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Research article

Molecular response to water stress in two contrasting Mediterranean pines (*Pinus pinaster* and *Pinus pinea*)

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ABSTRACT

Adaptation to water stress has determined the evolution and diversification of vascular plants. Water stress is forecasted to increase drastically in the next decades in certain regions, such as in the Mediterranean basin. Consequently, a proper knowledge of the response and adaptations to drought stress is essential for the correct management of plant genetic resources. However, most of the advances in the understanding of the molecular response to water stress have been attained in angiosperms, and are not always applicable to gymnosperms.

In this work we analyse the transcriptional response of two emblematic Mediterranean pines, *Pinus pinaster* and *Pinus pinea*, which show noticeable differences in their performance under water stress. Using microarray analysis, up to 113 genes have been detected as significantly induced by drought in both species. Reliability of expression patterns has been confirmed by RT-PCR. While induced genes with similar profiles in both species can be considered as general candidate genes for the study of drought response in conifers, genes with diverging expression patterns can underpin the differences displayed by these species under water stress. Most promising candidate genes for drought stress response include genes related to carbohydrate metabolism, such as glycosyltransferases or galactosidases, sugar transporters, dehydrins and transcription factors. Additionally, differences in the molecular response to drought and polyethylene-glycol-induced water stress are also discussed.

1. Introduction

Undoubtedly, one of the major driving factors of evolution and diversification of vascular plants since the Silurian (~430 MYA) is the adaptation to dry land environments, involving the development of water uptake and transport mechanisms and minimisation of water losses. Terrestrial plants display a whole panoply of constitutive and inducible anatomical and molecular adaptations to drought stress. These are particularly relevant in certain regions, and for perennial species such as trees, which most likely have to face water shortage several times during their lifespan. According to the current climatic forecast, drought stress will become even a more defining factor in great parts of the planet in the near future. For instance, Mediterranean regions will suffer a decrease of 10–20% of annual precipitations, with more frequent and severe drought periods, together with an increase in 3–3.5 °C in the mean annual temperature by the end of this century [1]. Thus, understanding adaptive responses with which tree species will have to face these situations is of the utmost interest.

Drought stress can limit plant growth and reproduction, and can lead to serious and eventually insurmountable difficulties to keep the homoeostatic equilibrium in cells: metabolism can be disrupted, leading to an increased production of free radicals and reactive oxygen species (ROS) that damage the membranes, especially the photosynthetic machinery. Higher order plants display protection mechanisms addressed to avoid desiccation and its deleterious effects.

It is well known that, at the molecular level, inducible response to drought, as well as to other abiotic stresses, is controlled by several genes, comprising multiple signalling pathways [2]. Most of the advances in the comprehension of the molecular response to drought stress have been achieved in angiosperms, which display peculiar

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characteristics that ease this kind of studies. For instance, they have smaller and simpler genomes than gymnosperms, so that the complete genome sequences of several herbaceous and woody angiosperm species are available, with a better annotation; they include also short-lived model species which allow the faster performance of repeated and serial experiments, etc. On the contrary, no such model species is available among gymnosperms. Additionally, due to the long time elapsed since both groups diverged (300 MYA), different genes and mechanisms can be expected to be involved in the response of gymnosperms to drought. The aim of this work is to help covering the gap in the current knowledge of the molecular response of conifers to water stress. Over the last decade several studies have reported on the identification of genes induced by drought stress in conifers, mainly *Pinus*, performing preliminary expression pattern analysis under moderate water stress [3–10]. Other studies have focused on the analysis of certain gene families presumably involved in the response to water stress, such as dehydrins [11–14]. In this work we compare the transcriptional response of two closely related pine species, *Pinus pinaster* and *Pinus pinea*, which show, however, noticeable differences in their performance under water deficit.

Both species thrive under the drought-prone conditions of the Mediterranean basin, and can be found on sandy soils, with low water retention capacity, where they play a major role in a characteristic, priority conservation habitat of the European Union, the "wooded dunes with *P. pinea* and/or *P. pinaster*". Their ecological requirements overlap to a great extent, and mixed stands of both species are frequent. Nevertheless, *P. pinaster*, although occupying a relatively small geographical range, in the Western Mediterranean basin, shows larger ecological amplitude, particularly in relation to water availability and has been used as model species for the study of the molecular response to drought stress in conifers in several studies [3,4,8,13,15]. Differences among provenances have been detected for this species, regarding mass allocation, water use efficiency under water stress [16,17] but, in general, it is considered as a drought-avoiding species which shows sensitive stomata and fast osmotic adjustment in response to water stress [18,19].

On its side, stone pine, *P. pinea*, shows a wider distribution, all around the Mediterranean sea, although displaying an extremely low neutral diversity [20]. *P. pinea* is a more thermophilic and xerophytic species, and is usually found on poorer soils. Additionally, and contrarily to *P. pinaster*, tolerates also shade rather well [21], the combination of shade and drought stress being one of the most restrictive conditions for plants in Mediterranean-type ecosystems [22].

In this work we have used microarray and RT-PCR techniques to analyse the expression pattern in both species of 1124 genes presumably involved in the response to water stress during a severe and prolonged drought, similar to the ones these trees have to face in nature, in order to identify the expression profiles associated to higher drought resistance in conifers.

2. Result and discussion

2.1. Water potential in leaves of P. pinaster and P. pinea during drought stress

Fig. 1 shows the evolution of leaf water potential (Ψ) in *P. pinaster* and *P. pinea* plants throughout the drought experiment. Notwithstanding the known differences in hydric requirements shown by both species, the evolution of Ψ during the experiment is noticeably similar in *P. pinaster* and *P. pinea*.

2.2. Genes induced by drought stress in P. pinaster and P. pinea

A total of 181 genes were significantly upregulated in needles, stems and/or roots in *P. pinaster* in response to the non-irrigation

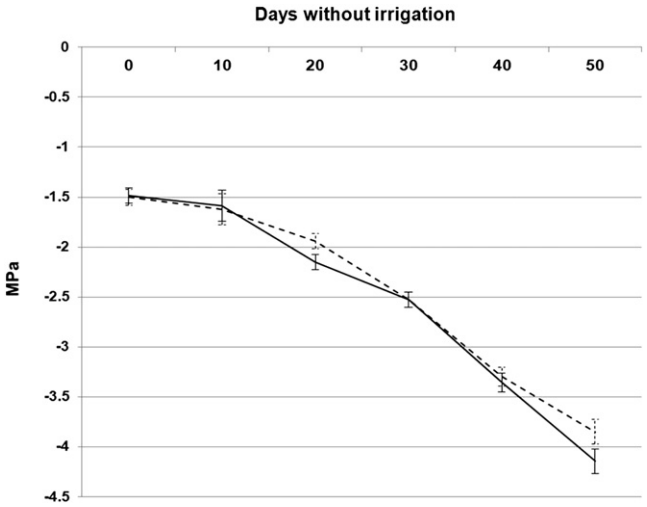

Fig. 1. Average midday water potential in needles along the drought treatment. *P. pinaster*: dashed line; *P. pinea*: continuous line. Bars represent standard errors.

treatment (Supplementary Table S1). Fifty three out of these genes were upregulated in the three organs. The highest number of overexpressed genes was found in stems with 116 genes, and 41 of them appeared significantly induced exclusively in this organ. 44 out of the 107 genes significantly overexpressed in needles were not detected as significantly induced in the other organs. Finally, 88 genes were significantly overexpressed in roots, and only 19 of them were identified exclusively for this organ (Supplementary Fig. S1).

The drought treatment in *P. pinea* led to the significant induction of 218 genes (Supplementary Table S2), and 67 out of them were upregulated in all the three organs. 123 genes were significantly upregulated in stems, and 23 of them exclusively in this organ. 144 genes were significantly induced in needles, and 40 out of them were not significantly induced in stems or in roots. Finally, up to 140 genes were significantly upregulated in roots, and 33 of them exclusively in this organ (Supplementary Fig. S2).

An enrichment analysis of the 113 genes significantly induced for both species (Fig. 2, Supplementary Table S3) yielded over-represented GO terms, such as response to water stimulus (GO:0009415) or response to hormone stimulus (GO:0009725). Most of them can be classified into four functional categories, according to FuncatDB [23] (Fig. 3): i) metabolism, ii) cell rescue and defence, iii) transport and iv) transcription related genes.

2.2.1. Metabolism related genes

A quarter of the genes significantly induced in both species corresponds to metabolism related genes, and up to one third of them are related to carbohydrate metabolism. Accumulation of sugars and other solutes is supposed to maintain turgor pressure and to protect structures from mechanical and metabolic stresses during dehydration, contributing to the acquisition of desiccation tolerance in plants [24]. Some of these genes have also been reported to be induced by PEG-induced water stress in *P. pinaster* [8], as for example an alkaline α-galactosidase (TC181331), a malate synthase (TC155104), a glycosyltransferase (TC156369), a beta-galactosidase (TC156138), a chitinase (TC157851) or an aldehyde dehydrogenase (TC158839). Another abundant group of genes is related to secondary metabolism, including genes involved in the synthesis of hormones such as ethylene (TCC182140, 1-aminocyclopropane-1-carboxylate (ACC) synthase; TC174045, ACC oxidase) or jasmonate (TC188679, 12-oxo-phytodienoic acid reductase).

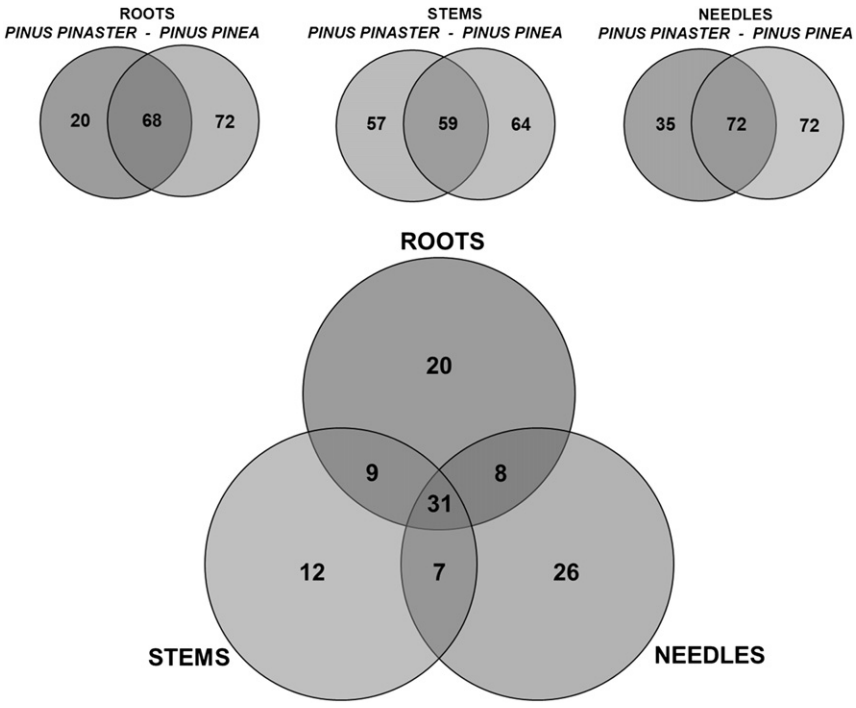

Fig. 2. Transcripts significantly upregulated shared between needles, stems and roots for both species. A total of 113 genes significantly upregulated were selected.

2.2.2. Cell rescue and defence genes

The most relevant genes within this category putatively encode late embryogenesis abundant (LEA) proteins and heat shock proteins (HSP). For instance, TC182917 and TC180126 are homologous to small HSP (sHSP), which are presumably involved in the maintenance of membrane integrity [25]. Although these genes have usually been related to other abiotic stresses such as heat or cold stress [26–28], it has also been reported that they can confer tolerance to drought and salt stress [29]. On its side, TC194781 corresponds to a HSP70, a type of HSP related to water stress resistance [30,31]. Among LEA genes, four different dehydrin genes (TC162509, TC179486, TC193003 and TC176703) have been detected as significantly upregulated in both species. Dehydrins are a complex, multigenic family involved in different stress response and ontogenic processes. An analysis of their structure and expression under drought stress in *P. pinaster* has recently been

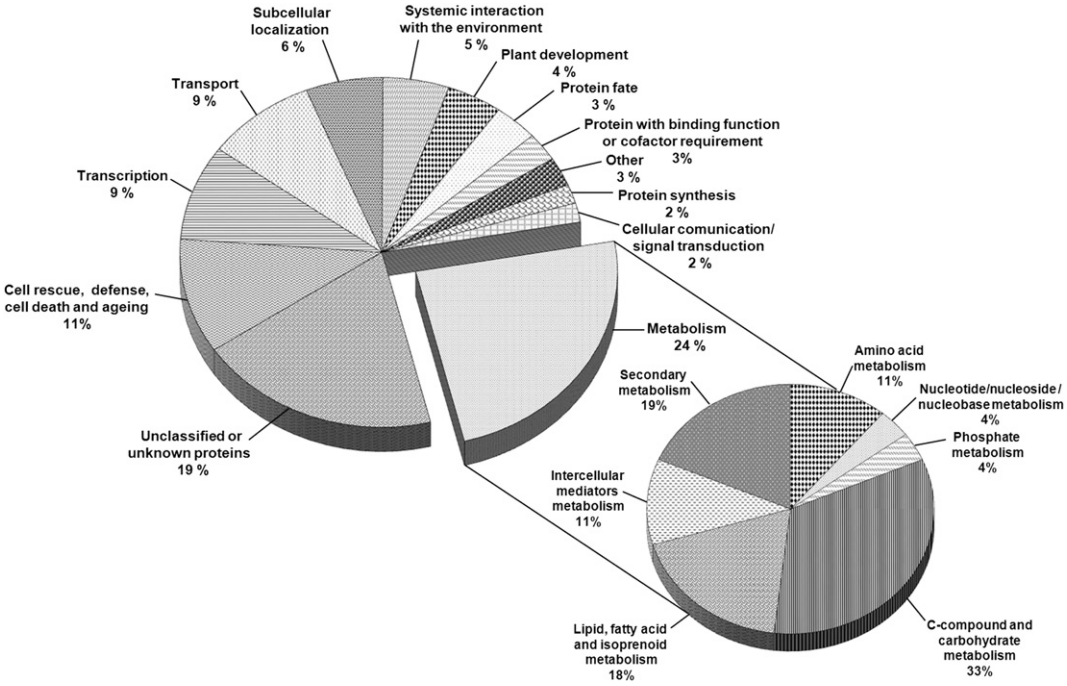

Fig. 3. Functional distribution of selected candidate genes obtained. A total of 113 genes were grouped according MIPS functional categories of *Arabidopsis thaliana*. The percentage of gene transcripts in each group is listed.

published [13]. TC168999 is homologous to *AtLEA14*, a gene that is upregulated by high light, drought, cold, and salt stresses in *Arabidopsis* [32].

Also in this category can be included TC176662, a putative U-box containing protein. This family, very abundant and diversified in plants, is supposed to be involved in ubiquitination under different conditions [33]. Thus, TC176662 could play a role in the degradation of proteins damaged under water stress. Interestingly, a recent rangewide study of *P. pinaster* populations has revealed a strong association of SNP allele frequencies for this gene (as well as for a putative heat shock factor, TC171120, see below) with temperature variables, suggesting the existence of variants adapted to local climatic conditions (González-Martínez et al., pers. comm.).

2.2.3. Transport

Approximately 9% of the genes induced by drought stress in both species are presumably related to the transport of sugars, anions and amino acids. These genes could act coordinately with inducible genes involved in metabolism, helping in adjusting osmotic pressure. For instance, two different hexose transporters (TC170434 and DR099938) and two inositol transporters (TC170498 and TC171882) can be found in this group. TC176635 corresponds to an amino acid permease, which could mediate the accumulation of free amino acids as proline, reported to confer resistance to desiccation [34]. Consistently, higher proline content in *P. pinaster* from xeric provenances has recently been reported [35]. On their side, TC173812 and TC167700 show homology with peroxisomal membrane proteins, which could be involved in the establishment of a ROS scavenging mechanism [36].

2.2.4. Transcription related genes

Another 9% of the genes induced both in *P. pinea* and *P. pinaster* show homology with transcription factors from different families, and could therefore be considered to play a key role in the transcriptional response of pines to drought. Thus, TC157919 is homologous to the DREB (drought responsive element binding proteins) subfamily. TC158167 and TC178542 correspond to the ERF (ethylene response factor) subfamily, which is supposed to be involved in gene regulation in both ethylene dependent and independent pathways [37]. A putative WRKY factor has also been detected (TC163430). These conserved plant transcription factors have been shown to play a critical role in ABA response [38] and their overexpression can increase water stress tolerance [39]. On its side, TC161257, significantly induced in the three organs of both species, has been identified as a bZIP transcription factor. The detection of a putative BEL1-like homeodomain transcription factor (TC170594) induced in roots, stems and needles of both species is also noteworthy. These regulatory elements, with a potential role as long distance signals [40], had never been described before as involved in the response to abiotic stress; however, TC170594 was also significantly upregulated in response to PEG-induced water stress in *P. pinaster* [8].

2.2.5. Other functions, unclassified or unknown proteins

Up to 19% of the genes induced by drought in both species show homology with genes of unknown function or even lack homologues in the databases. For instance, TC188788 and TC163698, which are overexpressed in the three organs both by drought and by PEG-induced water stress [8], are homologous to genes of the MtN3 nodulin family. No functional classification has been assigned to this family; however, recent studies on *Arabidopsis* and rice have reported their activity as sugar transporter, supporting import and efflux of sugars from the cells [41]. Overexpression of TC188788 has already been reported in response to drought in *P. pinaster* and *Pinus taeda* [3,4,6], as well as in response to cold stress in *Cupressus sempervirens* [42]. Therefore, this family could play an important role in the capacity of osmotic adjustment shown by many conifers, as *P. pinaster* [18]. On the contrary, this gene family never has been reported as drought responsive in angiosperms. Another remarkable example is the overexpression of TC197470, also induced by PEG treatment in *P. pinaster* [8] and corresponding to a tentatively annotated hydroxyproline-rich protein. The expression of proline rich proteins is stimulated by wounding and environmental stresses [43]; consistently, overexpression of a PRP under drought stress has been reported in *Pinus halepensis* [9].

These results are consistent with the ones reported previously for water stress induced in *P. pinaster* by the addition of polyethylene glycol (PEG) to a hydroponic culture [8]. However, several differences have also been detected. 24 out of the 67 genes reported as significantly induced by PEG in that work have not been detected here. A plausible explanation for this observation could be related to the toxicity of PEG. This substance, especially in its low molecular weight forms, can be absorbed by the roots, eliciting a specific response, in addition to the effect related to the decrease in water potential in the substrate. For example, significant overexpression of a putative soluble inorganic pyrophosphatase (*Ppter_DR_227* ∼ TC178028), gene associated to GO term GO:0010038 "response to metallic ions" was detected in roots during PEG-induced water stress but not in the experiments reported here, neither in *P. pinaster* nor in *P. pinea*. Similar results were observed for a gene (*Ppter_DR_162* ∼ TC160632) homologous to PDR ABC transporters, which are presumably involved in the response to different stresses [44]. In the same way, a significant enrichment in the GO term "binding unfolded ER proteins" (GO:0051082) has been detected among those 24 genes induced by PEG treatment. This term is related to chaperone activity, probably involved in the response to toxicity. Within this category, three high molecular weight heat shock proteins induced by PEG treatment (*Ppter_DR_136* ∼ spruceTC171037, *Ppter_DR_152* ∼ TC187027 and *Ppter_DR_235* ∼TC183705) were not detected here. Nevertheless, the failure to detect some of these 24 genes as significantly induced by the drought treatment could be also related to their time of response and to the different sampling schemes used in both studies, since the PEG treatment lasted for three weeks, with a more intensive sampling in the first 48 h. This could be the case for a putative heat-shock factor (TC171120), not detected as significantly induced by the drought treatment in any organ of *P. pinaster*, but in roots and stems of *P. pinea*, as well as in roots of *P. pinaster* under PEG treatment. In the same way, an embryo-abundant protein (*Ppter_DR_51* ∼ TC178394) showed overexpression in needles during the first steps of PEG-induced water stress, whereas remarkable repression has been detected in the present study, especially in roots (−25 to −45-fold the values of the unstressed plants). This gene could be involved in signal reception and modulation of changes in needles during the very first stages of water deficit.

2.3. Expression patterns in P. pinaster and P. pinea

Hierarchical clustering of the expression levels detected with microarray analyses led to the identification of 10 clusters in roots, 14 in stems and 9 in needles of *P. pinaster*, and of 15 clusters in roots, 19 in stems and up to 22 in needles of *P. pinea* (Supplementary Fig. S3 and S4). Microarray data are commonly validated by RT-PCR due to the higher accuracy attributed to this tool, especially for genes with low induction levels [45]. We have performed RT-PCR analysis for 16 genes, covering the main functional groups and expression patterns, and including some genes responsive to PEG-induced water stress [8] but not detected as significantly induced in the present study, using microarrays (TC183705; HSP90 and TC166071; unknown) (Fig. 4). Expression patterns detected with both techniques were fairly consistent, with Pearson

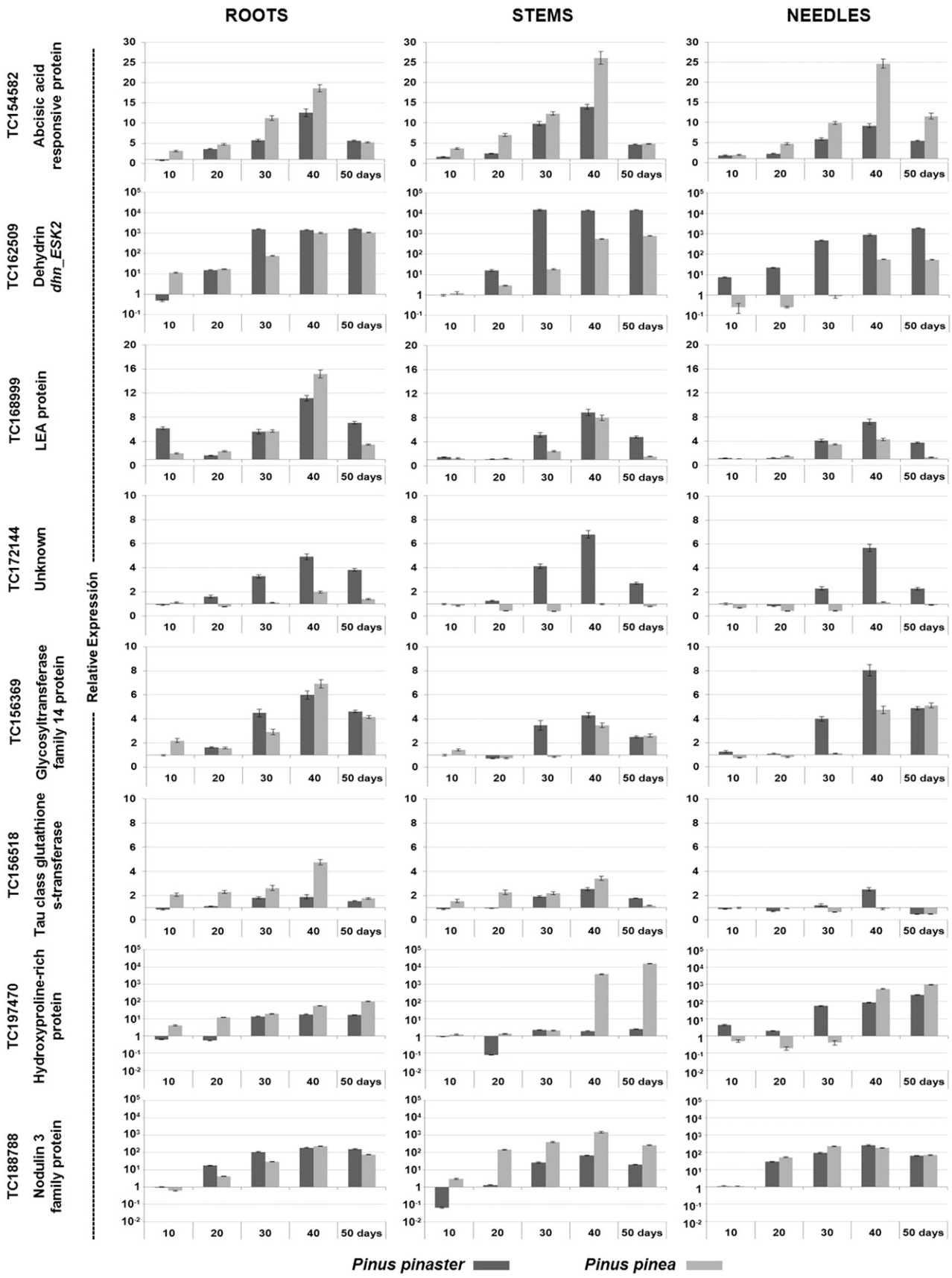

Fig. 4. Verification of microarray results by RT-PCR. Expression profiles along the drought stress treatment in roots, stems and needles from 16 genes involved in different functionalities.

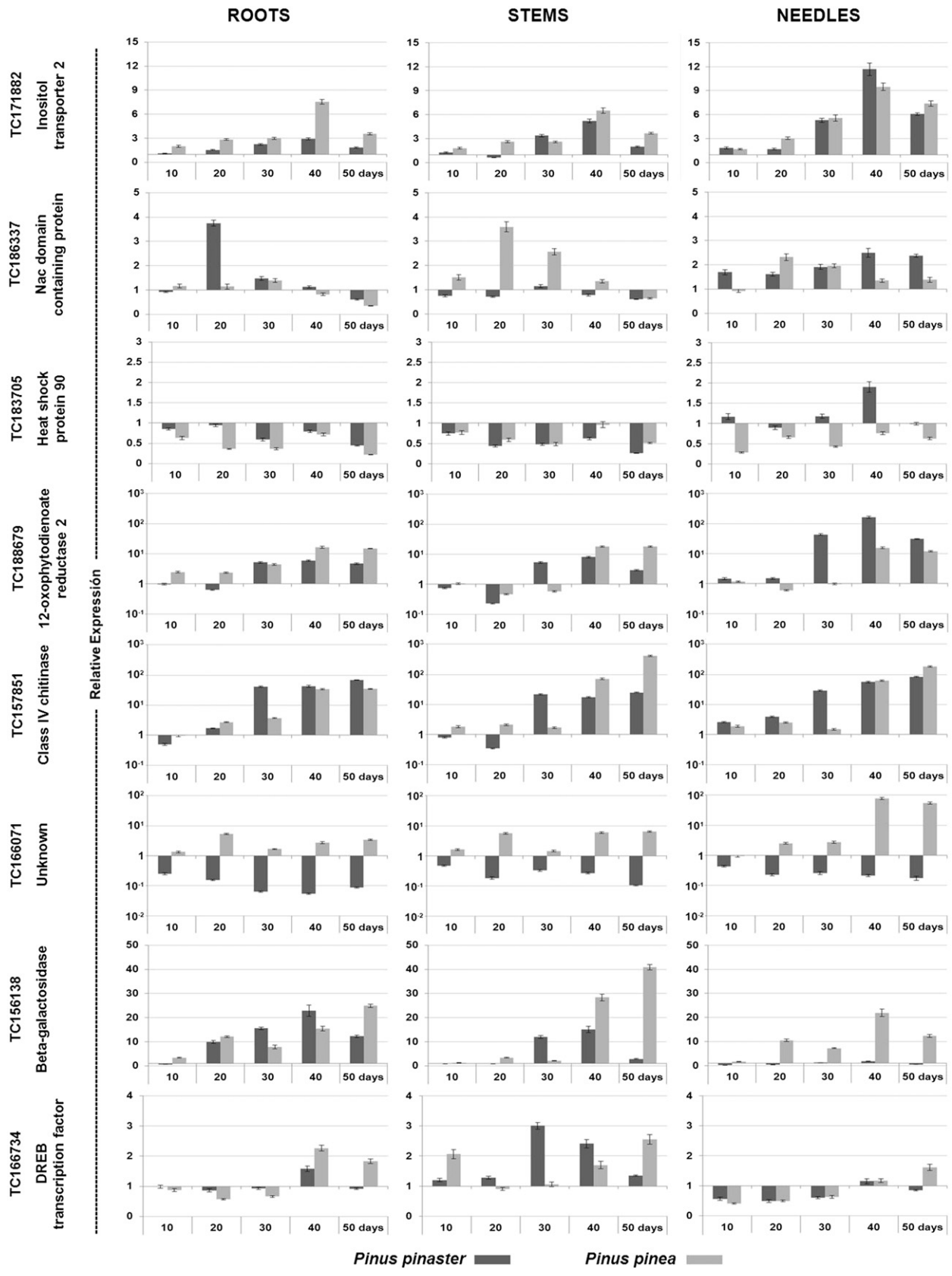

Fig. 4. (*continued*).

correlation values higher than 80% for 35 (*P. pinaster*) and 33 (*P. pinea*) of the 48 gene–organ combinations. As expected, lower correlations were obtained for the gene–organ combinations with lower induction levels, including those not identified as significantly overexpressed according to microarray analysis.

Genes overexpressed in both species can be grouped in three categories, according to their induction patterns detected by microarrays: a) genes induced in the first steps of the drought, (S1 and S2), whose expression level can decrease in further steps or can be kept stable during the rest of the treatment, b) genes whose expression constantly increases throughout the treatment and c) genes highly induced in later steps, S3 and, particularly, S4 and S5. In both species many genes are induced earlier in the roots than in the aerial parts, which is consistent with the role of roots in detecting and triggering the response to water stress. However, several differences can be observed between *P. pinaster* and *P. pinea*:

- A higher number of genes are significantly induced by drought stress in *P. pinea*, compared to *P. pinaster*, especially in roots and needles.
- Stronger inductions levels are detected in overexpressed genes in *P. pinea*, compared to *P. pinaster*. Thus, 83 inductions higher than 10-fold were detected for *P. pinea*, with a maximum of 72-fold for a putative hydroxyproline-rich protein (TC197470), whereas 43 inductions higher than 10-fold were detected for *P. pinaster*, with a maximum of 62-fold for a dehydrin (TC162509).
- A higher proportion of genes are induced in the first steps of the drought in *P. pinea* compared to *P. pinaster*, particularly for S1 (10 days without watering).
- On the contrary, certain genes show a delayed response in *P. pinea* (strong induction at S4-S5), compared to *P. pinaster* (strong induction at S2-S3). This is the case, for instance, for genes included in clusters 8, 9, 10, 11 and 12 in needles, cluster 1 and 9 in stems or clusters 4, 6 and 8 in roots of *P. pinea* and clusters 2, 4 and 6 in needles, clusters 4 and 8 in stems, and clusters 1, 2, 3 and 4 of roots in *P. pinaster*. These observations were confirmed, especially in stems and needles, for some genes studied with RT-PCR, for example for TC162509 (*Pper_dhn_ESK*$_2$ [13]), TC156369 (putative glycosyltransferase), TC197470 (putative hydroxyproline rich-protein), TC157851 (putative quitinase) and TC188679 (putative OPR).

Altogether, these results suggest *P. pinea* is a highly responsive species, displaying a faster and more intense transcriptional response to drought, compared to *P. pinaster*. Notwithstanding, several genes show a delayed induction in *P. pinea*. This result could be consistent with the ones reported recently by Sánchez-Gómez et al. (2011) [46], according to which, during the first steps of a moderate drought stress, the best performing clones of *P. pinea* showed a water-spending strategy, which could provide functional advantage in dry environments, out-competing other water-saving trees [47–49] as *P. pinaster* [19,50]. As the stress situation persists, *P. pinea* plants would switch swiftly to a water-saving behaviour, as reported for other water-spending species [51,52]. Further studies will be required to confirm this hypothesis.

The opposite expression pattern shown by some genes in both species is also noteworthy. TC177528 is strongly upregulated in *P. pinea* (59-fold in stems and 32-fold in needles at S4), while repressed in *P. pinaster* (−15-fold and −5-fold for the same points). This gene encodes a peptide presumably involved in ammonium nutrition in *P. pinaster* [53], which can have an indirect effect in the ABA-mediated reduction of stomatal conductance during drought [54]. On contrary, TC172144, a gene of unknown function, shows a moderate overexpression in *P. pinaster* in response to drought stress, whereas it seems repressed in *P. pinea*.

Increase in transcription of putative retrotransposons elements detected in roots of *P. pinaster* at 50 days of drought (Cluster 8; TC194362 and TC183415) is also remarkable. Enhanced transposition of such elements induced by stress, probably due to epigenetic transient modifications, is a well known phenomenon ([55] and references therein). Interestingly, CDT-1, a dehydration-inducible gene of resurrection plant *Craterostigma plantagineum* showing similarities with retrotransposons, has been reported to confer desiccation tolerance, putatively acting as regulatory non-coding RNA molecule [56]. None of these elements were detected as overexpressed in *P. pinea*, which could be due to the stringency of the criteria used, the specificity of the probes employed or inherent differences in response between the two species.

3. Conclusion

Since its introduction in 1995 [57] microarray technology has been used for the analysis of gene expression in different processes. Due to the high degree of conservation frequently found in coding regions, heterologous array analysis has often been applied in Pinaceae and other plant species [58–60]. While failure to detect significant overexpressions due to specificity of the probes, which would not hybridise properly with the heterologous cDNA, cannot be discarded, significant inductions revealed by microarray analysis are as reliable as for homologous samples. In this study, the oligo-array designed with candidate genes from maritime pine was successfully employed to study and compare the response to water stress displayed by *P. pinaster* and *P. pinea*.

Even though both *P. pinaster* and *P. pinea* are closely related and well adapted to drought, they display different patterns in the response to water stress, with *P. pinea* thriving even in more xeric conditions than *P. pinaster*. *P. pinea* appears as a more sensitive species, displaying a faster and stronger transcriptional response. Notwithstanding this fact, several genes show strong delayed induction compared to *P. pinaster*, or even show opposite expression patterns in both species. While induced genes with similar profiles between both species can be considered as general candidate genes for the study of drought response in conifers, genes with diverging expression patterns can underpin the differences displayed by these species in their performance under water stress. Further research must focus on the regulation of the expression of inducible genes, as well as in the epigenetic modifications likely involved in such regulation as well as in the intraspecific variability in the response. We can expect that fast adaptation to an increasingly drought-prone environment in the following decades will rely to a major extent on these epigenetic modifications, underlying plastic responses.

4. Material and methods

4.1. Plant materials and treatment conditions

Plant material from Oria (37°30'30″N 2°20'20″W, south eastern Spain) and Tordesillas (41° 30′ 6″ N, 4° 59′ 57″ W, central Spain) provenances was used for *P. pinaster* and *P. pinea*, respectively. Both species were grown using containers with peat:perlite:vermiculite (3:1:1 by weight). One year old plants were kept in a growth chamber for two months prior to the drought experiment, with a photoperiod of 16/8 (day/night), a temperature of 24 °C and 60% of relative humidity during the day and 20 °C and 80% of relative humidity during the night, and watered at field capacity.

Unstressed plants were harvested one hour after the last watering. The remaining plants were maintained without irrigation and collected at midday every ten days (five sampling points, S1–S5). Water potential in needles was measured at each sampling point (at midday) using a Scholander pressure chamber. Needles,

stem and roots from each plant were collected separately, immediately frozen in liquid nitrogen and stored at −80 °C.

4.2. Microarray design and hybridisation

A total of 1124 unigenes, 351 from a water stress SSH library reported by Perdiguero et al. (2012) [8] and 773 genes selected from public databases, were included in the microarray design (Agilent 8 × 15 K, Agilent Technologies, CA, USA). Genes are identified in this work with the code of the most homologous TC (*Tentative Consensus Sequence*) from Pine Gene Index. For each unigene, one to four 60-bp-long probes were designed and spotted at least three times on the slide. Probes designed for other pine, spruce and human ESTs available in public databases were included as negative controls. Four different unrelated genotypes for each species collected at each sampling point were used as biological replicates during the experiment. RNA from sampling points S1–S5 and control plants was hybridised to the microarrays. RNA amplifications, labelling and hybridisations, as well as data analysis was carried out as described elsewhere [8].

RNA amplification and labelling were performed as described by Adie et al. [61]. RNA was purified by using the Qiagen RNAeasy kit (QIAGEN, CA, USA). "The manual two-color microarray based gene expression analysis" protocol (Agilent Technologies, CA, USA) was followed for hybridisations. Images from Cy3 and Hyper5 channels were equilibrated and captured with a GenePix 4000B (Axon, CA, USA), and spots were quantified using the GenPix software (Axon, CA, USA).

4.3. Data analysis

Background correction and normalisation of expression data were performed using LIMMA (Linear Models for Microarray Data) [62,63]. LIMMA is part of "Bioconductor, an R language project" (www.bioconductor.org). For local background correction and normalisation, the methods "normexp" and loess in LIMMA were used, respectively. To achieve a similar distribution across arrays and consistency among arrays, log-ratio values were scaled using the median-absolute-value as scale estimator. Differentially expressed genes were evaluated by the non-parametric algorithm 'Rank Products' available as the "RankProd" package at "Bioconductor, an R language project" [64,65]. This method detects genes that are consistently high ranked in a number of replicated experiments independently of their numerical intensities. The results are provided in the form of *p-values* defined as the probability that a given gene is ranked in the observed position by chance. The expected false discovery rate was controlled to be less than 5%.

Changes in the expression of a gene relative to control plants were estimated using the average signal intensity across the four data sets (four genotypes). Based on the statistical analysis, a gene was considered to be significantly upregulated if it met all three of the following criteria: (1) FDR Rank Prod <0.05; (2) the fold change was ≥ 1.6 at any sampling point and in any organ and (3) the trend was consistent for all data. Hierarchical clustering of upregulated genes in the different organs was performed using the log ratio data and the Euclidean distance (complete linkage and threshold 2.5) options of the MeV 4.4 software [66].

4.4. Statistical analysis

4.4.1. Differential gene expression

Differential expression was performed to find the difference in the mean expression among the three organs (multi-class) or among species (two class) by using the *limma* package [67] implemented in Babelomics suite [68]. The gene expression pattern for each sample point was analysed, obtaining *P* values for each gene in the experiment. To account for multiple testing effects, *P* values were corrected using the false discovery rate. Significant differential expression was considered for *P* values <0.05.

4.4.2. Functional analysis

GO term enrichment for upregulated genes was analysed by using FatiGO software [69] implemented in the Babelomics suite [68]. This program executes a Fisher's exact test for 2 × 2 contingency tables and is used to check for significant over-representation of GO annotations. *Arabidopsis thaliana* was used as model species in order to identify over-representation of GO terms from upregulated genes with respect to the rest of annotated genome. Multiple test correction to account for the multiple hypotheses tested (one for each functional term) is applied. Significant enrichment of GO terms was considered for *P* values <0.01.

4.5. Real-time quantitative PCR

The expression pattern of several genes was confirmed by RT-PCR. For this purpose, the RNA was treated with DNAse Turbo (Ambion; Applied Biosystems, Life Technologies, CA, USA). First-strand cDNA was synthesised from 2 μg total RNA from each sample using PowerScriptIII reverse transcriptase (Invitrogen) according to the supplier's manual. 18S rRNA was used as a control, after verifying that the signal intensity remained unchanged across all treatments. The primers for experimental genes were designed using Primer Express version 3.0.0 (Applied Biosystems Life Technologies, CA, USA) and are shown in Supplementary Table S4. For those genes that showed low efficiency for *P. pinea* new pairs of primers were designed with specific sequences. Polymerase chain reactions were performed in an optical 96-well plate with a CFX 96 Detection system (BIO-RAD), using EvaGreen to monitor dsDNA synthesis. Reactions containing 2× SsoFast EvaGreen Supermix reagent (BIO-RAD, CA, USA), 12.5 ng cDNA and 500 nM of primers in a final volume of 10 μl were subjected to the following standard thermal profile: 95 °C for 3 min, 40 cycles of 95 °C for 10 s and 60 °C for 10 s. Three technical replicates were performed for each PCR run. To compare the data from different PCR runs or cDNA samples, the mean of the CT values of the three technical replicates was normalised to the mean CT value of Ri18S. The expression ratios were then obtained using the ΔΔCT method corrected for the PCR efficiency for each gene [70].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2013.03.008.

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