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Osmopriming-associated genes in *Poincianella pyramidalis*

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ABSTRACT

Osmopriming of seeds can increase seed vigour, allowing faster germination and field emergence, especially under adverse field conditions. The restricted imbibition during priming treatments reactivates metabolism although germination will be prevented. In order to identify and characterize genes that are involved in the improvement of vigour of *Poincianella pyramidalis* (Catingueira) seeds upon priming, we produced two different Suppression Subtractive Hybridization (SSH) cDNA libraries. These were a Forward and Reverse subtraction of cDNA samples isolated from untreated dry seeds (unprimed) and osmoprimed seeds. The two different libraries were sequenced by Illumina GAII next generation sequencing, resulting in almost 20 million reads that could be assigned to 5298 different contigs. Of these, 999 were only found in the unprimed seeds library and 2711 were specific for osmoprimed seeds. The contigs were annotated and subjected to gene set enrichment analysis and differential expression of several genes was confirmed by qRT-PCR analysis. The identified differentially expressed genes might play an important role in vigour improvement upon priming and may be potential markers for tolerance to water stress in *P. pyramidalis* seeds.

1. Introduction

Priming is a commercially used technique to improve seed germinability and seedling emergence. The most common priming techniques involve imbibition of seeds in water (hydropriming) or in osmoticum, e.g. polyethylene glycol - PEG (osmopriming), under controlled conditions which allow the reactivation of metabolism and initiation of early events of germination but preventing radicle protrusion, i.e. germination *per se* (de Castro et al., 1995, 2000; Jing et al., 1999; Ligterink et al., 2007). After priming seeds are dried back to their initial moisture content, but with faster and more synchronized germination upon sowing and re-imbibition towards improved cross-tolerance to abiotic stresses and crop establishment (Chen and Arora, 2013; Dawood, 2018; Heydecker and Gibbins, 1978; Lamichhane et al., 2018; Munné-bosch and Alegre, 2013; Wojtyla et al., 2016). Better germination performance after priming has been reported in many species, e.g. as soybean (*Glycine max* L. Merrill), tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus* L.) and pepper (*Capsicum annuum* L.), among others (de Souza et al., 2016; Jing et al., 1999; Korkmaz and Korkmaz, 2009; Ligterink et al., 2007; Zhuo et al., 2009).

Incubation of seeds in PEG solution results in reduction of water availability and using a low PEG concentration hampers seed germination (Cortez-Baheza et al., 2007). Genes related to osmopriming therefore might be the same as those involved in osmotic stress responses in general (Ligterink et al., 2007). Osmotic stress response genes code for proteins that protect plant cells against several abiotic stresses such as Heat Shock proteins (HSPs), Late Embryogenesis Abundant (LEA) proteins, osmoprotectants, antifreeze proteins, detoxification enzymes, free-radical scavengers, aquaporins and ion transporters (Blumwald, 2000; Bray et al., 2015; Ligterink et al., 2007). They may also encode proteins involved in signaling cascades and subsequent transcriptional control to regulate target genes, such as Mitogen-Activated Protein Kinases (MAPKs), Calcium-Dependent Protein Kinases (CDPKs), Salt Overly Sensitive (SOS) Independent Protein Kinases, phospholipases

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and multiple transcription factors (Ludwig et al., 2004; Rudrabhatla and Rajasekharan, 2002; Shinozaki and Yamaguchi-Shinozaki, 2000; Shinozaki et al., 2003; Zhu, 2001).

Several studies have reported on attempts to elucidate gene expression related to seed priming treatments, such as the increase in expression of genes coding for S-adenosyl-met synthetase, sugar epimerase, dioxygenase, glutathione transferase, stress related proteins and L-ascorbate peroxidase during osmopriming and germination of *Brassica oleraceae* seeds (Soeda et al., 2005); increased expression of transcripts related to LEA proteins, HSP proteins, proteinase inhibitors, replication and signal transduction in osmoprimed *C. annuum* seeds (Cortez-Baheza et al., 2007); whereas priming suppressed *LsNCED4* expression and enhanced expression of *LsGA3ox1*and *LsACS1* genes and alleviated thermoinhibition in *Lactuca sativa* seeds (Schwember and Bradford, 2010).

Alterations in the physiological state at the transcriptional level in plants submitted to water restriction stresses have been effectively analyzed by means of the Suppressive Subtractive Hybridization (SSH) approach, i.e. by using two populations of mRNA to identify genes that are expressed in one population (tester) but not in the other (driver) (Diatchenko et al., 1996; Lukyanov et al., 2007). SSH has been successfully used to identify genes related to dehydration stress in *Setaria italica* (Zhang et al., 2007), *G. max* (Rodrigues et al., 2012), *Hylocereus undatus*, *Vicia faba* and *Lolium multiflorum* (Abid et al., 2015; Fan et al., 2014; Pan et al., 2016; Rodrigues et al., 2012; Zhang et al., 2007) and also genes responsive to the stress related technique of osmopriming of *C. annuum* seeds (Cortez-Baheza et al., 2008, 2007). Compared to other techniques used in gene expression profiling, SSH needs relatively smaller amounts of the initial materials, with lower costs, and fewer false positives present within the results, whereas remarkable advancement has been achieved in the next-generation sequencing of transcripts through RNA-Seq (Podnar et al., 2014; Sahebi et al., 2015). Therefore, we describe in this manuscript the use of SSH combined with RNA-seq to identify genes that are correlated with the vigour improvement of osmoprimed *Poincianella pyramidalis* (Tul.) L. P. Queiroz seeds. *P. pyramidalis,* which has been recently renamed from *Caesalpinia pyramidalis* Tul. due to a taxonomic update (Gagnon et al., 2016), is a tree species popularly known as “Catingueira” that is endemic to the “Caatinga” biome that characterizes the northeastern semiarid region of Brazil and has a high plasticity to the harsh environmental conditions of this region. It is broadly used as folk medicine due to the presence of bioactive flavonoids and bioflavonoids in its leaves and bark (de Sousa et al., 2020; Falcão et al., 2015; Oliveira et al., 2016; Pereira Gomes--Copeland et al., 2018). The present study aimed at the elucidation of the molecular processes involved in the effects of priming on seed vigour and identification of molecular markers that are specific for stress resistance in *P. pyramidalis*.

2. Material and methods

2.1. Seed collection

The seeds were harvested from *P. pyramidalis* trees located at Petrolina county within the semi-arid region of Brazil (coordinates: 9° 30′ 21′’ S 40° 30′ 21′’W) in September 2008. Harvested seeds were dried under shade at room temperature for 3 days. Dried seeds presented 5.38 % moisture content (fresh weight basis) prior to further analysis.

2.2. Osmopriming

Seeds of *P. pyramidalis* with a moisture content of 5.38 % were osmoprimed in −1.2 MPa polyethylene glycol 8000 (PEG 8000 Sigma Aldrich, USA) for 7 days in a germination chamber at 25 °C and constant 24 h light (35 W m^{-2}). A roller system kept samples under agitation providing proper aeration. The osmotic potential was chosen based on a previous osmotic stress assay (Antunes et al., 2011). After incubation, the seeds were removed from the PEG solution, rinsed twice with distilled water and subsequently dried back to its original moisture content in an incubator at 20 °C and 30 % RH for 4 days (Nascimento et al., 2001; Powell et al., 2000). Seeds were germinated in paper rolls for 6 days at 25 °C and 24 h light. Germinability was tested by daily evaluations and determination of maximum germination percentage (G_{max}), germination speed as the mean time for 50 % germination (t_{50}) and germination uniformity (U_{84-16}) as the mean time between 16 and 84 % germination using the GERMINATOR software (Joosen et al., 2010).

2.3. SSH

SV Total RNA Isolation System Kit (Promega, Madison, USA) was used to extract total RNA from unprimed and osmoprimed seeds according to the manufacturer’s protocol. The Super Smart TMPCR cDNA Synthesis Kit (Clontech Laboratories, Inc., Canada) was used to make cDNA of those samples according to the manufacturer’s protocol. Two subtraction libraries were generated making use of PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories, Inc., Canada). In the forward library, cDNA from osmoprimed seeds was used as “tester” and cDNA from unprimed seeds as “driver” and vice versa for the reverse library.

2.4. Sequencing (RNA-seq) and Bioinformatics Analysis

Sequencing of plasmids from both libraries was performed by an Illumina GAII next generation sequencing platform according to manufacturer’s instructions. The reads were trimmed with a quality score limit of 0.01 (>Q20) and a minimum read length of 30 bp. The reads of the two libraries were assembled into contigs using the De Novo Assembler Velvet v1.07 package. Assembled contigs were blasted against the Genbank database (www.ncbi.nlm.nih.gov/genbank) using Blast X for annotation and homology search. The two highest scoring hits were kept for annotation. The reads of the individual samples were aligned onto the assembled contigs using the Tophat v.1.0.13 package (Trapnell et al., 2009). The corresponding expression values of contigs were expressed in FKPM (Fragments per Kilobase of Exon per Million fragments mapped) using Cufflinks v0.8.2 (Trapnell et al., 2011). The annotated differentially expressed sequences of each library were categorized based on Gene Ontology (GO- http://www.geneontology.org/).

2.5. Gene expression assay by quantitative reverse transcription PCR (qRT-PCR)

To validate SSH results, the expression of eight genes related to osmoprimed and unprimed libraries (see Supplemental Table S1) was evaluated by qRT-PCR. Total RNA was isolated from unprimed and osmoprimed seeds using Concert Plant RNA Reagent according to manufacturer’s instructions (Invitrogen, Carlsbad, USA). RNA samples were further purified by phenol-chlorophorm extraction followed by ethanol precipitation. A DNAse treatment was performed with help of RQ1 DNAse (Promega, Madison, USA) according to manufacturer’s instructions followed by phenol-chloroform extraction and ethanol precipitation. cDNA was synthesized from 2 µg total RNA using ImProm-II™ Reverse Transcription System Kit (Promega, Madison, USA) according to manufacturer’s instructions. Primers for qRT-PCR were designed with help of the Integrated DNA Technology website (http://www.idtdna.com/scitools/Applications/RealTimePCR). PCR reactions were composed of 10 µl of 2X SYBR® Green PCR Master Mix (Applied Biosystems, California, USA), 5 µl of cDNA, 0.5 µl of each primer (10 µM) and 4 µl of Milli-Q® (MQ) ultrapure water to a final volume of 20 µl. For the negative control, MQ water was used instead of cDNA. The reactions were performed in an Applied 7500 Fast Real Time PCR System (Applied Biosystems, California, USA). PCR conditions started with an initial step at 95 °C for 3 min, followed by 40 cycles with 15 s at 95 °C and 1 min at 60 °C. A melting curve analysis was performed to verify specificity of

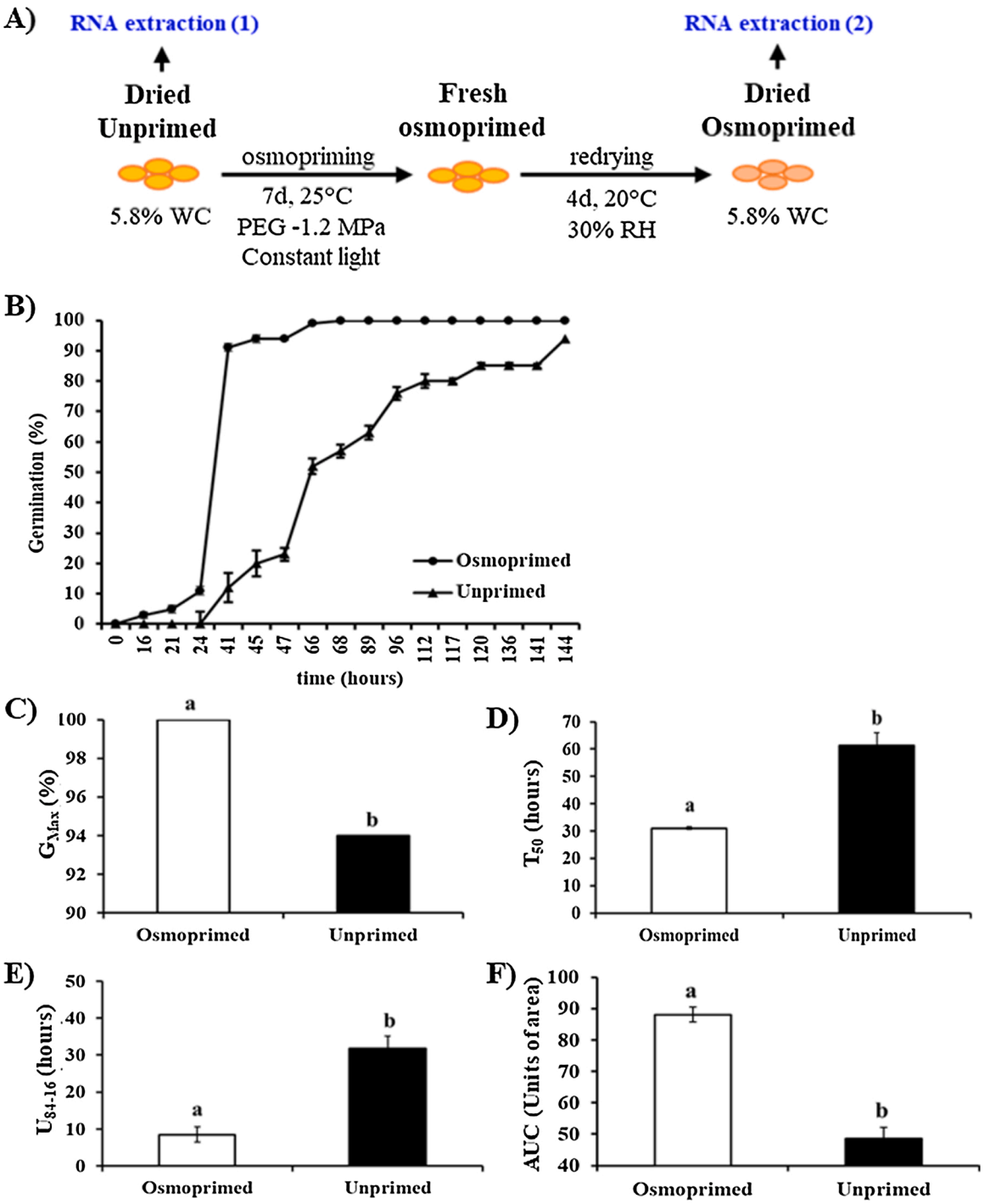

Fig. 1. Osmopriming treatment and germinability parameters of unprimed and osmoprimed *P. pyramidalis* seeds. (A) Schematic representation of the osmopriming procedure and samplings for RNA extraction; (B) Germination curves; (C) Final maximal germination (G_{max} %); (D) Germination rate (T_{50}, hours); (E) Germination uniformity ($U_{84\text{-}16}$, hours) and (F) Area under curve (AUC, units of area). Bars indicate standard error and different letters above the bars denote significant differences by Mann-Whitney test ($p < 0.05$).

amplification. Primer efficiency was tested using a standard curve for each gene, based on serial dilutions (1, 2, 4, 8 and 16x) of the cDNA. Based on homology to reference genes described for analysis of gene expression in *Arabidopsis thaliana* and *S. lycopersicum* seeds (Dekkers et al., 2012), we tested two genes (NODE 580 and NODE 45,602) (Supplemental Table S1) without differential expression in both libraries, to normalize the expression of the target genes. The stability of both genes was evaluated by geNorm ($M \leq 0.5$) (Hellemans et al., 2007). Relative expression of each target gene was calculated by the Pfaffl method (Pfaffl, 2001) with unprimed seeds as calibrator sample.

2.6. Experimental design and statistical analysis

Germination assays were conducted with 4 biological replicates of 25 seeds per treatment. The germination data did not present normal distribution by Shapiro Wilk test and therefore averages were compared by Mann Whitney test ($p < 0.05$). qRT-PCR assays were performed with 3 biological replicates of 10 seeds per replicate and 2 technical replicates.

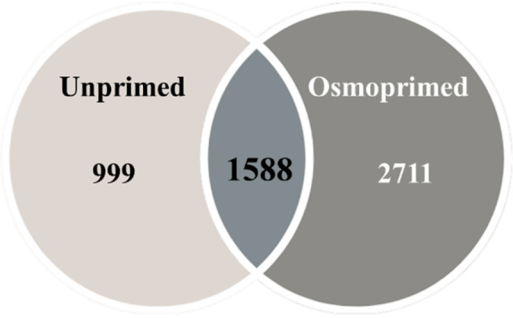

Fig. 2. Contigs number found in the unprimed and osmoprimed RNAseq libraries of *P. pyramidalis* seeds.

Relative expression values of each treatment were transformed in $\log_2$ and showed a normal distribution and were therefore compared by Student's *t* test ($p < 0.05$) with help of SISVAR software (Ferreira, 2011).

3. Results

3.1. Osmopriming improves P. pyramidalis seed vigour

The osmopriming treatment was done by incubating the *P. pyramidalis seeds* for 7 days in PEG solution (-1.2 MPa) under constant light and agitation, followed by re-drying the seeds to their original moisture content for 4 days at 20 °C and 30 % RH (Fig. 1A). The germination curve comparing unprimed and osmoprimed seeds showed the improvement of germinability parameters in favor of the osmoprimed seeds (Fig. 1B). Final maximal germination (G_{max}) (Fig. 1C) was significantly higher in osmoprimed seeds (100 %) as compared to unprimed seeds (94 %). Priming also had a significant positive effect ($p < 0.05$) on rate (T_{50}) and uniformity (U_{84-16}) of germination (Fig. 1D and E). The improvement in germinability parameters is also evident in the area under curve index (AUC) where the osmoprimed seeds had higher value than unprimed seeds (Fig. 1F).

3.2. Osmopriming represses expression of genes related to water deficit conditions

SSH was employed to produce subtracted cDNA libraries to identify genes differentially expressed in response to osmopriming. A set of forward and reverse subtracted cDNAs of high quality were obtained after 2 rounds of hybridization and amplification. Size distribution in subtracted samples was drastically reduced, indicating a good signal of efficient subtraction (data not shown).

Sequencing of the RNA libraries (RNASeq) resulted in almost 20 million reads of 75 bp that could be assembled into 5298 contigs. The average length of the contigs was 355 bp and maximal length was 1699 bp. Among the 5298 contigs, 999 were only found in the unprimed library and 2711 were specific for the osmoprimed library, while 1588 contigs were found in both libraries (Fig. 2).

The 30 most abundant genes in the osmoprimed library are presented in Table 1. The contigs NODE_44512, NODE_44444 and NODE_45893 didn't show homology to known genes, whereas other contigs had highest homology to genes in *G. max* (9 genes), *Lotus corniculatus* var. *japonicus* (3 genes), *Atriplex numulária* (1 gene), *Medicago sativa* (2 genes), *Medicago truncatula* (2 gene), *A. thaliana* (2 gene), *Oryza sativa* Japonica (2 genes), *Litchi chinensis* (1 gene), *Streptomyces* sp. C (1 gene), *Nicotiana benthamiana* (1 gene), *Mangifera indica* (1 gene), *Arachis hypogaea* (1 gene), *Brachypodium distachyon* (1 gene) and *Cicer arietinum* (1 gene).

Among them, eight contigs were homologous to uncharacterized or unknown genes (NODE_46535, NODE_46655, NODE_1217, NODE_44950, NODE_40249, NODE_46165, NODE_44008 and NODE_45002). Seven contigs were homologous to hypoxia response genes (NODE_44560, NODE_46673, NODE_44497, NODE_46464, NODE_45062, NODE_45451 and NODE_43241). Five contigs were homologous to gluconeogenesis or glycolytic process genes (NODE_46047, NODE_46498, NODE_45791, NODE_43241 and NODE_43340). Five contigs were homologous to oxidation-reduction processes genes

Table 1
The 30 most abundant genes specific for the osmoprimed library. The genes are ranked according to FPKM.

Contig	Accession	Description (BLASTX)	E-value	FPKM
NODE_46224	gi|356,549,751	PREDICTED: stem-specific protein TSJT1-like isoform 1 [*Glycine max*]	4,83E-17	20,510
NODE_44560	emb|AJ717414.1	mRNA for alcohol dehydrogenase [*Lotus corniculatus* var. *japonicus*]	1E-59	12,261
NODE_43340	gi|145,617,261	glyceraldehyde-3-phosphate dehydrogenase [*Medicago sativa*]	1,88E-33	10,813
NODE_46047	gb|U02886.1	glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA [*Atriplex numulária*]	8E-44	10,231
NODE_44512	Unknown	Unknown	Unknown	7791
NODE_46673	gi|18,397,956	maternal effect embryo arrest 14 protein [*Arabidopsis thaliana*]	4,07E-10	7319
NODE_44497	gb|BT092730.1	Hypoxia induced protein conserved region containing protein expressed [*Oryza sativa Japonica*]	2,90E-07	6932
NODE_46535	gi|351,728,107	uncharacterized protein [*Glycine max*]	9,60E-11	6623
NODE_44444	Unknown	Unknown	Unknown	6423
NODE_46655	dbj|AK245638.1	cDNA [*Glycine max*]	2E-19	6335
NODE_45006	gb|EU661924.1	NAC domain protein (NAC27) mRNA [*Glycine max*]	4E-13	5199
NODE_1217	gi|302,539,119	predicted protein [*Streptomyces sp. C*]	2,57E-06	4896
NODE_46464	gi|77,548,353	Hypoxia induced protein conserved region containing protein expressed [*Oryza sativa Japonica*]	8,60E-05	4656
NODE_44547	gi|116,871,386	Xyloglucan endotransglycosylase 3 [*Litchi chinensis*]	8,44E-15	4595
NODE_31770	IPR005050	Early nodulin 93 ENOD93 protein [*Arabidopsis thaliana*]	4,30E-21	4557
NODE_45062	gi|356,572,510	PREDICTED: pyruvate kinase, cytosolic isozyme-like [*Glycine max*]	9,87E-19	4534
NODE_46498	gi|380,505,034	glyceraldehyde-3-phosphate dehydrogenase, partial [*Nicotiana benthamiana*]	8,14E-20	4347
NODE_43966	gi|309,260,073	p-hydroxyphenylpyruvatedioxygenase [*Mangifera indica*]	6,43E-56	4329
NODE_44950	gb|BT092177.1	unknown mRNA [*Glycine max*]	8E-08	4292
NODE_45791	gi|356,505,332	PREDICTED: fructose-bisphosphatealdolase, cytoplasmic isozyme [*Glycine max*]	1,68E-22	4206
NODE_40249	dbj|AP010367.1	genomic DNA, chromosome 3 [*Lotus japonicus*]	0,000,007	4205
NODE_44626	gb|DQ889514.1	*Arachis hypogaea* double-stranded DNA-binding protein mRNA [*Arachis hypogaea*]	1E-127	4135
NODE_45451	gi|357,506,055	Pyruvate decarboxylase isozyme [*Medicago truncatula*]	1,31E-20	4091
NODE_45893	Unknown	Unknown	Unknown	3860
NODE_43241	gi|357,160,930	PREDICTED: pyruvate kinase, cytosolic isozyme-like [*Brachypodium distachyon*]	3,10E-41	3752
NODE_44164	gi|357,452,667	Thioredoxin-like protein [*Medicago truncatula*]	7,50E-55	3679
NODE_46165	gb|BT092470.1	unknown mRNA [*Glycine max*]	00,002	3386
NODE_44008	gb|BT093226.1	unknown mRNA [*Glycine max*]	0,000,001	3369
NODE_45002	dbj|AP010309.1	genomic DNA, chromosome 5 [*Lotus japonicus*]	0,000,001	3365
NODE_45595	emb|X79706.1	*capR1*, pathogenesis-related protein [*Cicer arietinum*]	000,001	3249

Table 2
The 30 most abundant genes specific for the unprimed library. The genes are ranked according to FPKM.

Contig	Accession	Description (BLASTX)	E-value	FPKM
NODE_3346	Unknown	Unknown	unknown	6003
NODE_4050	GQ997462.1	putative RF3 protein (ycf3) gene, chloroplast [*Lonicera japônica*]	1,00E-53	5919
NODE_33184	Unknown	Unknown	unknown	5181
NODE_590	gb|EU717241.1|	caseinolytic ATP-dependent protease (clpP) gene, chloroplast [*Lespedeza cuneata*]	4E-47	5130
NODE_4460	Unknown	Unknown	unknown	4639
NODE_2798	AAY34438.1	Kunitz-type chymotrypsin inhibitor [*Psophocarpus tetragonolobus*]	1,00E-12	4535
NODE_840	ref| XM_002517608.1|	processing-splicing factor, putative, mRNA. [*Ricinus communis*]	3E-60	4483
NODE_3807	gb|GQ998095.1|	cytochrome b6 protein (petB) gene, chloroplast. [*Cornus florida*]	2E-49	4393
NODE_753	gi|115,187,427|	Tonoplast intrinsic protein alpha TIP [*Arachis hypogaea*]	1,86E-46	4385
NODE_13392	Unknown	Unknown	unknown	4217
NODE_4048	gi|351,720,672|	Trypsin inhibitor p20 precursor [*Glycine max*]	2,70E-10	4125
NODE_1471	gb|DQ296034.1|	desiccation protectant protein LEA 14 mRNA [*Arachis hypogaea*]	1E-23	4036
NODE_11565	Unknown	Unknown	unknown	3997
NODE_665	gb|EU811880.1|	AtpB (atpB) gene and atpB-rbcL intergenic spacer; chloroplast [*Vachellia bravoensis*]	1E-113	3966
NODE_3209	Unknown	Unknown	unknown	3907
NODE_3870	gb|AF117723.1| AF117723	seed maturation protein PM27 (PM27) mRNA [*Glycine max*]	1E-35	3840
NODE_2365	AAC61881.1	11S storage globulin [*Coffea arabica*]	3,00E-16	3793
NODE_3529	gb|GQ435770.1|	Ycf5gene, chloroplast [*Caesalpinia sappan*]	1E-120	3742
NODE_8667	AEB33716.1	Conglutin beta 5 [*Lupinus angustifolius*]	2e-18	3707
NODE_2157	ref| XP_003610290.1|	Eukaryotic initiation factor iso-4 F subunit p82−34 [*Medicago truncatula*]	1e-57	3662
NODE_9371	CU223764.1	EST from leave [*Populus*]	2,00E-21	3640
NODE_2450	ref| XP_002269959.2|	PREDICTED: peptidylprolyl isomerase domain and WD repeat-containing protein 1 [*Vitis vinifera*]	7,00E-51	3625
NODE_752	gb|EU428748.1|	phytochrome A-3 (phyA) mRNA [*Glycine max*]	3E-61	3604
NODE_28630	emb|X58710.1|	mRNA for heat shock protein [*M. sativa*]	3E-60	3413
NODE_920	gb|EU436322.1|	Cogniauxia trilobata rpl20-rps12 intergenic spacer, chloroplast. [*Cogniauxia trilobata*]	5E-53	3399
NODE_3732	dbj|AB049336.1|	mRNA for dehydrin [*Nicotiana tabacum*]	2E-14	3336
NODE_5029	unknown	Unknown	unknown	3326
NODE_313	gi|356,562,555|	PREDICTED: eukaryotic initiation factor iso-4 F subunit p82−34-like isoform 1 [*Glycine max*]	6,39E-25	3185
NODE_1241	gi|357,513,109|	Pre-mRNA splicing factor [*Medicago truncatula*]	9,26E-20	3163
NODE_28884	emb|CU223764.1|	EST from leave. [*Populus*]	3E-34	3127

(NODE_46047, NODE_46498, NODE_43966, NODE_44164 and NODE_43340). One contig was homologous to xyloglucan metabolic process gene, positive regulation of transcription and aromatic amino acid family metabolic process gene, respectively (NODE_44547, NODE_45006 and NODE_43966). Three different glyceraldehyde-3-phosphate dehydrogenase genes were within the 30 most abundant genes (NODE_46047, NODE_43340 and NODE_46498) and two pyruvate kinase genes (NODE_45062 and NODE_43241) (Table 1).

The 30 most abundant genes in the unprimed library which apparently are strongly downregulated by osmopriming are presented in Table 2. The contigs NODE_3346, NODE_33184, NODE_4460, NODE_13392, NODE_11565, NODE_3209 and NODE_5029 didn't show homology to known genes, whereas other contigs showed highest homology to genes in *Lonicera japonica* (1 gene), *Lespedeza cuneate* (1 gene), *Psophocarpus tetragonolobus* (1 gene), *Ricinus communis* (1 gene), *Cornus florida* (1 gene), *A. hypogaea* (2 genes), *G. max* (4 genes), *Coffea arabica* (1 gene), *Caesalpinia sappan (*1 gene), *Lupinus angustifolius* (1 gene), *M. truncatula* (2 genes), *Populus* (2 genes), *Vitis vinifera* (1 gene), *M. sativa* (1 gene), *Cogniauxia trilobata* (1 gene) and *Nicotiana tabacum* (1 gene).

Among them, 8 contigs were homologous to uncharacterized or unknown genes (NODE_3346, NODE_33184, NODE_4460, NODE_13392, NODE_11565, NODE_3209 and NODE_5029). Other contigs were homologous to genes associated to seed storage (NODE_2365 and NODE_8667), seed maturation (NODE_3870), response to desiccation (NODE_1471 and NODE_3732), chaperone function (NODE_28630), photosystem (NODE_4050, NODE_590 and NODE_3807), detection of visible light (NODE_752), transmembrane transport (NODE_753), negative regulation of peptidase activity (NODE_4048), splicing (NODE_840 and NODE_1241), ATP synthesis coupled proton transport (NODE_665), heme transport (NODE_3529) and protein biosynthesis (NODE_2157 and NODE_313). Two contigs were homologous to Populus ESTs from leave (NODE_9371 and NODE_28884). Many of the contigs are related to cellular viability maintenance in the dry stage.

3.3. Gene ontology analysis of priming related P. pyramidalis genes

All contigs found in the the unprimed and osmoprimed seed libraries were categorized by Gene Ontology in the 3 different functional groups: Biological Process, Cellular Component and Molecular Function.

For Biological Process, there were twelve Gene Ontology categories with more than 1% of the contigs for at least one library. The osmoprimed seed library showed higher percentages for all these twelve categories compared to the unprimed seed library. The most contigs were found in immune system process (8 and 19 % in unprimed seed and

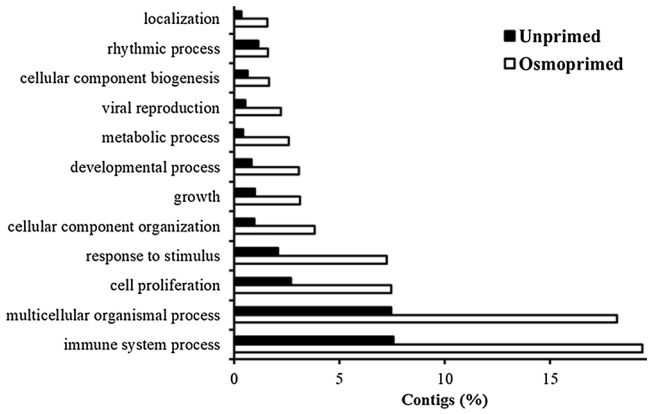

Fig. 3. Division of contigs from unprimed and osmoprimed seed libraries in different Gene Ontology (GO) categories for Biological Processes.

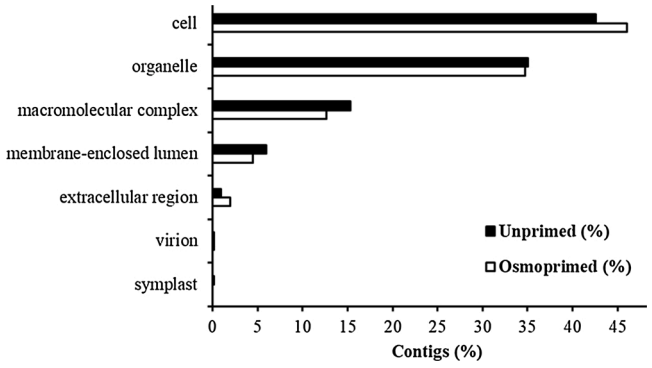

Fig. 4. Division of contigs from unprimed and osmoprimed seeds libraries in different Gene Ontology (GO) groups for Cellular Components.

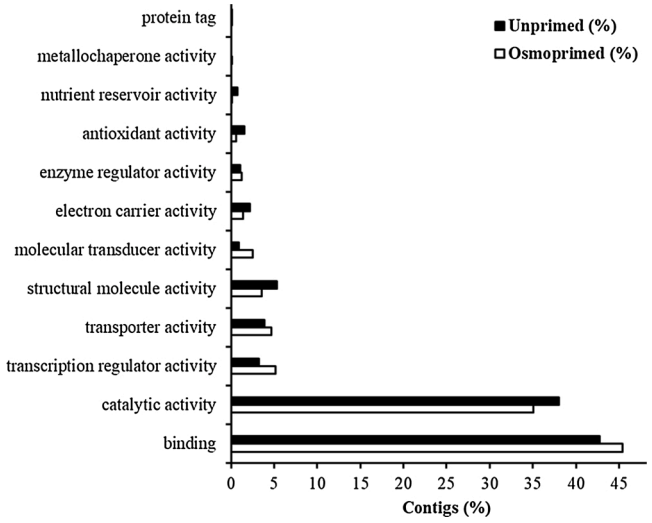

Fig. 5. Division of contigs from unprimed and osmoprimed seeds libraries in different Gene Ontology (GO) groups for Molecular Functions.

osmoprimed seed library respectively), multicellular organismal process (7 and 18 %), cell proliferation (3 and 7%) and response to stimulus (2 and 7%) (Fig. 3).

Among contigs related to Cellular Component found in the osmoprimed seed library, 46 % were grouped in cells, 35 % in organelles and 13 % in macromolecular complex. In the unprimed seeds library 43, 35 and 15 % were observed for the same categories, respectively. Contigs distributed in the symplast group, were only found in the unprimed seeds library (Fig. 4).

Contigs with Molecular function observed in the osmoprimed seed library were grouped in binding function (45 %), catalytic activity (35 %) and transcription regulator activity (5%). Percentages of the same contigs found in the unprimed seed library, were 43, 38 and 3%, respectively (Fig. 5).

3.4. qRT-PCR confirmed expression pattern of genes revealed by RNAseq

To validate expression data revealed by RNA-seq, 8 genes related to water stress (and consequently with osmopriming) with recognized differential abundance in a specific library were selected for confirmation of the results. Their relative expression in osmoprimed seeds was compared with that in unprimed seeds by qRT-PCR analysis (Fig. 6).

Seed maturation protein (NODE 1142) (FPKM unprimed/osmprimed = 3936/3); Metallothionein-II protein (NODE 1074) (FPKM unprimed/osmoprimed = 1102/11) and Ca^{+2}-binding EF hand protein (NODE 86) (FPKM unprimed/osmoprimed = 2369/907) were found with higher abundance in the unprimed seed library according to the RNA-seq data. These differences could not be confirmed by qRT-PCR analysis (Fig. 6A, C and D). Putative Oxidoreductase (NODE 46,022) (FPKM unprimed/osmoprimed = 36/384) and Glutathione S-Transferase Tau 25 (NODE 37,111) (FPKM unprimed/osmoprimed = 17/1918) presented higher abundance in the osmoprimed seed library. Also, these differences could not be confirmed by qRT-PCR analysis (Fig. 6B and E). Auxin Induced Glutathione S Transferase mRNA (FPKM unprimed/osmoprimed = 915/2843) presented higher abundance in the osmoprimed seed library but in the qRT-PCR assay show the opposite results showing higher expression in unprimed seeds (Fig. 6F).

Tonoplast intrinsic protein alpha (TIP, NODE 753) (FPKM unprimed/osmoprimed = 4385/0) and Seed maturation protein (NODE 3870) (FPKM unprimed/osmoprimed = 3840/0) contigs were exclusively found in the unprimed seed library (subtraction effect), and qRT-PCR analysis confirmed higher expression of these genes in unprimed seeds (Fig. 6G and H).

4. Discussion

4.1. How osmopriming improves P. pyramidalis seed vigour?

The effect of osmopriming on germination advancement is thought to be based on metabolic activities before radicle emergence, such as: DNA repair, DNA replication and mobilization of storage proteins (Chan et al., 2010; Heydecker and Coolbear, 1977; Powell et al., 2000). The pattern of water uptake during priming (slow and controlled) is also thought to be crucial to improve final germination (Varier et al., 2010). Osmopriming has been reported to improve the germination of *Cassia excelsa* seeds under stress conditions (Jeller and Perez, 2003). Better germination was also observed in *Stryphnodendron adstringens* and *Stryphnodendron polyphyllum* seeds after osmopriming in −0.5 MPa (Kissmann et al., 2010).

Improvement in rate and uniformity of seed germination by osmopriming is attributed to the additional repair time for the seeds in which they can recover the capacity to germinate under standard conditions and survive a further period of storage (Butler et al., 2009). The inert condition of PEG solutions effectively regulates entry of water into the seed (without causing injury) allowing a controlled reactivation, advancement and synchronization of germination reactions to a more or less fixed level determined by the water potential (-1.2 MPa in our case), therefore, promoting synchronization and better germination rate and uniformity, i.e. resulting in better seedling stand establishment within a population (Chen et al., 2013; Ligterink et al., 2007; Lopes et al., 2018; Parera and Cantliffe, 1994; Samfield et al., 1991).

Similarly, osmopriming showed to be an effective method for improving the germinability (performance) of *P. pyramidalis* seeds as it

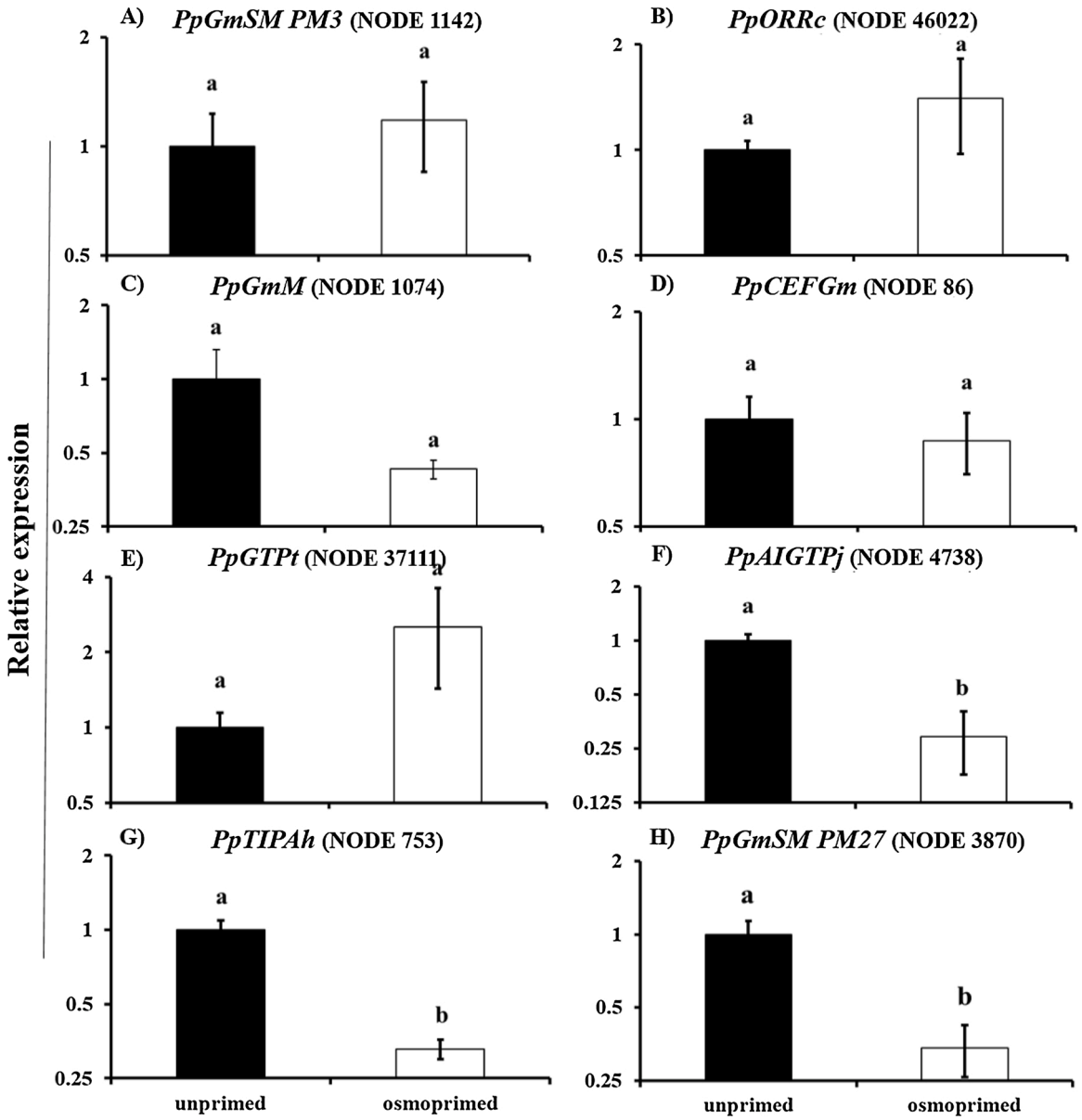

Fig. 6. Relative expression of different transcripts in unprimed and osmoprimed seeds determined by qRT-PCR. (A) Seed maturation protein (*PpGmSM PM3*; NODE 1142); (B) Putative oxidoreductase (*PpORRc*; NODE 46,022); (C) Metallothionein-II protein (*PpGmM*; NODE 1074); (D) Ca^{+2}-binding EF hand protein (*PpCEFGm*; NODE 86); (E) Glutathione S-Transferase Tau 25 (*PpGTPt*; NODE 37,111); (F) Auxin Induced Glutathione S Transferase (*PpAIGTPj*; NODE 4738); (G) Tonoplast intrinsic protein alpha TIP (*PpTIPAh*; NODE 753); (H) Seed maturation protein (*PpGmSM PM27*; NODE 3870). Bars indicate standard error and bars with different letters denote significant differences by Student's *t*-test ($p < 0.05$).

resulted in higher germination percentage, rate and uniformity.

4.2. Osmopriming induced genes control vigour improvement in *P. pyramidalis* seeds

Seed priming is a pre-sowing partial hydration process that often induces cross-tolerance to abiotic stresses and, therefore, improves crop performance (Chen et al., 201b; Lamichhane et al., 2018; Ligterink et al., 2007; Munné-bosch and Alegre, 2013). Once an osmotic agent, e.g. such as PEG, is used to control water uptake, seeds experience a water deficit situation which will alter gene expression leading to activation of germination related genes and also to specific induction of stress related genes (Ligterink et al., 2007; Romo et al., 2001). Some authors have successfully identified differentially expressed genes in different plant species under water stress or osmopriming conditions, combining SSH and Northern Blot analysis or SSH and microarrays (Cortez-Baheza et al., 2007; Irsigler et al., 2007; Rodrigues et al., 2012; Way et al., 2005; Zhang et al., 2007), while we used SSH in combination with RNA-seq to identify differentially expressed genes induced or repressed by osmopriming in *P. pyramidalis*, followed by qRT-PCR to validate some of those genes.

The 30 most abundant genes specifically found in the libraries of the osmopriming and unprimed seeds strongly differed. In general, in unprimed seeds, genes with high transcript abundance were associated with seed maturation, desiccation, and photosystem. These genes represent the transcriptome profile of dry seeds. For osmoprimed seeds, genes with high transcript abundance were involved in hypoxia response genes, gluconeogenesis or glycolytic process genes, and oxidation-reduction. The results suggest that osmopriming methods can program seeds to better respond to germination by the induction of genes involved in metabolic processes and genes involved in aerobic and anaerobic processes. Osmoprimed seeds showed improved germination

parameters during subsequent germination compared to unprimed seeds demonstrating the efficiency of the osmopriming treatment. We suggest that this beneficial effect is the result of changes in the seed transcriptome towards germination metabolism.

To validate the SSH results, expression differences of eight genes related to water stress with recognized differential abundance in each library were analyzed by qRT-PCR. The two genes used as reference (homologs of *G. max* serine/threonine protein phosphatase 2A (NODE 580) and *A. thaliana* Small GTP binding protein SAR 1 A (SAR 2) (NODE 45,602) were previously described as proper reference genes for expression analysis in tomato and Arabidopsis seeds (Dekkers et al., 2012). We show that they also can be used as good reference genes for gene expression studies in *P. pyramidalis* seeds with a geNorm M value of 0.399 for NODE 580 and 0.387 for NODE 45602.

PpAIGTPj (NODE 4738), *PpTIPAh* (NODE 753) and *PpGmSM PM27* (NODE 3870) were confirmed to be downregulated in osmoprimed seeds compared to unprimed seeds (Fig. 6F, G and H). *PpAIGTPj* (NODE 4738) was annotated as an Auxin Induced Glutathione S Transferase (GST), which compose a class of enzymes with potential antioxidant properties. They can act as antioxidant by tagging oxidative degradation products for removal or by directly scavenging peroxides. GSTs are also well known for the capacity to detoxify xenobiotics, i.e. such as herbicides, and respond to a range of biotic and abiotic stresses, i.e. such as water deficit (Dalton et al., 2009; Gong et al., 2005). We suggest that *the PpAIGTPj* is important to maturation and desiccation process scavenging peroxides generated during those processes. GST genes were upregulated at the end of the seed development in *C. arabica* corroborating with our results (Dussert et al., 2018). The *PpTIPAh* (NODE 753) was annotated as an alpha Tonoplast Intrinsic Protein (TIP), which are related to water transport, such as aquaporins which compose membrane water channels that facilitate cell-to-cell water movement as well as plant cell expansion and organ development (Chen et al., 2013b). Among Arabidopsis TIP orthologues, α-*TIP* is seed-specific and seems to play a role in maintaining the integrity of the tonoplast during the dehydration process of seed maturation (Gao et al., 1999; Maurel et al., 1997). High mRNA levels of TIP genes have also been found in dry seeds (Gattolin et al., 2011; Li et al., 2008). The expression of those genes is normally low during imbibition/ germination and probably that is why their expression is also low in *P. pyramidalis* osmoprimed seeds. The *PpGmSM PM27* (NODE 3870) was annotated as an Seed maturation protein PM27. The best *A. thaliana* homolog is the LEA gene AT1G22600, which is high expressed in dry seeds and downregulated during imbibition. LEA proteins are found in the seeds of many plant species and are associated with abiotic stress response (Hundertmark and Hincha, 2008).

We could confirm library specific transcripts with qRT-PCR. These results most likely point to the fact that also the other library specific genes are real differentials and as such are also good candidates for markers and further analysis of osmopriming effects.

5. Conclusions

SSH combined with RNA-seq analysis proved to be a powerful approach for the identification of a large number of differentially expressed genes in osmoprimed seeds of *P. pyramidalis*. We could confirm differential expression for genes that were unique to each library or had a high difference in abundance between the two libraries. With the results of the genes found in the unprimed library, but especially for those unique to the osmoprimed seeds library, we have potential markers for further analysis of the molecular pathways responsible for the beneficial effects of osmopriming of *P. pyramidalis* seeds with respect to seed germinability and vigour. This knowledge can ultimately be used for improvement of germination and seedling production protocols for the sustainable cultivation and/or exploitation of *P. pyramidalis*.

Author statement

RDdC, LGF, CRPC, PT, HWMH and WL conceived the study, CGCA, ACSSM and DT performed the experiments, CGCA, VGN, SAA and WL analysed the data. CGCA, RDdC, VGN and WL wrote the paper with help from all co-authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.envexpbot.2020.104345.

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