voesenek, L.A., Bailey-Serres, J., 2013. Flooding tolerance: O2 sensing and survival strategies. Curr. Opin. Plant Biol. 16, 647–653.
Wany, A., Kumari, A., Gupta, K.J., 2017. Nitric oxide is essential for the development of aerenchyma in wheat roots under hypoxic stress. Plant Cell Environ. 40, 3002–3017.
Wasternack, C., Hause, B., 2013. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann. Bot. 111, 1021–1058.
Watanabe, N., Lam, E., 2008. BAX inhibitor-1 modulates endoplasmic reticulum stress-mediated programmed cell death in Arabidopsis. J. Biol. Chem. 283, 3200–3210.
Yamauchi, T., Rajhi, I., Nakazono, M., 2011. Lysigenous aerenchyma formation in maize root is confined to cortical cells by regulation of genes related to generation and scavenging of reactive oxygen species. Plant Signal. Behav. 6, 759–761.
Yang, Y.X., Ahammed, G.J., Wu, C., Fan, S.Y., Zhou, Y.H., 2015. Crosstalk among jasmonate, salicylate and ethylene signaling pathways in plant disease and immune responses. Curr. Protein Pept. Sci. 16, 450–461.
Yoruk, R., Marshall, M.R., 2007. Physicochemical properties and function of plant phenol oxidase: a review. J. Food Biochem. 27, 361–422.
Youssef, M.S., Mira, M.M., Renault, S., Hill, R.D., Stasolla, C., 2016. Phytoglobin expression influences soil flooding response of corn plants. Ann. Bot. 118, 919–931.
Yuan, L.B., Dai, Y.S., Xie, L.J., Yu, L.J., Zhou, Y., Lai, Y.X., Yang, Y.C., Xu, L., Chen, Q.F., Xiao, S., 2017. Jasmonate regulates plant responses to postsubmergence reoxygenation through transcriptional activation of antioxidant synthesis. Plant Physiol. 173, 1864–1880.
Zhao, L., Gu, R.L., Gao, P., Wang, G.Y., 2008. A nonsymbiotic hemoglobin gene from maize, ZmHb, is involved in response to submergence, high-salt and osmotic stresses. Plant Cell Tissue Organ Cult. 95, 227–237.