Tree Genetics & Genomes (2014) 10:53–66
DOI 10.1007/s11295-013-0661-5

ORIGINAL PAPER

High-throughput analysis of transcriptome variation during water deficit in a poplar hybrid: a general overview

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Received: 31 May 2013 / Revised: 27 July 2013 / Accepted: 16 September 2013 / Published online: 27 September 2013

Abstract Poplar interspecific hybrids are one of the most important forest crops. In order to obtain data on molecular responses of forest trees to drought, Illumina sequencing technology was used to determine the sequence of most gene transcripts. This approach identified genes that contribute to tolerance to water-limiting environments, contributing to the long-term aim of developing strategies to improve plant productivity under drought. We generated 72,197,113 sequence reads, each 51 nt in length, encompassing 3.68 Gb of sequence from 12 cDNA libraries obtained from leaves of plants of a hybrid between *Populus deltoides* and *Populus nigra* subjected or not to moderate or severe drought. The expression of 41,335 poplar genes included in the *Populus trichocarpa* Phytozome database was studied by mapping Illumina cDNA reads on poplar unigene models. Expressed genes were characterised by gene ontology and by determining the metabolic pathway to which they belong. Most genes detected were expressed in control and drought-treated plants; however, a number of genes that were observed were significantly induced or repressed by drought. Induction or repression of most genes was more common after severe (relative water content around 55–60 %) than after moderate water deficit (around 85 %) even for genes that usually respond promptly to changes in environmental conditions, such as those encoding transcription factors. The dataset of expression profiles will be useful for future studies on responses to other stimula and for crop improvement of poplar.

Keywords Drought · Illumina sequencing · *Populus* hybrid · RNA-Seq · Transcriptome

Communicated by D. Neale

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Electronic supplementary material The online version of this article (doi:10.1007/s11295-013-0661-5) contains supplementary material, which is available to authorized users.

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Introduction

Recent advances in sequencing technology have dramatically changed perspectives of gene discovery and functional genomics. High-throughput "omics" technologies are facilitating the identification of new genes and gene function. In addition, network reconstructions at the genome-scale are keys to quantify and characterise the genotype to phenotype relationships (Feist and Palsson 2008). This "systems biology" approach allows a deeper understanding of physiologically complex processes and cellular functions.

A typical plant species has more than 30,000 genes and an unknown number of proteins, which can be subjected to more than 200 known post-translational modifications (Cramer et al. 2011). The molecular responses of plants to environmental changes are extremely complex. Environmental changes can severely reduce crop production. Lobell et al. (2011) estimated that only 3.5 % of the global land area is not affected by some environmental constraint as, for example, the reduction of arable land, the reduction of water resources, global warming, and other climate changes (Lobell et al. 2011).

Abiotic stress is defined as environmental conditions that reduce growth and yield below optimum levels (Cramer et al. 2011). The plant responses to stress are specific according to the stimulus and to the tissue or organ affected. For example, transcriptional responses to stress are tissue or cell-specific in roots and are quite different depending on the stress involved (Dinneny et al. 2008). Also the level and duration of stress (acute vs chronic) can have a significant effect on the

complexity of the response (Tattersall et al. 2007; Pinheiro and Chaves 2011).

Water deficit inhibits plant growth by reducing water uptake into the expanding cells and alters the rheological properties of the cell wall, for example, by the activity of reactive oxygen species on cell wall enzymes (Skirycz and Inzé 2010). Moreover, water deficit alters the cell wall non-enzymatically, for example, by the interaction of pectate and calcium (Boyer 2009). Water conductance to expanding cells is affected by aquaporin activity and xylem embolism (Boursiac et al. 2008). Such initial growth inhibition due to water deficit precedes any inhibition of photosynthesis or respiration (Hummel et al. 2010).

The plant's ability to osmotically adjust or conduct water is the limiting factor for growth. The epidermal cells can increase the water potential gradient by osmotic adjustment, eventually supplied by solutes from the phloem. Such solutes are produced by photosynthesis that is also supplying energy for growth and other metabolic functions in the plant. In the case of long-term water deficit, photosynthesis declines because of stomatal limitations for CO_2 uptake and difficulties in dissipating excess light energy, which leads to photoinhibition (Pinheiro and Chaves 2011).

One of the earliest metabolic responses to abiotic stresses and the inhibition of growth is the inhibition of protein synthesis (Good and Zaplachinski 1994) and an increase in protein folding and processing (Liu and Howell 2010). Energy metabolism is affected as the stress becomes more severe (Cramer et al. 2007; Pinheiro and Chaves 2011). Thus, there are gradual and complex changes in metabolism in response to stress.

The plant molecular responses to abiotic stresses involve interactions and crosstalk between many molecular pathways. One of the earliest signals in many abiotic stresses involves reactive oxygen species (ROS) and reactive nitrogen species (RNS), which modify enzyme activity and gene regulation (Wilkinson and Davies 2010; Mittler et al. 2011). Hormones are also important regulators of plant responses to abiotic stress. The two most important are abscisic acid (ABA) and ethylene (Goda et al. 2008).

ABA is a major regulator of many plant responses to environmental stresses, especially osmotic stresses (Chinnusamy et al. 2008; Hubbard et al. 2010). Its signalling can be very fast without involving transcriptional activity, as in the case of the control of the stomatal aperture by ABA through the biochemical regulation of ion and water transport processes (Kim et al. 2010). Slower responses to ABA involve transcriptional changes that regulate growth, germination, and protective mechanisms. Studies of the transcriptional regulation of dehydration stress have revealed both ABA-dependent and ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki 2006). Cellular dehydration under limited water availability increases endogenous ABA levels that trigger downstream target genes encoding signalling factors, transcription factors, metabolic enzymes, and others (Yamaguchi-Shinozaki and Shinozaki 2006). Ethylene is also involved in many stress responses (Yoo et al. 2009), including drought. There are known interactions between ethylene and ABA during drought (Wilkinson and Davies 2010).

Forest crops are especially susceptible to drought stress that can seriously affect biomass production. The genus *Populus* is an important crop and a model system to understand the molecular processes of growth, development, and responses to environmental stimuli in trees. At the transcriptome level, hundreds and/or even thousands of differentially expressed genes involved in drought response in poplars have been documented via the production of expressed sequence tag (EST) libraries and/or via microarray analysis.

Nanjo et al. (2004) sequenced over 30,000 ESTs from *Populus nigra* leaves treated with dehydration, chilling, high salinity, heat, ABA, or H_2O_2 and discovered over 4,500 non-redundant full-length cDNAs. Other EST libraries from drought-stressed plants and/or organs were produced and described in *Populus trichocarpa*, the model species for this genus, whose genome has been sequenced and deeply analysed at both structural and functional levels (Tuskan et al. 2006; see also Cossu et al. 2012). Also, differential EST libraries have been widely produced, for example, from cold-treated *Populus alba* leaves (Maestrini et al. 2009). Microarray-based variations in transcriptome were described in drought-stressed *Populus balsamifera* (Hamanishi and Campbell 2011; Cohen et al. 2010; Raj et al. 2011) and *Populus euphratica* plants (Yan et al. 2012). Hybrid poplar genotypes were found to have different transcriptional responses to drought at different times of the day (Wilkins et al. 2009). Poplars from the same clone previously exposed to different environmental conditions developed differences in transcript abundance patterns and DNA methylation levels in response to the same drought treatment (Raj et al. 2011). We can conclude that *Populus* trees did not have a common genus-specific drought-driven transcriptome (Wilkins et al. 2009; Cohen et al. 2010; Hamanishi and Campbell 2011).

The recent development of high-throughput sequencing technologies are changing the way transcriptomes and genomes are discovered and defined, including the 454-Roche (http://www.454.com; Margulies et al. 2005), ABI-SOLiD (http://www.appliedbiosystems.com; Pandey et al. 2008), and Solexa/Illumina (http://www.illumina.com; Bentley et al. 2008) technologies. For example, the Illumina system can yield millions of short reads and is therefore more suitable for tag-based transcriptome sequencing and digital gene expression analysis, achieving a superior accuracy and precision compared to older methods (Wu et al. 2010; Wang et al. 2010; Yang et al. 2011a), especially for genes showing low levels of expression (Schmidt et al. 2012). Recently, transcriptome sequencing using 454 cDNA sequencing technology was performed for deep gene expression profiling of *Populus deltoides* (Yang et al. 2011b). Solexa/Illumina deep

transcriptomic data from genome-wide analyses showed thousands of genes activated by salt stress-driven metabolism in *P. euphratica* and *Populus simonii* x *P. nigra* (Qiu et al. 2011; Chen et al. 2012), which was far more than previously reported. In another study, Illumina deep sequencing was used to reveal fine differences in response to drought between male and female trees of *Populus yunnanensis* (Peng et al. 2012). In light of this information and with the aim of contributing to the construction of a meta-transcriptome for the poplar genus, we have performed a genome-wide analysis of the transcriptome in leaves of an interspecific hybrid between *P. deltoides* and *P. nigra* (one of the most cultivated poplars in Northern America and Northern Europe) in response to water deficit using Illumina high-throughput cDNA sequencing.

Materials and methods

Sample preparation and sequencing

Rooted cuttings of hybrids between *P. deltoides* (L155-079, female) and *P. nigra* (71077-2-308, male), produced at INRA, Orleans (France), were cultivated in 20×20 cm^2 pots in the greenhouse, under natural daylight conditions (750 μm m^{-2} s^{-1}, maximal photon flux density), with air temperature maintained at 17–29 °C, and relative humidity from 55 to 90 %.

In May 2011, some hybrid plants 50–70 cm in height were normally watered and watering of others were suspended for 8 and 13 days. Since in diverse plant species, including *Populus*, transcriptome responses to drought are shaped by the time of day (Wilkins et al. 2009; Hamanishi and Campbell 2011), all materials were sampled at the same time of the day (11.00 a.m.). Fully expanded leaves at 6–8 internodes from apex (see Supplementary material 1) were collected.

Leaf water loss during the experiment was followed by relative water content measurement [RWC=100 (FW−DW) / (TW−DW)], where FW is the fresh weight, DW the dry weight, and TW the turgid weight. One fully expanded leaf was collected from each plant and divided into two portions; one was used for RNA isolation and the other was used to measure tissue hydration by determining the RWC. The experimental design was as follows: 2 clones (biological replicates)×3 treatments (control, C; moderate, D1; and severe drought, D2)×2 hybrids (obtained from the same parents).

The total RNA was isolated from leaves of single plants with different RWC, according to the method described by Logemann et al. (1987), followed by DNAse I (Roche) treatments according to the manufacturer's instructions, to completely remove genomic DNA contamination. Finally, RNA was purified by phenol/chloroform extraction and was precipitated following standard procedures.

A RNA-Seq library was generated using the TruSeq RNA-Seq Sample Prep kit, according to the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). Poly-A RNA was isolated from the total RNA and chemically fragmented. First- and second-strand cDNA syntheses were followed by end repair and adenosines were added to the 3′ ends. Adapters were ligated to the cDNA and 200±25 bp fragments were gel-purified and enriched by PCR. The library was quantified using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and run on the Illumina HiSeq2000 (Illumina Inc.) using version 3 reagents. Single-read sequences with length of 51 bp were collected. Low-quality bases, empty reads, and adapter sequences were removed by using CLC-BIO Genomic Workbench, version 5.1 (CLC-BIO).

Mapping and analysis of Illumina reads against the *P. trichocarpa* unigene model database

Reads were mapped with CLC-BIO Genomic Workbench 5.1, using the *P. trichocarpa* unigene model database version 9.1, available at the Phytozome site (http://www.phytozome.net/poplar) (Tuskan et al. 2006). The following parameters were used for alignments: maximum number of mismatches=2, minimum number of reads=10.

Gene expression level was calculated and expressed as reads per kilobase per million reads mapped (RPKM) as described in Mortazavi et al. (2008).

Expression profiles were evaluated considering RPKM values in C, D1, and D2 plants using Baggerly's test (Baggerly et al. 2003). Weighted proportion fold changes among severely droughted plants, moderately droughted plants, and control plants were treated as follows: when values were higher in droughted than in control plants (or in D2 than in D1 plants), they were reported as positive, when they were higher in control than in droughted plants (or in D1 than in D2 plants) as negative, thus leading to a "+" value in the case of above-control average expression levels and a "–" value in the case of below-control average expression levels.

The weighted proportion fold changes between treatments were considered as significant when the weight of a sample was at least twofold higher or lower than the other, with an FDR-corrected p value≤0.05, according to Baggerly's test. Gene expression profiles were subdivided into nine groups: those remaining constant, those increasing their expression in D1 or in D2, or in both drought treatments, those reducing their expression in D1 or D2, or in both treatments, those increasing their expression in D1 and reducing in D2, and vice versa.

RT-PCR analyses

Reverse transcription polymerase chain reaction (RT-PCR) experiments were carried out on specific genes, selected according to RNA-Seq data. First-strand cDNA synthesis was performed with 3 μg of purified total RNA using M-MLV reverse transcriptase RNase H−(Solis Biodyne)

according to the manufacturer's instructions. Forward and reverse specific primers were designed for seven differentially expressed genes (Potri.003G079500.1, Potri.009G133500.1, Potri.010G012100.1, Potri.014G029700.1, Potri.016G065300.1, Potri.012G032700.1, and Potri.016G070200.1) and three genes with constant expression level (Potri.001G162200.1, Potri.004G152900.1, and Potri.002G195200.1) (see Supplementary material 2). Equal template amounts used in these experiments were verified by RT-PCR performed with three genes with constant expression level by stopping cycle amplification during the exponential phase of PCR and checking the amounts of products by separating them in 2 % agarose and GelRedTM staining.

The PCR amplifications were carried out in a 15 μl volume by using 3 μl of 1:4 dilution of RT reaction as template and a HOT FIREPol DNA Polymerase (Solis Biodyne) in non-saturating conditions. PCR involved a 95 °C step hold for 15 min, followed by 30 cycles at 95 °C for 30 s, 55–60 cycles (depending on primer Tm value) for 30 s, and 72 °C for 30 s. The PCR products were separated in 2 % agarose and GelRedTM stained. The RT-PCR experiments were repeated three times.

Functional profiling of differentially expressed genes

The web tool gProfiler (http://biit.cs.ut.ee/gprofiler/) was used for analysis of gene enrichments in leaves of droughted plants compared to control plants. GO terms from the categories "biological processes", "molecular function", and "cellular activities" with $p<0.05$ (adjusted using g:SCS threshold) were considered over represented in the subsets of genes analysed.

The web tool BLAST2GO (http://www.blast2go.com/b2ghome) was used for annotation of differentially expressed genes, and for grouping and counting gene ontology (GO) classes for each of two gene expression profiles (activated in D1 and/or D2, repressed in D1 and/or D2), without counting the three root classes (Cellular Component, Biological Process, and Molecular Function). The difference in the occurrence of GO terms between activated and repressed genes was statistically analysed by applying Fisher's test, at the same website. The same analysis was performed for genes showing strong gene expression (RPKM>100) in C, D1, and D2 leaves.

Functional characterisation of differentially expressed genes

For the display of metabolic pathways in which induced or repressed genes are involved, we have used the MapMan 3.5.1R2 tool (Thimm et al. 2004; Usadel et al. 2009). Functional classifications of the genes were assigned into Mapman's bins using Ptrichocarpa_v3.0_210_peptide.m02 (http://mapman.gabipd.org/web/guest/mapmanstore) for mapping.

Only data related to genes with RPKM ratio higher than +2, or lower than −2 (with FDR-corrected $p \leq 0.05$, as discussed above) in at least one treatment were exported to MapMan.

Results

Global analysis of gene expression

To obtain a global view of the transcriptome during water deficit in a poplar hybrid, we used an Illumina Genome Analyzer to perform high-throughput tag sequencing analysis on cDNAs from 12 libraries (three culture conditions per two hybrids per two clones of the same hybrid as biological replicates, Table 1). Control leaves (C) RWC ranged from 92.58 to 95.75; moderately stressed leaves (D1) showed a RWC ranging from 84.89 to 86,31; severely stressed leaves (D2) RWC ranged from 52.78 to 61.83. We generated 72,197,113 sequence reads, each 51 nt in length, encompassing 3.68 Gb of sequence data. The total number of tags per library ranged from 3.52 to 14.90 million, a tag density sufficient for quantitative analysis of gene expression (Morin et al. 2008).

The sequence reads were aligned on the *P. trichocarpa* Phytozome unigene database (Tuskan et al. 2006, version 9.1), using the CLC-BIO software set to allow two base mismatches. The distribution of total and distinct tag counts over different tag abundance categories showed very similar tendencies for all libraries (Table 1). Of the total reads, 67.6 % matched either to a unique (60.9 %) or to multiple (6.7 %) unigene sequences; however, 32.4 % of the tags could not be mapped to the gene sequences.

We evaluated the expression of 41,335 gene models included in the *P. trichocarpa* Phytozome database in the three treatments, by measuring the number of reads per exon kilobase per million of mapped sequence reads (RPKM, Mortazavi et al. 2008). CLC-BIO can count unique reads and discard multi-reads, or distribute multi-reads at similar loci in proportion to the number of unique reads recorded. In the first case, the expression of genes that have closely related paralogs would be underestimated. Hence, we decided to include in the analysis, beside the unique reads, also reads that occur up to ten times, a strategy that should allow a correct estimation of activity also for paralogous genes (Mortazavi et al. 2008).

We found many genes whose expression was detectable in controls and/or during moderate or severe drought treatments. The analysis was limited to genes mapped by at least one read per million in at least one of the two clones of the two hybrids (see Chen et al. 2010). By this method, we identified 26,133 genes that were expressed at one stage at least. We also calculated the number of genes that were significantly expressed (i.e. mapped by at least one read per million, as above) in C, D1, and D2, or whose expression was detectable only in one or two of the three conditions, by comparing the mean RPKM value of each gene (Fig. 1). It can be seen that out of 26,133 genes that were expressed in at least one condition, the vast majority (76.1 %) was expressed in all conditions tested. However, a number of genes were specifically expressed in leaves of moderately and/or severely droughted

Table 1 Number of Illumina reads matching to the *P. trichocarpa* unigene database (45,033 CDS sequences) for each library

Library	RWC	Total reads	Counted reads		Uncounted reads (%)
			Unique (%)	Non-specific (%)	
Hybrid 85, clone 3 (C)	95.51	14,896,108	9,463,461 (63.5)	1,045,628 (7.0)	4,387,019 (29.5)
Hybrid 85, clone 4 (C)	92.58	3,627,754	2,384,665 (65.7)	263,098 (7.3)	979,991 (27.0)
Hybrid 89, clone 6 (C)	95.75	8,721,936	5,491,313 (63.0)	647,397 (7.4)	2,583,226 (29.6)
Hybrid 89, clone 8 (C)	95.40	3,844,897	2,271,486 (59.1)	262,839 (6.8)	1,310,572 (34.1)
Hybrid 85, clone 12 (D1)	86.31	4,401,794	2,856,492 (64.9)	325,341 (7.4)	1,219,961 (27.7)
Hybrid 85, clone 24 (D1)	85.64	4,832,559	2,865,518 (59.3)	333,838 (6.9)	1,633,203 (33.8)
Hybrid 89, clone 10 (D1)	84.89	6,004,464	3,623,105 (60.3)	399,280 (6.6)	1,982,079 (33.0)
Hybrid 89, clone 15 (D1)	86.30	7,110,515	4,358,483 (61.3)	488,750 (6.9)	2,263,282 (31.8)
Hybrid 85, clone 42 (D2)	54.78	3,537,379	2,046,338 (57.8)	213,296 (6.0)	1,277,745 (36.1)
Hybrid 85, clone 45 (D2)	61.83	5,522,530	2,880,593 (52.2)	319,017 (5.8)	2,322,920 (42.1)
Hybrid 89, clone 20 (D2)	52.78	3,954,156	2,156,908 (54.5)	191,480 (4.8)	1,605,768 (40.6)
Hybrid 89, clone 35 (D2)	59.69	5,743,021	3,596,156 (62.6)	334,911 (5.8)	1,811,954 (31.6)
Total		72,197,113	43,994,518 (60.9)	4,824,875 (6.7)	23,377,720 (32.4)

For each sample the leaf RWC is reported

C control, *D1* moderate water deficit, *D2* severe water deficit

plants (overall, 3,170 genes, 12.1 %), showing a significant change in the transcription pattern. Finally, a low number of genes were expressed in leaves of control and severely droughted plants (1.1 %).

Of the 41,335 poplar gene models, 23.2, 24.1, and 26.6 % did not have any detectable transcriptional activity in control and moderately and severely droughted plants, respectively, indicating that either these models were not expressed in any of the three conditions examined in the present study or that these gene models of *P. trichocarpa* have diverged in *P. deltoides* and *P. nigra* and so have gone undetected. Additional transcriptome profiling across different developmental stages/tissues (e.g. apical and vegetative meristems, developing inflorescences) and/or different abiotic/biotic environmental variables will be required to further assess the transcriptional activity of these genes. The global analysis of gene expression is reported as an excel file, available at the Department of Agriculture, Food, and Environment of Pisa University repository website (http://www.agr.unipi.it/Sequence-Repository.358.0.html), in which each gene was represented by its absolute expression level in control, moderately dehydrated, and severely dehydrated leaves.

Drought-induced gene expression

The expression profiles of the differentially expressed genes were determined. Genes were subdivided into nine clusters based on their expression modulation (Fig. 2). Genes that were positively or negatively modulated for the whole time course (clusters 1 and 2) were relatively few (only 0.26 and 0.65 %, respectively). Genes that were already induced or repressed with moderate water deficit (clusters 3 and 4) were a bit more frequent (1.37 and 0.74 %, respectively). Interestingly, clusters 5 and 6 that include genes that were positively or negatively modulated only by severe water deficit were the most numerous (5.84 and 7.98 %, respectively). A small number of genes were transiently modulated, i.e. induced or repressed only after moderate drought (0.15 and 0.39 %, clusters 7 and 8, respectively). However, the vast majority of genes were expressed at the same levels in the three stages (82.63 %, cluster 9).

Though the method based on RNA-Seq has been reported as highly reliable (Zenoni et al. 2010), we have performed reverse transcription PCR on ten mRNAs that were differentially expressed in response to moderate or severe stress or showed a constant expression level to confirm the expression data. Results indicated a very good correspondence between RT-PCR and RNA-Seq data (Fig. 3).

Ontology of genes up- or down-regulated by drought

For a functional analysis of gene expression, functional categories were assigned to all predicted poplar genes using the GO terms assigned to *P. trichocarpa* sequences.

First, using gProfiler, we determined whether any GO terms were significantly over- or under-represented among the genes differentially expressed between D1 and control or between D2 and control (Supplementary material 3). The over-represented GO terms in D1 included those related to response to water deprivation and response to lipid (GO biological process, $p = 7.17e{-}04$ and $p = 1.16e{-}02$), as well as DNA binding (GO molecular function, $p = 9.24e{-}03$). The

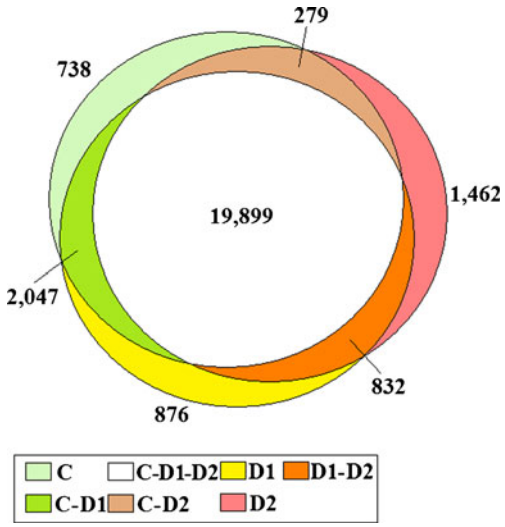

Fig. 1 Venn diagrams representing genes with RPKM>1 (see text) in leaves of control (*C*), moderately droughted (*D1*), and severely droughted (*D2*) poplar hybrid plants

GO categories related to carbohydrate catabolic process (GO biological process, $p=2.04e-07$), coenzyme binding (GO molecular function, $p=3.16e-03$) and membrane-enclosed lumen (GO cellular component, $p=6.75e-47$) were among the over represented in genes that were up-regulated in D2 compared to control leaves. Over-represented GO terms in D1 down-regulated genes include many metabolic and biosynthetic processes (for example, single-organism metabolic process, GO biological process, $p=6.38e-09$), transporter activity (GO molecular function, $p=2.56e-05$), and vacuole (GO cellular component, $p=4.10e-05$). Down-regulated genes in D2 are also enriched in GO terms related to metabolic and biosynthetic processes (for example, carbohydrate metabolic and biosynthetic process, GO biological process, $p=5.25e-04$ and $p=6.09e-07$, respectively). Other GO terms include photosynthesis (GO biological process, $p=1.41e-51$), and organelle (GO cellular component, $p=2.78e-37$).

Expression profile	Number of genes	%
1 	67	0.26
2 	171	0.65
3 	358	1.37
4 	193	0.74
5 	1,525	5.84
6 	2,086	7.98
7 	38	0.15
8 	102	0.39
9 	21,593	82.63

Fig. 2 Number of unigene models per expression profile as indicated on the left. *C* control, *D1* moderate water deficit, *D2* severe water deficit

In another analysis, the GO term occurrence in the different profiles of expression was calculated using BLAST2GO for two groups of genes showing opposite expression profiles, i.e. activated (showing increasing expression at least in one drought condition compared to the control) or repressed (showing decreasing expression at least in one drought condition), listed in Supplementary material 4. The results of this analysis are shown in Fig. 4, in which for the sake of simplicity, only low-level GO terms were reported. Functional classes were sorted by molecular function, cellular component, and biological process. Within each root class, differences were seen (Fig. 4). Within the molecular function class, "catalytic activity" and "binding" were the most abundant terms, as already observed in drought-stressed poplars by Cohen et al. (2010). For the class catalytic activity, the high percentage of GO terms indicated that many genes involved in catalytic activity were present in both down- and up-regulated gene lists, although they are different and specific for each list. Water deprivation should activate a number of genes involved in catalytic activity and also repress a number of genes that were active in the control plants. This trend can be suggested also for the molecular function class binding; the large percentage of GO terms in both expression profiles suggested a significant modification of the activity of such genes during drought treatment.

Within the cellular component class, six GO terms occurred at significantly higher frequency in the list of up-regulated genes than in that of down-regulated ones. This should indicate a general increased activity in the related genes. Finally, in the biological process class "metabolic process" and "cellular process" were the most frequent terms in both expression profiles, confirming the occurrence of deep metabolic variations in response to drought. Many GO terms, including "response to stimulus", were more represented in up-regulated than in down-regulated genes, suggesting an increase of the related activities during drought treatment.

Ontology of the highly expressed genes

Gene ontology of highly expressed genes (RPKM>100) in C, D1, and D2 plants was also analysed in some details. The GO terms of these three groups of genes are summarised in Fig. 5 in which for the sake of simplicity, only level 2 GO terms were reported, as described above.

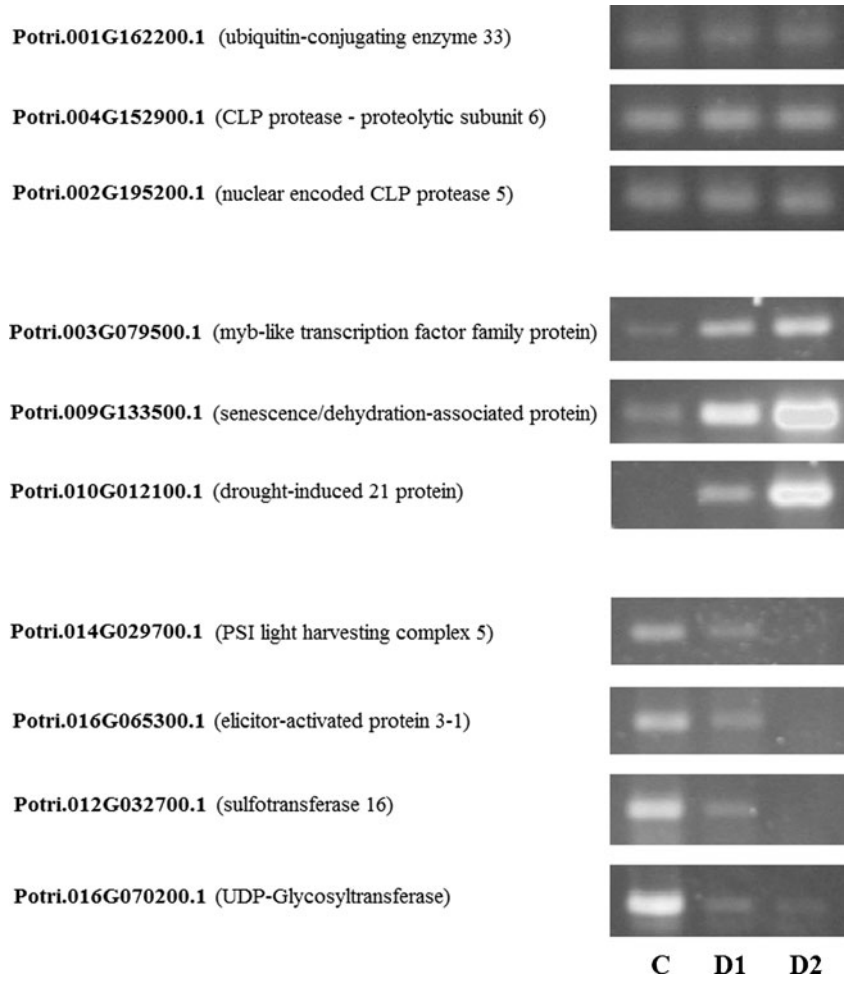

Fig. 3 RT-PCR analysis of the expression of ten genes, selected according to their expression profile (three with stable expression, three with increasing expression, and four with decreasing expression). For each gene, the putatively encoded protein is indicated in parentheses. *C* control (plant 85_4), *D1* moderate drought (plant 85_12), *D2* severe drought (plant 85_42)

For terms related to biological processes, it can be observed that genes involved in binding and catalytic activity were by far the most frequent among highly expressed genes in all the analysed conditions.

Two ontologies related to cellular component "cell" and "macromolecular complex" were more represented in genes highly expressed in D2 than in those highly expressed in D1 conditions. For terms related to molecular function, it is worth noting that genes involved in response to stimulus were more represented in moderately than in severely droughted plants. On the contrary, GO terms related to metabolic process and cellular processes were more represented in D2 than in D1 conditions.

Biochemical pathways regulated by drought

With the objective of displaying differentially expressed genes in pathways and obtaining an overview of genes affected in response to drought in *P. deltoides* x *P. nigra*, the MapMan 3.5.1R2 tool was used on 5,860 genes for which differential expression values compared to controls (i.e. weighted proportions fold change >2 or<−2, with FDR-corrected $p \leq 0.05$) were observed at moderate and/or severe water deficit. MapMan allowed the assignment of 5,995 genes, being some of the genes mapped to multiple pathways, into a total of 34 of 35 functional categories. Whilst a large number of genes (1,431) were classified as unknown, or not assigned a category, the remaining 4, 564 genes were identified as belonging to known metabolic pathways or large enzyme families. Amongst gene categories, 3,571 (78.2 %) genes belonged to ten categories, which included protein metabolism (1,000 genes), RNA metabolism (631 genes), miscellaneous enzyme families (400 genes), signalling (348 genes), transport (316 genes), stress (199 genes), development (188 genes), photosystems (171 genes), hormone metabolism (166 genes), and cell organisation (152 genes).

We explored gene categories that were presumably activated during drought response using the Image annotator module of the MapMan application. We selected genes related to transcription regulation, stress responses, energy metabolism, and secondary metabolism that were well documented to be responsive to a wide array of stresses.

Many genes (537) assigned to transcription factors of different classes were identified and mapped. They are shown in Fig. 6 and in Supplementary material 5. For instance, genes

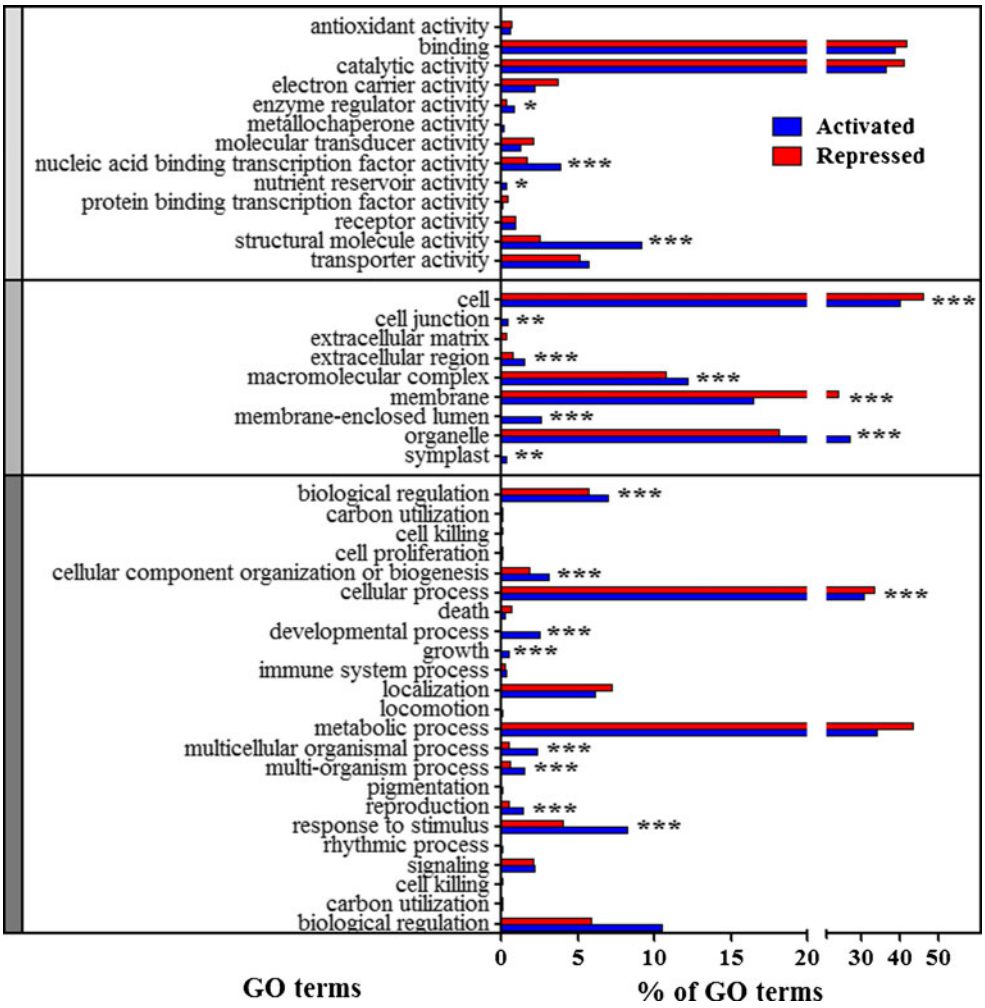

Fig. 4 General functional characterisation of poplar expressed genes. Genes were categorised hierarchically according to two possible expression behaviours, i.e. repressed (showing a reduction in moderate and/or severe drought, compared to the control; profiles 2, 4, and 6, see Fig. 2) or activated (showing an increase in moderately and/or severe drought compared to the control; profiles 1, 3, and 5) and according to level 2 terms of the three principal gene ontologies, biological processes, cellular components, and molecular functions (indicated in the *y*-axis by the vertical bar, with *dark grey*, *medium grey*, and *light grey*, respectively). *Asterisks* indicate significant differences between the two groups of genes (*$0.01<p<0.05$; **$0.001<p<0.01$; ***$p<0.001$ according to Fisher's test)

encoding early response to dehydration-related proteins (Mapman code AP2-EREBP) were highly expressed after both moderate and severe drought, as reported previously (Yan et al. 2012). One NAC domain protein encoding gene, known to be involved in poplar drought response (Brinker et al. 2010), was found to be induced in moderately droughted plants. Considerable expression variation was observed within many transcription families including MYB domain-containing family, bHLH family protein, WRKY, zinc-finger (C2C2, C2H2, and C3H) families, as reported previously (Abe et al. 1997; Sakamoto et al. 2004; Xiang et al. 2008; Wang et al. 2009). A general overview (Fig. 6) revealed a larger number of transcription factors encoding genes more strongly activated (in blue) or repressed (in red) in D2 than in D1 plants. If complete repression of such genes during severe drought was obvious, then it is less obvious that many transcription factors encoding genes increased their expression with increasing water deficit. Presumably, these genes encode transcription factors that are necessary not only at the onset of treatment, but also when stressful conditions are sustained over prolonged time intervals.

In terms of energy metabolism, changes in the magnitude of enzymes and metabolites of carbon and energy cycles have been shown to play crucial roles in cellular metabolism during the response to stress (Apel and Hirt 2004). The induction of mitochondrial respiratory activity and ATP release during these reactions help to initiate tolerance events under stress conditions, for example during hypoxia (Kreuzwieser et al. 2009). Seventy eight genes related to energy metabolism were identified in our analyses (Table 2), of these, two were induced with moderate water deficit (encoding an alternative oxidase and a mitochondrial carrier-like protein) and 58 were activated after severe water deficit. For example, genes encoding mitochondrial electron transporters, isocitrate dehydrogenase (six genes), succinate dehydrogenase (two genes), etc. were induced in severely droughted plants. Only 19 of genes involved in these metabolic pathways were repressed.

Secondary metabolites such as flavonoids and isoflavonoids are known to play a significant role in plant defence responses to pathogens (Dixon and Steele 1999; Uppalapati et al. 2009). The expression of 123 genes related to secondary metabolism were observed in our analysis (Table 2); of these, 21 were

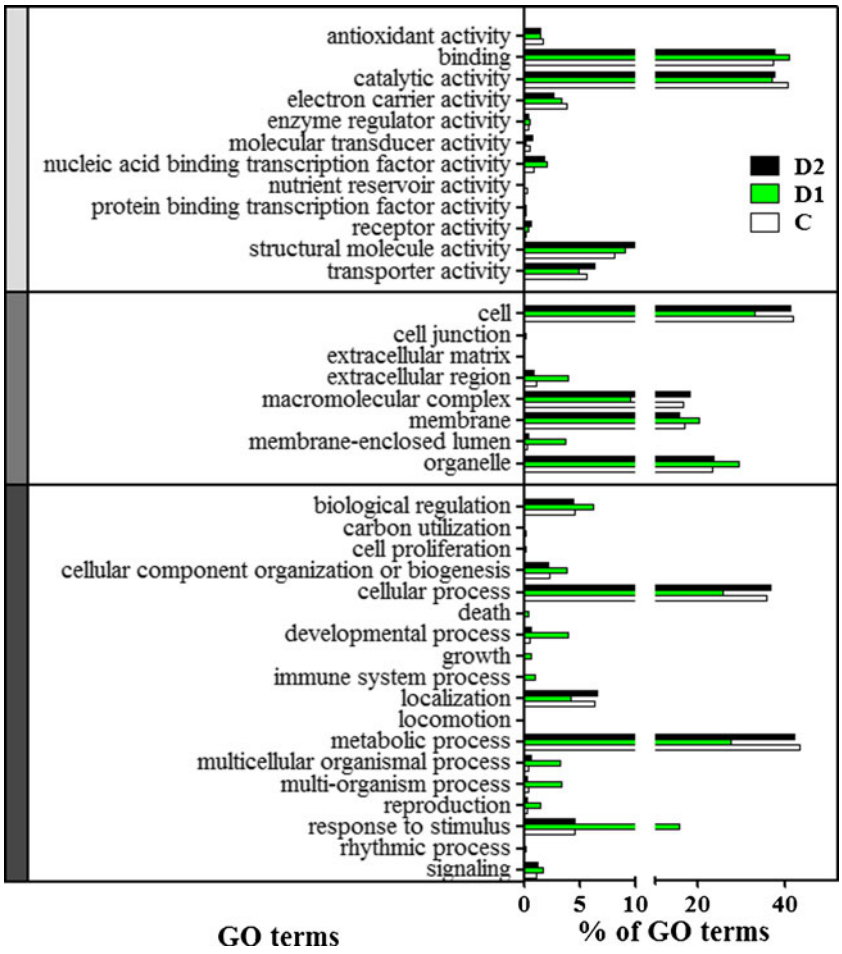

Fig. 5 General functional characterization of the most expressed genes (RPKM>100) in poplar leaves of control (*C*) and moderately (*D1*) and severely droughted (*D2*) plants. Genes were categorised hierarchically according to level 2 terms of the three principal gene ontologies, biological processes, cellular components, and molecular functions (indicated in the *y*-axis by the vertical bar, with *dark grey*, *medium grey*, and *light grey*, respectively). The number of analysed genes for each culture condition is reported in parentheses

induced in response to both moderate and severe water deficit, and another 28 were activated by severe water deficit. Genes related to phenylpropanoids (39 genes, for example encoding enzymes of the shikimate *O*-hydroxycinnamoyl-transferase family), flavonoids (22 genes, for example a cynnamoyl-CoA-reductase encoding gene), and isoprenoid metabolism (43 genes, for example an encoding hydroxy-phenyl-pyruvate dioxygenase) were up-regulated, especially by severe drought. By contrast, many genes involved in secondary metabolism were found to be repressed, after either moderate (35 genes) and severe water deficit (73 genes).

Genes responding to stress factors such as heat shock, anaerobiosis, plant pathogens, oxygen-free radicals, heavy metals, water stress, and chilling in plants have been assessed in various plant species (Matters and Scandalios 1986). In our study, 113 genes with stress-related annotations (either biotic or abiotic) were identified as affected positively or negatively by drought. As observed in the above-mentioned classes, induced stress-related genes were more frequent after severe than after moderate drought (Table 2). However, some dehydration- and heat shock protein (HSP)-related genes were already activated with moderate water deficit, as expected, because they had responded to drought effects. Genes whose expression was increased by both moderate and severe drought included one encoding heat shock factor (HSF), two HSP-binding, and three DNAJ heat shock protein. Many stress response-related genes were inactivated by severe drought.

Analysis of ABA-related genes

Special attention was paid to the expression of genes of poplar involved in ABA-related drought response (13 gene families), or involved in ABA-independent signalling pathways (2 gene families), selected according to Cohen et al. (2010) and to their occurrence among genes mapped by more than one read per million (see above discussion).

For each gene family, the number and the annotation of different members expressed in our experiments are reported in Table 3 and in Supplementary material 6, respectively.

Nearly all genes analysed were modulated by drought. Many gene sequences were up-regulated by severe and, to a lesser extent, by moderate drought. On the contrary, only two genes belonging to two gene families were down-regulated by moderate drought. Many genes were repressed after severe water deficit, probably in relation to deep metabolic changes that would have occurred after prolonged drought (Table 3).

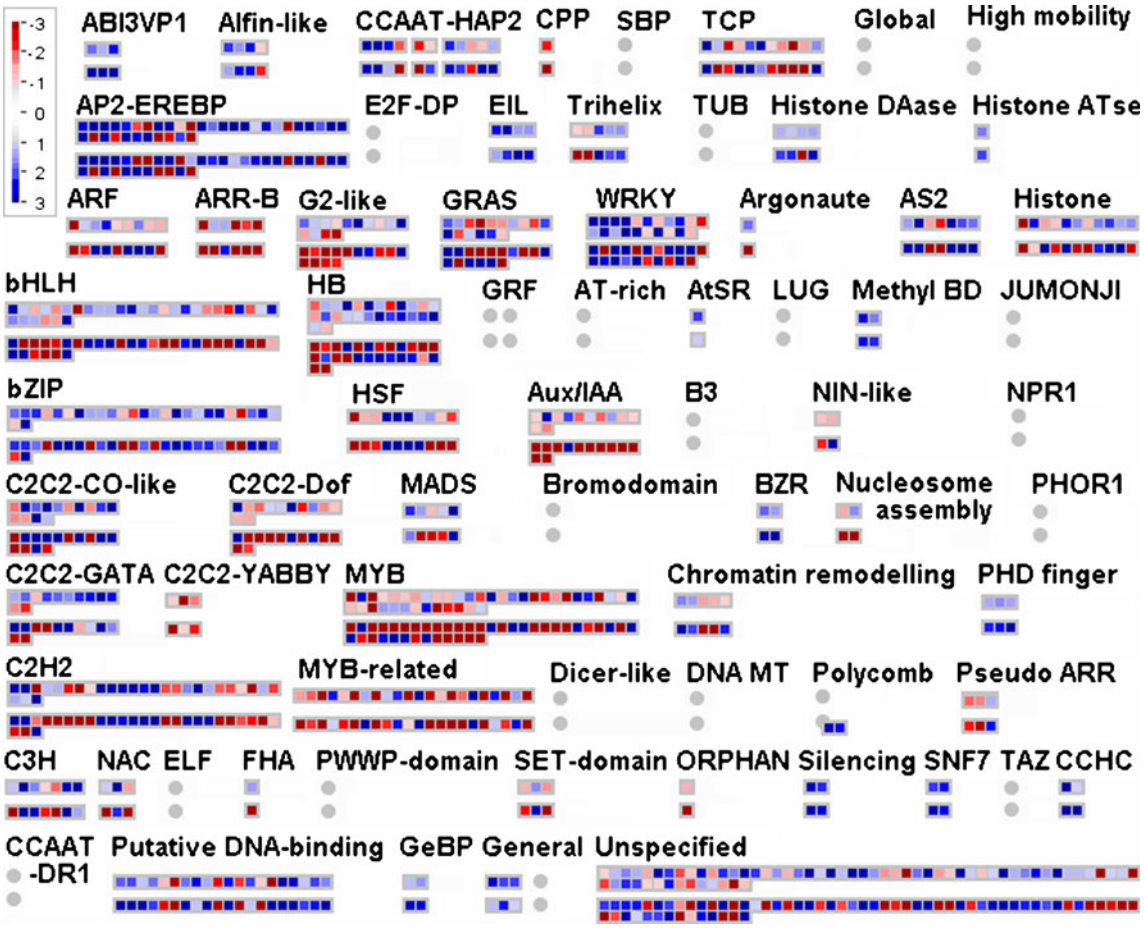

Fig. 6 Schematic global representation of expression levels of genes involved in regulation of transcription obtained using Mapman software on genes induced or repressed (RPKM ratio >2 or<−2) by moderate (upper lines) or severe (lower lines) water deficit. *Grey dots* indicate the absence of genes belonging to that family in the samples. The scale of expression is reported on the *top left*. Each transcription factor family is indicated by its Mapman code

Discussion

Drought-responsive genes were identified using Illumina sequence data generated from leaves of droughted plants of two *P. deltoides* x *P. nigra* hybrids. Illumina sequencing has been shown to be very efficient in the identification of differentially expressed genes (Hoen et al. 2008). Rare and low-abundant transcripts can be detected, resulting in analysis of a comparatively greater number of genes than using other technologies. Moreover, differently from other technologies such as microarrays, cross-hybridization artefacts are avoided and the sequence-based analysis does not require background correction. On the other hand, the appropriate mapping of short reads onto annotated regions and assignment of multi-mapping sequences are still critical challenges, although the development of new algorithms for analysis will rapidly overcome these difficulties (Mortazavi et al. 2008; Shendure 2008). On the whole, transcriptomic analysis can benefit from being re-evaluated using high-throughput sequencing methods (Yan et al. 2012). We used Illumina sequencing technology to determine the sequence of most gene transcripts and to identify genes that contributed to poplar tolerance to water-limiting environments, with a long-term aim of developing strategies to improve plant productivity under drought.

The expression of 41,335 poplar genes included in the *P. trichocarpa* Phytozome database was studied by mapping Illumina cDNA reads, collected from leaves of plants at two water deficit levels, on poplar unigene models. This global analysis of gene expression provided a comprehensive dataset (see "Data archiving statement"). We observed 4,540 genes with significant expression changes, for which expression profiles during progressive drought and gene ontology annotations were established. It was also possible to have a general overview of the metabolic pathways that are activated or repressed by drought.

Most genes were highly expressed in leaves of control and droughted plants, suggesting that they were either not affected, or only moderately affected, by drought. On the other hand, a number of genes were significantly induced or repressed by drought and may constitute a useful dataset for further studies.

Table 2 Number of *P. trichocarpa* unigene models involved in energy metabolism, secondary metabolism, and stress response, according to the Mapman pathway database that shows at least twofold expression variation between control and moderately (*D1*) or severely (*D2*) droughted *P. deltoides* x *P. nigra* plants

Metabolic pathway	Category	No. of genes identified	Up-regulated		Down-regulated	
			D1	D2	D1	D2
Energy metabolism	Respiratory enzymes	34	0	28	3	6
	Metabolite transporters	25	1	15	4	9
	Mitochondrial electron transporter	19	1	15	1	4
Total		78	2	58	8	19
Secondary metabolism	Flavonoids	22	3	5	11	12
	Phenylpropanoids	39	10	22	11	27
	Isoprenoids	43	2	9	8	28
	Shikimate pathway	11	1	8	3	3
	Wax	8	5	5	2	3
Total		123	21	49	35	73
Stress response	Dehydration/salt-related	19	6	10	2	9
	Cold-related	10	1	5	0	5
	HSP and HSP-related	43	8	14	9	29
	Pathogenesis-related	17	4	10	1	7
	Unspecified abiotic stress	24	3	11	5	12
Total		113	22	50	17	62

Analysis of expression of genes under moderate (RWC about 85 %) or severe (RWC 55–60 %) water deficit showed that only a small number of genes are already induced or repressed under moderate drought. On the contrary, induction or repression of most genes was more common after severe drought, even for genes that are usually described as responding promptly to changes in environmental conditions, such as those encoding transcription factors. This data

Table 3 Number of poplar genes belonging to gene families involved in ABA-related drought response, or in ABA-independent signalling pathways, selected according to Cohen et al. (2010), that were up- or down-regulated in moderately (*D1*) or severely (*D2*) droughted *P. deltoides* x *P. nigra* plants

ABA-related gene families[a]	Total genes	Up-regulated		Down-regulated	
		In D1	In D2	In D1	In D2
ABA biosynthesis					
1-deoxy-D-xylulose-5-phosphate synthase (DXS)	3	0	2	0	1
Phytoene synthase (PSY)/desaturase (PDS)	5	0	1	1	4
Lycopene cyclase (LYC)	5	0	2	0	3
9-*cis*-Epoxycarotenoid dioxygenase (NCED)	3	0	2	0	1
Zeaxanthin epoxidase(ZEP)	2	0	0	0	2
ABA-mediated signalling pathway					
ABRE binding factors	2	0	2	0	0
SNF1-related protein kinase (OST1/SNRK2-6)	3	1	2	0	1
Negative regulation of ABA signalling pathway					
ABI5 binding protein(ABF)	2	1	1	0	0
Early responsive to dehydration (ERD15)	2	0	2	0	0
DEAD-box RNA helicase	3	0	1	0	2
Response to ABA stimulus					
Nuclear factor Y (NF-YA)	3	2	1	0	1
Cold-regulated 413-plasma membrane (COR413-PMs)	2	0	1	0	1
Remorin (REM)	5	0	3	0	2
ABA-independent signalling pathway					
C2H2-type zinc finger (ZAT12)	4	1	2	1	2
Nuclear factor Y (NF-YB)	2	0	1	0	1

[a] Only genes mapped by more than one read per million were analysed

indicates that for many poplar genes, a moderate reduction of relative water content (as that observed in D1) was not the signalling event in determining change in gene expression, confirming the data reported for *P. euphratica*, in which the highest number of up- and down-regulated genes was observed in severely droughted plants compared to plants coping with lower stress conditions (Yan et al. 2012).

The transcriptome data obtained in our study of drought response in a poplar hybrid were compatible with the drought transcriptomes observed for most other plants, including poplar (Yan et al. 2012). However, some differences were seen, especially at gene family level, for which some members of one and the same family were up-regulated by drought and others were down-regulated. Similarities and differences in microarray-analysed transcriptomes of poplar species, tissues, and organs, have been classified in order to obtain a meta-transcriptome, i.e. to determine what genes are always activated, for example, in drought response and what genes are, on the contrary, specifically activated in one tissue or one species (Cohen et al. 2010). The activation or repression of these latter genes might determine the phenotypic differences amongst individuals and/or species in the response to drought. Re-analysis of transcriptomes using high-throughput sequencing technologies should facilitate such comparative studies, allowing a fine distinction amongst members of one and the same gene family.

Systems biology approaches have given us a more holistic view of the molecular responses. The responses of plants to abiotic stimula are dynamic and complex and cannot be based only on the analysis of gene expression; they also require the integration of multiple omics studies (Cramer et al. 2011). Many gene categories revealed by MapMan analyses showed that within a family, some paralogous genes are repressed and others are induced. For example, secondary metabolism was deeply affected by drought, not only in inducing the activity of some genes, but also in repressing other genes. For ABA-related genes, the activation of most gene families by drought was in general agreement with the findings of Cohen et al. (2010). Only zeaxanthin epoxidase encoding genes show a different regulation compared to data reported by Cohen et al. (2010). However, it is worth noting that other genes belonging to this gene family (i.e. encoding the same product) resulted as up-regulated in our experiments, though at a fold change lower than 2. On the whole, fine regulation of expression in the different gene families, with both up- and down-regulated elements, can be seen from our data.

Structural genomic studies are necessary to establish whether such differences between members of one and the same gene family can be ascribed to differences in *cis*-regulatory sequences. It is also possible that even different alleles of the same gene may produce different responses to the same drought levels, so contributing to a variation in tolerance to drought among different poplar genotypes.

Differences in gene expression can also be related to epigenetic regulation by the environment. Great changes in DNA methylation have been observed amongst poplar clones, possibly influencing their response to drought (Raj et al. 2011). High-throughput sequencing technologies provide new opportunities to analyse non-coding RNAs and can clarify aspects of epigenetic regulation of gene expression (Gregory et al. 2008; Zhang et al. 2006). Such analyses have elucidated the global transcriptomes of plants exposed to abiotic stresses such as dehydration, cold, heat, high salinity, osmotic stress, and ABA (Matsui et al. 2008; Zeller et al. 2009), indicating that these stresses not only increase or decrease transcript abundance of stress-responsive genes, but also transcription of thousands of unannotated non-protein-coding regions. Matsui et al. (2008) estimated that approximately 80 % of previously unannotated up-regulated transcripts arise from antisense strands of sense transcripts. In our analyses, 32.4 % of Illumina reads could not be mapped to previously annotated genes. It is possible that many of these do represent antisense transcripts, whose biological function is still to be clarified, but could probably be involved in the epigenetic regulation of drought tolerance. A number of drought-related microRNAs have recently been identified in *P. euphratica* (Li et al. 2011). This dataset and specific microRNA datasets of *P. deltoides* x *P. nigra* hybrids will be useful for clarifying the nature and the function of unannotated transcripts.

Acknowledgments This research work was supported by PRIN-MIUR, Italy, project "Verso la delucidazione delle basi molecolari dell'eterosi nelle piante coltivate: variazione cis-regolatoria ed espressione genica in ibridi di pioppo".

Thanks are due to Dr. Catherine Bastien (INRA-UAGPF, Orleans, France) for ensuring availability of the hybrid plants, to Dr. Michele Braidotti (Arizona Genomics Institute) for collaboration on data handling and treatment, and to Dr. John A. Walsh (Warwick University, UK) for critical reading of the manuscript.

Data archiving statement All cDNA raw Illumina sequences used in this work are available at the NCBI Sequence Read Archive under the accession number SRP024267 (Submission: Populus x canadensis RNAseq).

The global analysis of gene expression is reported as an excel file, available at the Department of Agriculture, Food, and Environment of Pisa University repository website (http://www.agr.unipi.it/Sequence-Repository.358.0.html), in which each gene was represented by its absolute expression level in control, moderately dehydrated and severely dehydrated leaves.

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