# Adaptive mechanisms and genomic plasticity for drought tolerance identified in European black poplar (*Populus nigra* L.)

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Running title: ADAPTATION TO drought in poplar

# Abstract

Summer droughts are likely to increase in frequency and intensity across Europe, yet long-lived trees may have limited drought resilience. It is therefore critical that we improve our understanding of phenotypic plasticity to drought in natural populations for ecologically and economically important trees such as *Populus.* A common garden experiment was conducted using approximately 500 wild *Populus nigra* trees, collected from 11 river populations across Europe. Phenotypic variation was found across the collection, with southern genotypes from Spain and France characterised by small leaves and limited biomass production. To examine the relationship between phenotypic variation and drought resilience, six genotypes with contrasting leaf morphologies were subjected to a water deficit experiment. ‘North eastern’ genotypes were collected at wet sites and responded to water deficit with reduced biomass growth, slow stomatal closure and reduced water use efficiency (WUE) assessed by Δ13C. In contrast, ‘southern’ genotypes originating from arid sites showed rapid stomatal closure, improved WUE and limited leaf loss. Transcriptome analyses of a genotype from Spain (Sp2, originating from an arid site) and another from northern Italy (Ita, originating from a wet site), revealed dramatic differences in gene expression response to water deficit. Transcripts controlling leaf development and stomatal patterning, including *SPCH, ANT, ER, AS1*, AS*2, PHB, CLV1, ERL1–3* and *TMM* were down-regulated in Ita but not Sp2 in response to drought.

**Keywords:** Black poplar (*Populus nigra*),carbon isotope discrimination (Δ13C), stomatal number, drought, water deficit, microarray

# Introduction

Forests in Europe and elsewhere are likely to experience unprecedented rises in temperature and increases in the frequency and intensity of summer droughts in the future (Lindner et al. 2010; IPCC, 2014). The capacity for long-lived forest trees to adapt to such a rapidly changing climate is determined by adjustments to functional traits including both morphological and physiological levels. This phenotypic plasticity allows trees to respond to a rapidly changing climate and thus provides a mechanism for acclimation (Bussotti et al., 2015). Although recent droughts in Europe have had major effects on forest tree mortality ([Solberg 2004](#_ENREF_75); [Bréda & Badeau 2008](#_ENREF_9); Allen et al. 2010), high phenotypic plasticity could enable populations to survive in a changing environment (Benito-Garzón et al., 2011), where moderate droughts will be increasingly common. As such, understanding phenotypic responses to drought provides an important insight into likely long-term genetic adaptations (Alberto et al. 2013).

The traits which underpin drought tolerance are complex and may vary in importance depending on severity, duration and timing of the drought (Bréda et al., 2008; Tardieu & Tuberosa 2010), but they include reduced leaf size and number, ABA-dependent and -independent signalling, lowered stomatal aperture and numbers, reduced stomatal conductance (gs), decreased leaf growth and altered patterns of root development (Tardieu & Tuberosa 2010). Improved water use efficiency (WUE), is also associated with severe drought, where WUE is the ratio between net carbon assimilation (A) and transpiration rate (E), and is negatively associated with carbon isotope discrimination (Δ13C) or positively correlated with carbon isotope composition (δ13C) ([Farquhar & Richard 1984](#_ENREF_26); [Farquhar et al. 1989](#_ENREF_25); [Condon et al. 2002](#_ENREF_19)). How these traits link to their underlying regulatory gene and metabolism networks is being elucidated in poplar, where gene expression in response to drought has been followed in *P. deltoides* and *P. trichocarpa* ([Streetet al. 2006](#_ENREF_77)), *P. euphratica* ([Bogeat-Triboulotet al. 2007](#_ENREF_7)), *Populus* clones of *P. deltoides* x *P. nigra* ([Cohenet al. 2010](#_ENREF_18)), *P. balsamifera* (Hamanishi et al. 2010; Hamanishi et al. 2015) and *P. nigra* x *P. maximowiczii* ([Wilkinset al. 2009](#_ENREF_90)).

Furthermore, microarray studies on drought tolerance in *Populus balsamifera* have identified variation in the pattern of transcript abundance between genotypes, which was correlated to growth maintenance after a water deficit (Hamanishi et al. 2010). These important studies generally focus on using commercial tree genotypes to elucidate gene expression changes which may be involved in determining water deficit responses. However, there is a limited understanding of genomic responses to drought in wild collections, which may harbour genetic potential for adaptation and increasingly provide the focus for large-scale genomic and genetic analysis of links between traits and genes.

Natural collections of trees obtained from diverse climatic zones have traditionally been used to establish common garden experiments for phenology trials (Rohde et al. 2011), but their use for more complex genomic analysis is now emerging. Recently, the potential to exploit natural genetic variation has been recognised in *Arabidopsis* with genome wide association studies for traits becoming routine (Atwell et al. 2010), but the relevance of *Arabidopsis* for understanding tree adaptation may be limited (Taylor 2002). Drought tolerance is an obviously multi-genic trait and genomic technologies allow the investigation of such traits, in contrast to traditional single gene studies which can limit the focus to the interaction between a small number of genes and therefore impede the identification of different pathways involved in drought response and adaptation.

European black poplar (*Populus nigra*) is a riparian species that is widely distributed in Europe, North Africa, and Central and West Asia ([Vanden Broeck 2003](#_ENREF_84)). It has many economic uses, including domestic plantations and breeding programs ([Vanden Broeck 2003](#_ENREF_84)). Ecologically, *P. nigra* is a keystone riparian species ([Vanden Broeck 2003](#_ENREF_84)), threatened by river drainage, water management ([Gaudet et al. 2008](#_ENREF_30)) and climate change. Understanding phenotypic plasticity in response to drought of *P. nigra* is important. *Populus* is also widely accepted to be a model tree since it is fast-growing, its genome is fully sequenced and there are a wide array of applicable genomic and genetic resources available ([Taylor 2002](#_ENREF_78); [Tuskan et al. 2004](#_ENREF_83); [Tuskan et al. 2006](#_ENREF_82); [Jansson & Douglas 2007](#_ENREF_39)). Although poplars are considered sensitive to drought as they are abundant in riparian environments and often have a high demand for water ([Dreyer et al. 2004](#_ENREF_23); [Street et al. 2006](#_ENREF_77)), considerable variation in response to water deficit has been observed between genotypes of *Populus* ([Marron et al. 2002](#_ENREF_47); [Monclus et al. 2006](#_ENREF_51); [Street et al. 2006](#_ENREF_77); [Huang et al. 2009](#_ENREF_38); [Regier et al. 2009](#_ENREF_65); [Cocozza et al. 2010](#_ENREF_17); Viger et al. 2013).

The aims of this study were; 1) to quantify natural variation in a wide natural collection of black poplar for traits related to drought tolerance, 2) to elucidate phenotypic plasticity in response to drought in a group of genotypes based on this study and 3) to determine the transcriptomic differences underlying drought tolerance in extreme genotypes from this natural collection. Alongside these aims, the relationship between drought tolerance and tree adaptation to their region of origin, which differ particularly in precipitation was examined. We have used this three-fold approach to identify the genomic basis of drought tolerance and adaptation in this important riparian tree species.

# Material and Methods

## Common garden experiment

### Plant material and growth conditions

Cuttings from wild trees of *P. nigra* of 479 genotypes were collected from five different European countries: Spain, France, Italy, Germany and The Netherlands (Table S1). Genotypes were grouped into 11 populations related to the river system near the collection (Fig. 1). Hardwood cuttings were planted in a common garden in Belgium, Geraardsbergen (50º 46‟51.23”N) in the spring 2004, cut at the base in the spring 2005, and side stems removed so that trees grew as single stems in June 2005. The experiment followed a randomised block design with six blocks each containing one replicate of each genotype with a double row of *Populus* ‘Muur’ planted around the six blocks. The trees were planted at 0.75 m x 2 m spacing. The site was rain-fed and not fertilised between March and September, but it was weed controlled and treated with fungicides every three weeks during this period.

### Assessing phenotypic traits in the *Populus nigra* collection

Each replicate was assessed for twelve morphological traits over three growing seasons (2005, 2006 and 2007). The youngest fully mature leaf was harvested, traced while fresh and placed in a paper bag. Leaf outlines were scanned using an Umax Astra 6700 scanner and assessed using Image J software (Image J.1.32.j, Wayne Rasband, USA). Leaf outlines were measured for leaf area (mm2), leaf length (mm) and leaf width (mm), from which the leaf ratio (length:width) was calculated. Leaves collected in the second growing season (2005) were placed in paper bags, dried for 48h at 80oC, and weighed to calculate specific leaf area (SLA) as the ratio of leaf area (mm2) to leaf dry weight (mg).

Epidermal cell number and size was measured using cell imprints taken in 2006 from the first interveinal region of the abaxial surface of the first fully mature leaf following the methods of [Gardner et al. (1995](#_ENREF_29)). Images of cell imprints were assessed in Image J ([Abramoff et al. 2004](#_ENREF_1)) to count the number of cells and stomata per unit area and average cell area of ten cells per leaf. From these measures stomatal density (ratio of stomata number per unit area), stomatal index (ratio of the number of stomata per total cell number as a percentage), and cell number per leaf, estimated as the ratio of leaf area (mm2) to cell area (mm2), were calculated. Two measures indicative of biomass were also assessed for each replicate. Height was recorded following the second year of growth (2005), and circumference 1 m above ground level was assessed following the second (2006) and third year of growth (2007).

Wood was collected in March 2007, with 30 cm sections cut from 40 cm above ground, then put in a plastic bag and stored in a cold room. Samples were debarked and cut into small pieces for Δ13C measurements. Samples were dried in the oven for 48h at 80ºC before being ground using a ball grinder (Glen Creston ball, Retsch MM300, London, UK) and stored in a glass container. 1 mg of material was weighed and placed into a 6 x 4 mm tin capsule (Elemental Microanalysis, Devon, UK). Samples were analysed using a SerCon 20-20 Stable Isotope Analyzer with ANCA-GSL Solid/Liquid Preparation Module (SerCon, Crewe, UK). Carbon isotope composition was determined by δ13C (‰) = δplant = [(Rsample - Rreference) / Rreference] × 1000, where Rsample and Rreference are the 13C/12C ratios of the sample and the reference respectively, in VPDB (Vienna Pee Dee Belemnite) units ([Scrimgeour et al. 2004](#_ENREF_70)). Carbon isotope discrimination was calculated as Δ13C (‰) = [(δair - δplant)/(1+( δplant /1000)] with δair assumed to be -8‰ ([Farquhar & Richard 1984](#_ENREF_26); [Monclus et al. 2006](#_ENREF_51)).

## Drought experiment

### Plant material and growth conditions

In order to examine phenotypic plasticity related to water deficit, a subset of trees were chosen for a moderate drought glasshouse experiment in Southampton, UK. Six genotypes were selected from the *P. nigra* collection (Table S2): four from the extreme “leaf size” genotypes (two Spanish ‘small leaf’, Sp1, Sp2; one Italian ‘large leaf’, Ita and one from the Netherlands ‘large leaf’, NL) and two from the Drôme population in France (Fr1, Fr2). These genotypes were chosen to test the hypothesis that ‘small leaf’ genotypes are indicative of adaptation to low water availability. The French genotypes were selected to represent a range of temperatures and precipitation patterns, since the French river populations span a diverse range of climatic conditions between Spain, Italy and the Netherlands. Cuttings were planted in John Innes No. 2 (John Innes, Norwich, UK) without fertilisation in January 2007 in a glasshouse and cut back in November 2007 at 10 cm from the base. They were watered daily and put into dormancy conditions (natural light, 15 ºC:13 ºC day:night). In May 2008, the temperature in the glasshouse was set at 22 ºC:16 ºC, day:night. During the experiment, photoperiod was maintained 16 h:8 h, light:dark with a minimum photosynthetic active radiation at the top of the plants of 150 μmol m-2 s-1, supplementing natural daylight. The number of replicates for each genotype varied between 5 and 10 per condition (Table S2). The trees were randomised in 10 blocks containing one replicate per genotype per treatment.

At the start of the experiment on September 1st 2008, 200 ml of water was added to each tree and the pots were then covered in aluminium foil to prevent water evaporation. The first mature leaf and the first emerging young leaf were tagged with cotton string. Over the next month (30 days), soil moisture content was measured every morning with a Delta-T ML2x ThetaProbe connected to an HH2 moisture meter (Delta-T Devices, Cambridge, UK). Well-watered trees (control) were watered to field capacity and drought stressed trees were kept between 15–20 % percent volume soil moisture as determined as a suitable moderate drought treatment for poplar by Street and colleagues (2006). Using a repeated measurements test over time, soil moisture content showed significant differences between treatment (F1,50=363.17, p<0.001) but no significant differences between genotypes (F5,50=1.06, p=0.392) and no genotype x treatment interaction effect (F5,50=0.82, p=0.543), meaning all the genotypes had their soil moisture decreased equally under drought (Fig.3, Fig. S1, Fig. S2).

### Physiological and growth measurements

Biomass measurements were conducted on September 1st 2008 (0 day after drought (DAD)) and September 17th 2008 (16DAD). Measures included height (cm), stem diameter (mm, measured using digital callipers at 10 cm from the stem base), the number of branches, and the number of leaves. Height and stem diameter growth were calculated as the difference between 0DAD and 16DAD. The number of branches and leaves developed during the experiment was calculated as the difference between the 0DAD and 16DAD for branch number and leaf number respectively. Leaves newly developed (NLN) during the experiment above the tag on the first emerging leaf were also counted at 16DAD and used with the total number of leaves at 0DAD and 16DAD to calculate the number of fallen leaves, as senescence = (NL16DAD-NL0DAD)-NLN. The third mature leaf (counting from the uppermost mature leaf) was sampled at 27DAD, traced, and dried as described above. Dried leaves were used to calculate SLA, the ratio of leaf area in cm2 (prior to drying) to leaf dry mass in grams ([Marron *et al.* 2005](#_ENREF_48)).

The first three leaves which emerged on the main stem during the experiment were followed for leaf area using the leaf tagged on 0DAD. The contour of the leaves was traced onto paper before the images were scanned and processed using ImageJ ([Abramoff et al. 2004](#_ENREF_1)). Stomatal conductance (*g*s) was measured on the first mature leaf tagged at 0DAD, 5DAD and 15DAD, using a steady-state porometer (LI-1600; LICOR, Inc. Lincoln, Nebraska, USA). In order to examine variation in WUE, a young leaf (third leaf from the top) of each tree was placed in a paper bag on 19DAD and oven dried. Δ13C was measured as described for the wood collected in Belgium.

### Gene expression analysis

Young leaves were sampled on 19DAD for gene-expression analyses (microarrays and real-time PCR). Two genotypes – one from Spain (Sp2) and one from Italy (Ita) were selected for microarray analysis based on being the most extreme genotypes in terms of morphology. Each sample (the first two unfurled leaves) was flash frozen in liquid nitrogen and stored at -80°C for further analysis. RNA was extracted following the CTAB protocol from [Chang et al. (1993](#_ENREF_15)). Eight RNA samples, corresponding to two biological replicates of both well-watered and drought treatments per genotype, were sent to the European Arabidopsis Stock Centre (NASC, Loughborough, UK) microarray service for the cDNA synthesis, fragmentation, array hybridization and scanning using Affymetrix GeneChip Poplar Genome Arrays (Affymetrix, Santa Clara, USA). Affymetrix CEL files were imported into R software (Core Team, 2014, R Foundation for Statistical Computing, http://www.R-project.org). Probe sets exhibiting no signal intensity were filtered out by a Present call procedure as described by McClintick and Edenberg (2006). Briefly, CEL files were normalised using the MAS5 algorithm with default parameters (affy package, v1.48.0, Gauthier et al, 2004). MAS5 provides a detection call, Absent (A), Present (P) or Marginal (M), which indicates whether the specific transcript is detectable. For each probe set, the percentage of Present calls in each condition was calculated. Probe sets that exhibited a percentage of Present calls of 100% in at least one condition for both genotypes were kept. The other probe sets were removed from the analysis. This procedure also allowed probe sets that hybridized exclusively to one genotype to be discarded (Cohen et al., 2010). Finally, 31084 validated probe sets were retained. In order to compute differential gene expression, CEL files were then normalised using the RMA algorithm with default parameters (affy package, v1.48.0). Differential expression was calculated as log2(Fold Change) between drought and control samples for the 31084 validated probe sets. Statistical significance of differential expression was tested using moderated t-tests implemented in the eBayes function (limma package v3.24.12, Smyth, 2004) and FDR corrections for multiple-testing were applied. Thresholds of |Log2(FC)|≥1 and corrected p-value<0.05 were used to identify differentially expressed genes. Probe sets were annotated using the Poparray website (http://aspendb.uga.edu/poparray) and assigned to a Populus gene model (v. 3.0) and its closest *Arabidopsis* homolog, and GO (Gene Ontology) biological process, cellular component and molecular function classifications.

The software Mapman ([Thimm et al., 2004](#_ENREF_369)) was used for pathway analysis. Statistics (Wilcoxon Rank Sum Test with a Benjamini-Hochberg FDR correction) were implemented in Mapman to reveal BINs exhibiting a significant difference in expression profile behaviour compared to the other BINs. Gene ontology enrichment was also studied using the PAGE tool on AgriGo web site ([Du et al. 2010](#_ENREF_24)) with default parameters using validated probe sets as the reference.

Results of the microarray experiment were confirmed using quantitative real-time PCR (qPCR) for a set of differentially expressed candidate genes. Forward and reverse primers were designed, from the *Populus trichocarpa* genome (v1.2), specifically to each gene (Table S3). Reverse transcription of RNA to cDNA was performed using the ImProm-II Reverse Transcription kit (Promega UK, Southampton, UK) following the manufacturer’s instructions. Each qPCR reaction was composed of 5 µL 2X Precision-SY Master Mix (PrimerDesign Ltd, UK), 5 pmol forward and reverse primers and 25 ng diluted cDNA. Plates were run on a Chrom4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, USA). Reactions were incubated at 95 °C for 10 minutes and then 40 cycles of 15 seconds at 95 °C, 1 minute at 60°C and a plate read. An incubation at 72 °C for 10 minutes followed. A melting curve was then performed from 60 °C to 95 °C with a read every 0.2 °C and 1 second hold, in order to check for primer dimers, DNA contamination and secondary products. Values were exported with the software Opticon Monitor 3.1 (Bio-Rad Laboratories, Hercules, USA). Amplification efficiency was measured following the equation from Liu & Saint (2002):

E = (Rn,A / Rn,B) ^ [1/CT,A-CT,B) ] + 1

where Rn,A and Rn,B are Rn at arbitrary thresholds A and B in an individual curve, respectively, and CT,A and CT,B are the threshold cycles at these arbitrary thresholds ([Liu & Saint 2002](#_ENREF_45)).

Ratios were calculated as $\frac{E^{\left(control-drought\right)target}}{E^{\left(control-drought\right)reference}}$

## Statistical analysis

Data from the Belgium common garden experiment were analysed using the SPSS software package (SPSS, Chicago, USA). Kolmogorov-Smirnov tests were used to test for normality and transformation (log10) was carried out when required. A GLM tested the effects of block and river population:

*Y ij = µ + α i + β j + ε*

where *Yij* is the phenotype in the *i*th block and in the *j*th river population, α*i* is the block effect, β*j* is the river population effect, and ε is the residual error. A comparison of means was carried out between river populations using a Student-Newman-Keuls post-hoc test.

A GLM was also performed to test genotype effects:

*Y i = µ + α i + ε*

where *Yi* is the phenotype in the *i*th genotype, α*i* is the genotype effect and ε is the residual error.

Climatic data from the region of origin for genotypes were correlated with and among phenotypic traits as measured in the common garden was tested using Spearman’s rho in SPSS v19.0 (SPSS, Chicago, USA).

Data from the glasshouse experiment were also analysed for genotype and treatment effect (and their interaction) using the SPSS software package (SPSS, Chicago, USA). Normality (Kolmogorov-Smirnov test) and block effects were checked before performing an ANOVA GLM. Data were transformed using a natural log when required. A GLM tested the effects of genotype and treatment:

*Y ij = µ + α i + β j + ε*

where *Yij* is the phenotype in the *i*th genotype and in the *j*th treatment, α*i* is the genotype effect, β*j* is the treatment effect, and ε is the residual error. A comparison of means was carried out among genotypes using a Student-Newman-Keuls post-hoc test. A test for repeated measurements was used for leaf area over time for each leaf number. Phenotypic plasticity in drought response was quantified using the equation $\frac{drought-control}{control} x 100 $ from Street et al. (2006).

# Results

## Common garden

The collection of nearly 500 genotypes of *P. nigra* selected from contrasting climatic zones across Europe was used to study natural variation in wood carbon isotope discrimination (Δ13C), leaf, cell and biomass traits for trees grown under well-watered conditions in a Belgium common garden field site (Fig. 1). Significant differences in plant morphology were observed between natural populations. Leaf area, stem circumference and Δ13C varied significantly between river sites (*F*10,482=129.8, p<0.001; *F*10,453=35.2, p<0.001; *F*10,466=33.5, p<0.001 respectively with Type III sums of squares). For stomatal index (SI), although the difference was not significant (p<0.05), a trend was apparent with northern populations (Italy, The Netherlands and Germany) exhibiting a high SI, while genotypes from French populations showed a lower SI. In contrast to other measurements, the Spanish genotypes were intermediate in their ranking (Fig. 1e). Leaf area and stem circumference were highest in the northern river populations (Italy, The Netherlands and Germany), while Spanish and Southern French genotypes had the smallest leaves and stem circumference (Fig. 1d and 1f). Δ13C tended to decrease along this north-south latitudinal gradient, although there were exceptions, such as for genotypes from west Loire (Fig. 1g), but suggests that, under well-watered conditions, water use efficiency was higher for genotypes from northern latitudes, when grown in the Belgian common garden.

Correlation between leaf, cell, and biomass traits within and among growing seasons showed that leaf area correlated with tree height and circumference, both of which are woody biomass traits (Table S4, Fig. 2). In the third year of growth (2006), when leaf cell traits were measured, there was a strong positive correlation between cell number per leaf and leaf area (Table S4, Fig. 2, R2 = 0.927, *P* < 0.0001) but a weak negative correlation between cell area and leaf area (Table S4, Fig. 2, R2 = -0.235, *P* < 0.0001). Furthermore, stomatal patterning correlated strongly with all biomass traits with the exception of stomatal index showing no relationship to leaf shape ratios in either 2005 or 2006 (R2 = -0.059, *P* < 0.85; R2 = 0.025, *P* < 0.429 respectively). Precipitation at the genotype site of origin correlated with leaf and stem phenotypic traits with higher precipitation (mean annual, minimum and maximum) correlated with increased leaf areas, which are made up of a greater number of smaller cells per leaf with more stomata, higher SLA, and increased stem height and circumference (Table S4). Additionally, higher temperatures (mean annual, minimum and maximum) correlated with leaf shape ratio and SLA. The temperature of the coolest month seems most important with respect to leaf area and cell number per leaf, as well as stem height and circumference in 2005 and 2006 respectively. However, mean annual temperature and the temperature of the warmest month also correlated with reduced leaf cell size and stem circumference and increased abaxial stomatal density (Table S4).

## Drought experiment

Six contrasting genotypes were selected from the common garden trial to further elucidate phenotypic plasticity in response to drought, and how this varied across genotypes adapted to local drought conditions. These genotypes were subjected to a moderate drought in a controlled environment glasshouse in southern England (Fig. 3a). Variation in response to drought was observed across the six selected genotypes (Table 1). Interaction between genotype and treatment was significant for Δ13C, from a two-way ANOVA and close to significant (p<0.10) for stem growth. Five of the nine traits measured, showed both genotype and drought main ANOVA effects, whilst highly significant drought effects were observed for stomatal conductance, Δ13C, leaf production and growth traits (Table 1). Furthermore, SLA varied significantly between genotypes but was unaffected by the drought treatment (Table 1).

### Biomass

Images taken on 20DAD revealed the main morphological variation in response to drought across the six selected genotypes (represented by four genotypes in Fig. 3b). Biomass production was also measured (Table S5, Fig.4) and revealed that height growth decreased for all genotypes in response to drought (genotype: *F*5,85=6.6, p<0.001; treatment: *F*1,85=37.1, p<0.001) with the largest decrease (-86%) for the Ita genotype (Fig. 4a). Fr1 and Sp2 maintained some height growth under drought with only moderate reductions apparent (-32% and -37% respectively).

Differences between genotypes were apparent for both leaf production (formation) and leaf loss (senescence). Leaf production differed significantly between genotypes (*F*5,91=16.2, p<0.001). In addition, leaf production was significantly impacted by drought, particularly in Ita, Fr2 and NL (*F*1,91=25.0, p<0.001, Fig.4c). One Spanish genotype (Sp2) continued to develop approximately the same number of new leaves during exposure to drought (an average of 6.0 leaves) – similar to that in well-watered conditions (6.8 leaves). In the well-watered treatment, Sp1 developed the most new leaves during the experiment (8.88) while trees from Italy only formed an average of 3.89 new leaves. Leaf senescence and loss on the main stem increased significantly under drought (*F*1,86=5.2, p=0.025) but significant genotype effects were also apparent (*F*5,86=2.5, p=0.036). French and Spanish genotypes lost more leaves (Fig. 4d) while trees from Italy and The Netherlands largely retained leaves. Sp2 also developed two to four more branches on average in drought compared to well-watered conditions (Fig. 4e). However, this trait did not show any significant genotype (*F*5,81=0.697, p=0.627) or treatment effects (*F*1,81=0.948, p=0.33). Genotypes NL, Ita and Fr2 developed no branches in response to water deficit. SLA was measured at the end of the experiment and revealed a significant genotype (*F*5,87=10.5, p<0.001) but not a treatment effect (*F*1,87=3.0, p=0.09)

### Stomatal conductance and carbon isotope discrimination

Stomatal conductance was measured during the progression of drought (Table S5, Fig. 4g,h). Early after the onset of drought (5DAD, Fig. 4g), Spanish and French genotypes reacted quickly to water deficit with stomatal conductance declining rapidly by -54 and -36%, respectively (genotype: *F*5,96=5.1, p<0.001, treatment: *F*1,96=15.9, p<0.001). In contrast, the Ita genotype showed a small increase in stomatal conductance in response to drought (3.4%) and NL a moderate decline (-17%). After 15 days of drought (Fig. 4h), these contrasting early responses of stomata to drought were no longer apparent and all genotypes showed a significant decline in stomatal conductance (*F*1,92=103.1, p<0.001). Young leaves developed during the experiment were collected to measure Δ13C (Fig. 4i). Δ13C showed significant variation between genotypes (*F*5,58=5.9, p<0.001), a highly significant effect of drought (*F*1,58=7.5, p=0.008) and a significant interaction of genotype x treatment (*F*5,58= 2.6, p=0.037), indicating that the response to drought differed depending on genotype. While Sp1, Sp2 and Fr1 decreased their Δ13C by around 10% during the drought treatment, possibly indicating an increase in water use efficiency (WUE), Fr2 showed no variation between treatment and Ita increased Δ13C under drought.

### Leaf growth

Leaf area was measured for the first three leaves emerging from 1–19DAD (Fig. 5). Genotype had a significant effect on leaf area for all leaf numbers (Leaf 1: *F*5,82=7.538, p<0.001; Leaf 2: *F*5,54=6.162, p<0.001; Leaf 3: *F*5,36=6.328, p<0.001). The effect of treatment was also significant (Leaf 1: *F*1,82=21.75, p<0.001; Leaf 2: *F*1,54=26.86, p<0.001; Leaf 3: *F*1,36=23.69, p<0.001) but genotype and treatment did not interact. For the trees under well-watered conditions, both Spanish genotypes had the smallest leaves (1700 mm2 and 1000 mm2 on average respectively) and the Italian had the largest leaves (4700 mm2 on average for Leaf 1). This rank order and size distribution was consistent with that observed in the common garden experiment, indicating the greenhouse conditions did not change the phenotypic differences in these plants. Sp2 showed the smallest reduction in leaf area (-21.2 %) and Fr2 the largest reduction (-66.3%) in response to drought.

### Transcriptome response to drought

Dramatic differences were apparent in the transcriptomic responses to drought in the contrasting Spanish and Italian genotypes selected for gene expression analysis (Table S9). In the Northern Italian genotype (Ita), 8857 probe sets displayed a significant two-fold change in intensity in response to drought (3610 up-regulations and 5247 down-regulations, Fig. 6a,b). In contrast, for the Spanish genotype (Sp2), only 1067 probe sets exhibited a two-fold differential expression between control and drought conditions (338 up-regulations and 729 down-regulations, Fig. 6a,b). Only 258 probe sets were commonly up-regulated between the two genotypes under drought and 643 were commonly down-regulated in response to drought (Fig. 6c, Table S9).

A combination of pathway analysis from Mapman and PAGE analysis from AgriGO allowed functional enrichments to be identified (Table 3, Fig. 8). Only three bins were significant in the Mapman analysis for the Spanish genotype in response to drought (Table 3, Table S7 for full details): Cell (p= 0.0000003), secondary metabolism (p=0.01) and transport (p=0.000062). The Italian genotype had 24 Mapman bins which were significant (p<0.05) including DNA, RNA, cell, stress, transport, hormone metabolism and signalling (Table 3, Table S7 for full details).

PAGE analysis of drought responsive genes confirmed the results from Mapman and allowed 453 and 115 significantly enriched GO terms to be highlighted for Ita and Sp2, respectively (Table S8). Eighty three GO terms were commonly enriched for both genotypes. Among the 31 common down-regulated biological processes (Table S8), 50% were related to cell division (e.g “mitosis”, “DNA metabolic process”, “chromosome organisation”, “cell cycle”, etc.). Other negatively regulated processes were also found such as “regulation of gene expression” and “secondary metabolic process”. Additionally, GO analysis revealed enrichment of up-regulated biological processes related to transport (GO:0006810, GO:0006812, GO:0006811), response to stress and stimuli (GO:0006950, GO:0042221, GO:0050896, GO:0009628), and carbohydrate catabolism (GO:0016052, GO:0019320, GO:0006007, GO:0046365, GO:0006090) for both Ita and Sp2 (Fig. 8, Table S8). For the Spanish genotype only, functional enrichment was detected for repressed processes such as phenylpropanoid and flavonoid biosynthesis, and for induced ones involved in nucleotide and lipid metabolisms. Among the 247 biological processes enriched specifically for Ita, 135 are up-regulated including GO terms assigned to response to hormone (abscisic acid, auxin, cytokinin, salicylic acid and jasmonate), response to abiotic and biotic stress (e.g “response to water deprivation, “response to osmotic stress”, response to oxidative stress, “response to biotic stimulus”, etc.), metabolism and catabolism of amino acid, and to transport (ion, carbohydrate peptide, etc.). Finally, 112 down-regulated biological processes were found to be enriched for Ita only and are predominantly related to growth, development, cell division and morphogenesis. Among these down-regulated developmental processes, of particular interest were “stomatal complex development” and its parent term “organ development” that encompassed drought responsive genes. Among drought responsive genes, of particular interest were those related to stomatal development and patterning (Fig. 7) and leaf development (Table 2), since these showed marked differences between genotypes in response to drought.In Sp2, only four genes were significantly down-regulated in response to drought; two *Erecta* genes *(ERECTA),* one *Erecta-like* coding gene (*ERL2)* and *Mute* (*MUTE*), an ortholog of *Speechless* which did not lead to a functional enrichment.In contrast, eight transcripts determining stomatal patterning were down-regulated in Ita in response to drought, including two *Speechless* orthologs (*SPEECHLESS* and *MUTE*), two *Erecta* coding genes(*ERECTA*), three *Erecta-like* coding genes(*ERL1, ERL2 and ERL3)* and *Too Many Mouths* (*TMM*). Transcripts controlling the activity of the shoot apical meristem and leaf development were also down-regulated in the Italian genotype in response to drought (Table 2), such as five close homologs of *Asymmetric leaves* coding genes(*AS1,* *Potri.017G112300, Potri.006G085900, Potri.004G102600* and *AS2, Potri.010G177100 , Potri.008G079800*), six homologs of *Phabulosa* (*PHB*), *CLAVATA1* (*CLV1*) and five homologs of *Aintegumenta* (*ANT*). Two of the same homologs of *AS1* and *AS2* were down-regulated in Sp2, as well as in one of the *PHB* homologs, but in general, as for stomatal patterning transcripts, there were far fewer changes in Sp2 then in Ita for leaf development transcript response to drought.

### Variation under well-watered conditions

To elucidate constitutive differences in gene expression between the Spanish and Italian genotypes that are present in well-watered conditions, a comparison was also completed for the control dataTable S6). 252 up-regulated and 284 down-regulated transcripts were identified in Sp2 compared to Ita (Fig. 6c). The AgriGO analysis showed enriched GO terms differentially expressed between Sp2 and Ita in well-watered conditions, and these were generally related to secondary metabolism. Also up-regulated in the Spanish genotype were *ERD1* (*EARLY RESPONSE TO DEHYDRATION 1*) AND *RD21* (*RESPONSIVE TO DEHYDRATION 21*).

### Real-time qPCR

Microarray results were validated by real-time qPCR on four candidate genes selected after microarray analysis. Gene expression was quantified for additional genotypes that were not included in the microarray experiment: Fr1 from France and NL from the Netherlands (Fig. S3). Real-time qPCR values were expressed in response to drought for each genotype. *SPEECHLESS* expression ratios were lower in response to drought in both Ita and Sp2, although this response was greater in Ita (*F*3,32=9.311, p<0.001, Figure S3).The expression ratios of ERECTA were reduced in response to drought with no significant difference between genotypes (*F*3,32=0.845, p=0.48, Figure S3).

# Discussion

Our analysis has revealed significant natural variation between populations of black poplar originating from contrasting climatic conditions within Europe. By combining a common garden approach with manipulative experiments and genome-wide gene expression, this study provides considerable insight into the intra-specific variation in drought tolerance for this important keystone riparian tree species. We have identified transcriptome and trait differences that suggest important adaptive mechanisms that exist within the species.

When grown at a single site in northern Europe under well-watered conditions, leaf, cell, and stem size traits differed among genotypes of *P. nigra* (Fig. 1), indicating significant genetic variance. Further, significant correlation among traits were found in established *P. nigra* trees (Table S4, Fig. 2). Consistent with previous studies in *Populus* ([Rae et al. 2004](#_ENREF_64); [Monclus et al. 2005](#_ENREF_50)), leaf area in *P. nigra* correlated with stem circumference and plant height, indicating leaf size is an early diagnostic indicator of biomass (Rae et al. 2009), with genotypes originating from areas of higher precipitation characterised by larger and more deltoid leaves (Fig. 1, Table S4). Smaller leaf morphotypes from Spain and southern France, which originate from regions of lower precipitaion had smaller, more rhomboid leaves. In addition to being an early diagnostic indicator for yield, leaf expansion is known to be highly sensitive to water availability and breeding programs use variation in leaf size as a drought related trait ([Levi et al. 2009](#_ENREF_43); [Ashraf 2010](#_ENREF_4)). Therefore, we hypothesise that Spanish and southern French genotypes have smaller leaves as an adaptation to drought in their native environment. Similar observations have been drawn for two other genotypes of *P. nigra* from contrasting northern and southern (water limited) environments in Italy ([Regier et al. 2009](#_ENREF_65); [Cocozza et al. 2010](#_ENREF_17)).

Although *Populus* is often defined as sensitive to drought, large variation in traits related to drought tolerance and water stress response have been reported, but generally in F1 or F2 hybrids of commercial value, and not for a wild collection such as described here. For example, osmotic adjustment varies across F1 and F2 genotypes ([Marron et al. 2002](#_ENREF_47); [Tschaplinski et al. 2006](#_ENREF_81)), as does leaf expansion ([Street et al. 2006](#_ENREF_77)), leaf abscission ([Street et al. 2006](#_ENREF_77)), WUE ([Rae et al. 2004](#_ENREF_64); [Monclus et al. 2005](#_ENREF_50); [Monclus et al. 2006](#_ENREF_51); [Voltas et al. 2006](#_ENREF_85); [Dillen et al. 2008](#_ENREF_22)) and Δ13C (Monclus et al. 2012). Stomatal traits linked to improved drought tolerance are complex and related to both stomatal function (opening and closing) and stomatal development and patterning. For the population of *P*. *nigra,* genotypic variation was clear in Δ13C and varied with site of origin (Fig. 1). Wood Δ13C was lower in populations from the north and east of Europe, such as The Netherlands, Germany, and northern Italy, and this indicates higher water use efficiency (WUE). However, these trees were collected from wet environments in Europe, comparable to the conditions in the common garden, suggesting that they are particularly well adapted to the Belgian climate. On the other hand, and perhaps counter-intuitively, Spanish and southern French populations had the highest Δ13C, suggesting a lower WUE and poor control of water loss without a reduction in photosynthesis or lowered photosynthetic rates but with unchanged stomatal conductance. However, estimating WUE from Δ13C in this study is complicated by the use of bulk wood (with potential variability in lignin:cellulose ratio) and no quantification of leaf anatomy, which can have different internal conductance to CO2 thus impacting the isotopic model of WUE. It is possible that the lower WUE in genotypes originating from areas of low precipitation is due to different ecological strategies of these genotypes when they are grown in an environment where water is not limited. Although the relationship between Δ13C/WUE varies with climate and can respond positively to precipitation ([Ferrio et al. 2003](#_ENREF_27); [Otieno et al. 2005](#_ENREF_56)), WUE generally decreases with increasing levels of precipitation ([Li et al. 2007](#_ENREF_44)), as we have also shown here in the glasshouse in all but the Ita genotype. In contrast to Δ13C, no significant differences were observed between populations in stomatal index (a measure of stomatal patterning), although there was a trend of increased stomatal numbers in northern and eastern genotypes. Given the potential for stomatal patterning and related genes to affect stomatal conductance and thus WUE ([Woodward et al., 2002](#_ENREF_91); Masle et al., 2005; Roussel et al., 2009), this lack of significance was surprising. Any adaptation to water deficit by the small-leaf morphotypes, characteristic of Spanish trees, is likely, however, to involve additional physiological pathways that are distinct from those controlling stomatal development. It is also possible that stomatal patterning is phenotypically plastic, with differential stomatal patterning occurring in leaves in response to water deficit and our data for gene expression from the drought experiment would support this contention.

Contrasting genotypes were identified from the moderate drought experiment on genotypes from four locations (Fig. 3a), with different adaptive mechanisms apparent for response to drought stress. The ‘northern eastern’ genotype is characteristic of the northern Italian and Netherlands genotypes, originating from areas of high precipitation, where tree productivity and leaf area development is generally high but where height growth and new leaf formation decreased dramatically following the onset of drought. No branches were developed in drought and SLA remained unchanged. At the same time, the Ita and NL genotypes lost few leaves through abscission in response to drought. Stomata responded slowly to drought, and carbon isotope discrimination increased and decreased in Ita and NL, respectively. In contrast, a ‘southern’ genotype, from a region of low precipitation, is characterised by the Spanish and southerly French populations. Slow-growing with small leaves, these genotypes responded to drought with rapid stomatal closure, with the maintenance of leaf expansion (for Sp2) and formation (at least in the extreme example of Sp2), but with some leaf loss. Leaf senescence and loss in response to drought allows remobilisation of nutrients from mature leaves ([Abreu & Munné-Bosch 2008](#_ENREF_2)) and reduced whole-plant transpiration, improving drought tolerance ([Chaves et al. 2003](#_ENREF_16); [Munné-Bosch & Alegre 2004](#_ENREF_52)). Rapid stomatal closure only five days after drought in French and Spanish genotypes supports the idea that variation in stomatal behaviour can exist within species, as was shown by Sparks & Black (1999) in four populations of *Populus trichocarpa* originating from contrasting environments.

Stomatal closure is a biological process to avoid water loss in the event of drought stress but can have other physiological consequences as it can inhibit photosynthesis ([Cornic 2000](#_ENREF_20)). There is a trend in our results which indicates that Sp2 closed stomata more in response to drought when compared to Ita (5DAD and 15DAD) and this correlated with reduced Δ13C under drought, suggesting an increase in WUE in droughted conditions. In a study of δ13C in beech planted in different sites throughout Europe, the highest values (thus the lowest values of Δ13C) were observed in the most southern location in France ([Keitel et al. 2006](#_ENREF_40)). Monclus et al. (2005) studied different genotypes of Populus (tolerant and non-tolerant to drought) and showed that the drought tolerant trees tended to decrease in Δ13C, but the inverse was observed for the non-tolerant genotypes. Thus there is much evidence to show Δ13C varies between tolerant and non-tolerant genotypes and we suggest that this may be due to stomatal responsiveness in tolerant genotypes. However, if this is the case and rapid stomatal closure is an important drought response mechanism, the consequences of this phenotypic plasticity for tree productivity at plantation-scale remain to be elucidated.

Genotypes from Spain and Italy were selected for gene expression analyses because their sites of origin differed markedly in rainfall but not temperature, and thus likely represented contrasting strategies for response to soil water deficits. Given the controlled application of water deficit, with controlled constant temperature in this experiment, it was surprising to see that gene expression changes differed so markedly between the two genotypes, with more than twice the number of differentially expressed genes observed in the Ita compared to the Sp2 genotype. It is remarkable that only 901 transcripts were commonly expressed in response to drought for both genotypes, considering more than 8000 changes in gene expression were observed in total. This result strongly suggests that the Spanish and Italian genotypes are differentially adapted to drought stress and that this involves considerable plasticity in gene expression – manifested in contrasting phenotypic acclimation to the imposed stress. This result is similar to that from Mediterranean species, which has suggested that phenotypic plasticity is lower in plants from low resource environments as part of a conservative resource-use strategy (Valladares et al. 2000). These contrasting patterns of gene expression in *P. nigra*, and their associated phenotypes, provide important clues to aid our understanding of adaptation. This will help to ensure the availability of a resilient gene pool as drought stress increases across Europe, which is a valuable resource for future management and conservation of black poplar.

A larger number of Gene Ontology groups related to ‘response to stimulus’ were significantly expressed in Ita compared to in Sp2, suggesting a highly water stress responsive gene expression pattern in the Ita trees (Fig. 9). Similar conclusions were drawn for salt-stressed ([Walia et al. 2005](#_ENREF_86); [Walia et al. 2007](#_ENREF_87)) and drought stressed ([Degenkolbe et al. 2009](#_ENREF_21)) rice genotypes. When comparing two genotypes of potatoes, [Schafleitner et al. (2007](#_ENREF_69)) observed only 186 up-regulated and 77 down-regulated genes in common while 1713 genes were expressed in total in response to drought.

There are two important phenotypic traits, which underpin drought tolerance and appear to be key to understanding genomic plasticity and adaptation in these contrasting genotypes of black poplar. These traits are linked to leaf development and stomatal patterning and contribute to drought tolerance. Leaf size determines leaf and canopy transpiration ([Radin et al. 1994](#_ENREF_63); [Levi et al. 2009](#_ENREF_43); [Ashraf 2010](#_ENREF_4)) and is also tightly related to yield, an important trait linked to fitness ([Rae et al.. 2004](#_ENREF_64); [Monclus et al.. 2005](#_ENREF_50)). Leaf production and leaf loss represent important adaptive mechanisms enabling long-lived trees to moderate the amount of transpiring leaf surface area. Furthermore, stomatal aperture and stomatal number both contribute to the control of transpiration, leaf-level water use efficiency and drought tolerance ([Nilson & Assmann 2007](#_ENREF_54)). Whilst both genotypes showed reduced leaf expansion in response to drought, for the Ita genotype this reduction was dramatic (< 50%), whilst for Sp2 it was moderate (< 20%). Changes in gene expression concur with these different developmental responses to drought. Transcripts determining the balance between shoot apical meristem activity and the initiation and development of leaf primordia were down-regulated for the Italian genotype in drought (Table 2), including *ANT, PHB, AS1, AS2* and *CLV1* while only *AS1* and *AS2* were down-regulated in Sp2. These data suggest that cell proliferation, rate of leaf expansion and leaf size would be reduced for the Ita genotype in drought, confirming our observations, with less impact on the Sp2 genotype. Ita expression is thus more concentrated in reacting to stress rather than maintaining leaf development (Fig. 9) and this is supported by a drastic down-regulation of processes linked to cell division revealed by gene ontology analysis.

Similarly, striking differences in genes controlling stomatal initiation and number were observed in response to drought for Spanish and Italian genotypes. Stomata regulate CO2 and water-vapour exchange between leaves and the atmosphere ([MacAlister et al. 2007](#_ENREF_46)) and prevent water loss through partial stomatal closure. Although the genetic control of stomatal initiation and patterning is now well documented ([Barton 2007](#_ENREF_5); [Gray 2007](#_ENREF_31); [Casson & Hetherington 2010](#_ENREF_14); Torii, 2015), less is known about how the environment interacts with the control of stomatal patterning, although genes regulating the development of stomata have also been discovered in response to light ([Casson et al. 2009](#_ENREF_13)), CO2 ([Gray et al. 2000](#_ENREF_32); [Hu et al. 2010](#_ENREF_37)) and drought ([Masle et al. 2005](#_ENREF_49)). Unfortunately stomatal patterning was not measured here but our on-going research suggests patterning differs depending on genotype (Smith, unpublished).

Several stomatal patterning genes that negatively regulate stomatal number were down-regulated in response to drought for the Italian genotype including *TMM (TOO MANY MOUTHS), ERECTA, ERL1 (ERECTA-LIKE 1,* Fig. 7*).* In particular, there is strong evidence thatincreased transcript abundance in *ERECTA* is linked to declining stomatal numbers, and that *ERECTA* acts to regulate the initial decision of cells to enter the stomatal developmental pathway ([Shpak et al. 2007](#_ENREF_74)). Two positive regulators of stomatal development were also down-regulated – *SPEECHLESS* and *MUTE*. Overall, the down-regulation of *ERECTA, ERL1* and *TMM* in the Italian genotype suggests that the formation of stomata was stimulated in response to drought. Few changes in gene expression for genes that regulate stomatal numbers were apparent for the Spanish ecotype; only the *ERECTA* gene showed any response to drought, and this could still be significant. The stomatal patterning phenotype remains to be tested in these *P. nigra* trees.

Although our prediction is for increased stomatal numbers in response to drought for Ita, this has not yet been tested but is the subject of future research alongside RNA-Seq analysis of guard cell and epidermal gene expression. The stimulation of stomatal initiation in response to drought is somewhat counter-intuitive and recent reports for *P. balsamifera* showed reduced stomatal numbers following drought treatment ([Hamanishi et al. 2012](#_ENREF_33)). These authors also assessed expression of several stomatal patterning genes and differences between two commercial genotypes were apparent, although they were often inconsistent across several sampling times. Nevertheless, they reinforce the concept that the regulation of stomatal numbers varies intra-specifically and may be an important control point to elucidate differences in adaptation to drought in the genus *Populus* (Roussel et al., 2009).

In summary, we have identified significant differences in response to drought for black poplar genotypes collected from dry and wet environments across Europe. ‘Southern’ Spanish trees are well adapted to slow growth in droughted conditions, producing small leaves and partially closed stomata, with a higher intrinsic water use efficiency, whilst Italian and ‘north eastern’ trees demonstrate a dramatic response to drought with reduced growth and increased stomatal formation. We hypothesise therefore that each of these strategies may be of value, depending on the likely frequency and duration of drought in a particular environment. Importantly here, we have identified a suite of genes that will be the focus of our future research using reverse genetic approaches and testing material in the field in contrasting drought environments. Thus, screening for functional genomic and genetic variation in genotypes from diverse geographic locations under drought stress is a powerful strategy to inform the conservation and management of germplasm resources in a future, changing climate and should be exploited more widely in these difficult-to-study, long-lived but critical plants that contribute to timber, fuel, fibre and ecosystem service provision on a global scale.

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# Tables

Table 1: Summary of statistical results presenting the F-value and p-value for each trait using a GLM test for the main effects genotype and treatment and the interaction genotype x treatment. Bold values are significant (p<0.05)

|  |  |  |  |
| --- | --- | --- | --- |
|  | Genotype | Water treatment | Genotype x Water treatment |
| Trait | F | p-value | F | p-value | F | p-value |
| gs 5DAD | 5.078 | **<0.001** | 15.860 | **<0.001** | 1.344 | 0.252 |
| gs 15DAD | 1.469 | 0.207 | 103.092 | **<0.001** | 1.912 | 0.100 |
| Δ13C | 5.893 | **<0.001** | 7.511 | **0.008** | 2.567 | **0.037** |
| Height growth | 6.579 | **<0.001** | 37.086 | **<0.001** | 0.726 | 0.606 |
| Stem diameter growth | 2.116 | 0.071 | 14.77 | **<0.001** | 1.989 | 0.088 |
| Branches formation | 0.697 | 0.627 | 0.948 | 0.333 | 0.639 | 0.670 |
| New leaf development | 16.216 | **<0.001** | 24.964 | **<0.001** | 0.523 | 0.758 |
| Leaf senescence | 2.502 | **0.036** | 5.182 | **0.025** | 0.839 | 0.526 |
| SLA | 10.538 | **<0.001** | 2.977 | 0.088 | 0.923 | 0.470 |

Table 2: Candidate genes involved in leaf development differentially expressed under drought in the Italian (Ita) and Spanish (Sp2) genotypes. Details include the name of the gene and probe set ID, the poplar (v3.0) and Arabidopsis gene models, the log2 expression ratio for each genotype (in bold if p<0.05) and a brief description of its function.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Name** | **Probe Set ID** | **Poplar gene model (v3.0)**  | **Arabidopsis gene model** | **Ita Log2(FC)** | **Sp2 Log2(FC)** | **Description** |
| *AS1* | PtpAffx.163978.1.S1\_at | Potri.004G102600 | AT2G37630.1 | **-2.70** | **-1.87** | Involved in specification of the leaf proximodistal axis |
| *AS1* | PtpAffx.2947.1.S1\_at | Potri.017G112300 | AT2G37630.1 | **-2.92** | -0.24 | Involved in specification of the leaf proximodistal axis |
| *AS1* | PtpAffx.2947.2.A1\_at | **-1.66** | -0.31 |
| *AS1* | Ptp.4356.1.S1\_at | Potri.006G085900 | AT2G37630.1 | **-4.93** | -0.92 | Involved in specification of the leaf proximodistal axis |
| *AS2* | PtpAffx.207814.1.S1\_at | Potri.008G079800 | AT1G65620.1 | **-2.91** | **-2.20** | Required for formation of a symmetric flat leaf lamina |
| *AS2* | PtpAffx.209221.1.S1\_at | Potri.010G177100 | AT1G65620.1 | **-1.82** | -1.23 | Required for formation of a symmetric flat leaf lamina |
| *AS2* | PtpAffx.44821.1.A1\_s\_at | **-2.07** | -1.04 |
| *CLV1* | PtpAffx.201597.1.S1\_at | Potri.002G019900 | AT1G75820.1 | **-2.06** | -0.52 | Controls shoot and floral meristem size |
| *PHB* | Ptp.548.1.S1\_at | Potri.001G372300 | AT2G34710.1 | **-3.29** | -0.46 | Controls adaxial –abaxial patterning |
| *PHB* | Ptp.548.1.S1\_x\_at | **-3.14** | -0.37 |
| *PHB* | PtpAffx.38907.1.S1\_at | Potri.011G098300 | AT2G34710.1 | **-2.21** | -0.79 | Controls adaxial –abaxial patterning |
| *ANT* | PtpAffx.1799.1.A1\_at | Potri.014G008100 | AT4G37750.1 | **-4.26** | -1.19 | Required for control of cell proliferation |
| *ANT* | PtpAffx.211416.1.S1\_at | **-3.23** | -0.77 |
| *ANT* | PtpAffx.147010.1.A1\_at | Potri.002G114800 | AT4G37750.1 | **-1.41** | -0.80 | Required for control of cell proliferation |
| *ANT* | PtpAffx.34524.3.A1\_a\_at | Potri.005G148400 | AT4G37750.1 | **-4.35** | -2.21 | Required for control of cell proliferation |

Table 3: Description of the significant bins from the microarrays transcripts list in response to drought for the Spanish and the Italian genotypes. The probability (p-value) was calculated using a Wilcoxon Sum of Rank test with a Benjamini Hochberg correction in MapMan (Thimm *et al.*, 2004). Examples of significant transcripts are given for several significant bins with the probe set ID, Poplar gene model, a brief description and log2. Complete list is in Table S7.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Genotype** | **Bin code** | **Bin name** | **Probe Set ID** | **Poplar gene model** | **Arabidopsis gene model** | **Brief description** | **log2 (FC)** |
| **Ita** | **28** | **DNA (128 probes, p=7.87E-15)** |   |   |   |   |   |
|  | 28.1 | DNA synthesis /chromatin structure | ptpaffx.200289.1.s1\_at | Potri.001G074000 | AT5G44635.1 | Minichromosome maintenance (MCM2/3/5) family protein | -4.97 |
|  | 28.1.3 | DNA synthesis /chromatin structure histone | ptp.4194.1.s1\_x\_at | Potri.017G123700 | AT3G45980.1 | Histone superfamily protein | -1.62 |
|  | 28.2 | DNA repair | ptp.1405.1.s1\_at | Potri.014G128500 | AT2G47590.1 | Photolyase/blue-light receptor 2 | -1.48 |
| **Ita** | **31** | **Cell (213 probes, p=9.91E-18)** |   |   |   |   |   |
|  | 31.2 | Cell division | ptpaffx.204723.1.s1\_at | Potri.009G089200 | AT3G19590.1 | Transducin/WD40 repeat-like superfamily protein | -4.20 |
|  | 31.3 | Cell cycle | ptpaffx.200879.1.s1\_at | Potri.001G272000 | AT2G26760.1 | Cyclin B1;4 | -4.69 |
|  | 31.4 | Cell vesicle transport | ptpaffx.2864.2.s1\_at | Potri.003G177700 | AT1G04760.1 | Vesicle-associated membrane protein 726 | 1.80 |
| **Ita** | **20** | **Stress (148 probes, p=0.004)** |   |   |   |   |   |
|  | 20.1 | Stress biotic | ptp.6055.1.s1\_at | Potri.007G043500 | AT4G37000.1 | Accelerated cell death 2 (ACD2) | 1.65 |
|  | 20.2.1 | Stress abiotic heat | ptpaffx.210289.1.s1\_at | Potri.012G017600 | AT5G42020.1 | Heat shock protein 70 (Hsp 70) family protein | 1.38 |
|  | 20.2.3 | Stress abiotic drought/salt | ptpaffx.208807.1.s1\_x\_at | Potri.010G094100 | AT1G26850.1 | S-adenosyl-L-methionine-dependent methyltransferases superfamily protein | -1.83 |

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Genotype** | **Bin code** | **Bin name** | **Probe Set ID** | **Poplar gene model** | **Arabidopsis gene model** | **Brief description** | **log2 (FC)** |
| **Ita** | **27** | **RNA (559 probes, p=0.04)** |   |   |   |   |   |
|  | 27.3.3 | RNA regulation of transcription AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family | ptpaffx.211416.1.s1\_at | Potri.014G008100 | AT4G37750.1 | ANT, Integrase-type DNA-binding superfamily protein | -3.23 |
|  | 27.3.6 | RNA regulation of transcription bHLH | ptpaffx.210224.1.s1\_at | Potri.012G031800 | AT5G53210.1 | SPCH, basic helix-loop-helix (bHLH) DNA-binding superfamily protein | -2.70 |
|  | 27.3.22 | RNA regulation of transcription HB transcription factor family | ptpaffx.38907.1.s1\_at | Potri.011G098300 | AT2G34710.1 | PHB, Homeobox-leucine zipper family protein/lipid-binding START domain-containing protein | -2.21 |
|  | 27.3.25 | RNA regulation of transcription MYB domain transcription factor family | ptpaffx.212699.1.s1\_at | Potri.015G041100 | AT1G22640.1 | myb domain protein 3 | -1.61 |
|  | 27.3.32 | RNA regulation of transcription WRKY domain transcription factor family | ptpaffx.203170.1.s1\_at | Potri.003G111900 | AT2G30590.1 | WRKY DNA-binding protein 21 | 2.06 |
|  | 27.3.50 | RNA regulation of transcription General Transcription | ptpaffx.200328.1.s1\_s\_at | Potri.001G082700 | AT4G24150.1 | Growth-regulating factor 8 | -2.42 |
| **Sp2** | **31** | **Cell (57 probes, p=3.34E-7)** |   |   |   |   |   |
|   | 31.1 | Cell organisation | ptpaffx.148282.1.s1\_s\_at | Potri.002G111900 | AT1G50010.1 | Tubulin alpha-2 chain | -1.52 |
|   | 31.2 | Cell division | ptpaffx.212842.1.s1\_at | Potri.015G090600 | AT3G25100.1 | Cell division cycle 45 | -1.41 |
|   | 31.3 | Cell cycle | ptpaffx.63679.1.a1\_s\_at | Potri.005G181400 | AT1G44110.1 | Cyclin A1;1 | -2.56 |

# Figure legends

Figure 1: Association population information and measurements from the common garden experiment in Belgium: mean annual rainfall and temperature per river population **(a)**, map of the 11 river populations of *Populus nigra* collected in five European countries **(b)**, leaf size and shape variation between populations **(c)**, Leaf area in mm2 **(d)**, stomatal index in % **(e)**, stem circumference in cm **(f)** and wood carbon isotope discrimination in ‰ **(g)**. Same letter indicates no significant difference at 5% level, Student-Newman-Keuls post-hoc testing. Each value with bars represents the average ± standard error.

Figure 2: Correlations of biomass traits of interest: leaf area and height in 2005 **(a)**, leaf area and cell area in 2006 **(b)** and leaf area and cell number per leaf in 2006 **(c)**. Leaf traits are based on the youngest fully mature leaf from each tree. Spearman’s rho (rs) and the probability that it differs from zero (p) is provided for each correlation.

Figure 3: Soil moisture content (%) over time (days after drought) for each genotype **(a)**. Black symbols represents well-watered (control) and open symbols are for drought treatments. Each value with bars represents the average ± standard error. Photographic representation of the morphological effects of drought on the trees grown in the greenhouse **(b)**.

Figure 4: Percentage difference of biomass using the formula [(drought - control)/(control x 100)] from Street *et al.* (2006): Height growth in mm **(a)**, stem diameter growth in mm **(b)**, new leaf formation **(c)**, leaf senescence **(d)**, branch formation **(e)**, Specific Leaf Area in cm2.g-1 **(f)**, stomatal conductance in μmol m-2 s-1 after 5 **(g)** and 15 **(h)** days of drought and carbon isotope discrimination in ‰ **(i)**.

Figure 5: Leaf area development over time (days after drought) for the first emerging

leaf (■), the second leaf emerging (●) and the third leaf emerging (▼) under well-watered conditions (full lines and white symbols) and drought stress (broken lines and black

symbols) for each genotypes: Sp1 **(a)**, Sp2 **(b)**, Fr1 **(c)**, Fr2 **(d)**, Ita **(e)**, NL **(f)**. Percentage difference in leaf area corresponds for the 1st emerging leaf after 18 DAD following the formula [(drought - control)/(control x 100)] from Street *et al.* (2006).

Figure 6: Venn diagram representing the Affymetrix ID probe sets which were two-fold up **(a)** and down **(b)** regulated in response to moderate drought – differentially expressed between the Spanish Sp2 (white) and the Italian Ita (grey) genotypes. Numbers in the circle overlap indicate the number of transcripts common to both genotypes and numbers outside the overlap indicate the number of transcripts exclusive to the genotype indicated. Circles **(c)** indicate the number of transcripts up-regulated and down-regulated in Sp2 compared to Ita in well-watered conditions.

Figure 7: Gene expression changes for Sp2 and Ita in response to water deficit for stomatal patterning candidate genes: *ERECTA*, *ERL1, ERL2, ERL3,* *TMM*, *SPCH* and *MUTE.* Values are in log2.

Figure 8: Z-score values of the main groups for Sp2 and Ita genotypes transcripts in response to drought using the PAGE analysis from AgriGO (Du *et al.,* 2010). Full analysis is in Table S9.

Figure 9: Summary of the response to drought in two genotypes of *Populus nigra*.

# Supplementary documents:

Table S1: Details of the populations of *Populus nigra*, their location and climates. Temperature and precipitation data were collected from the website <http://www.worldclim.org/>. Range of temperatures and precipitations are given for the population collected at different locations (e.g. along a river system).

Table S2: Provenance of the six *P. nigra* genotypes used in the drought experiment.

Table S3: Forward and Reverse primers for each candidate gene (5’ to 3’).

Table S4: Climatic data from the region of origin and phenotypic correlation among traits measured in a common garden study of *P. nigra* from natural populations across western Europe. Correlation coefficients were estimated as Spearman’s rho, with p-values corrected for multiple comparisons using the sequential Bonferroni (asterisks).

Table S5: Summary of the measurements for each genotype under well-watered (control) and drought treatments. Average ± standard error.

Table S6: Normalised microarray expression matrix using the complete list of transcripts.

Table S7: Complete Mapman analysis. Description of the significant bins from the microarray transcripts list in response to drought for the Spanish and the Italian genotypes. The probability (p-value) was calculated using a Wilcoxon Sum of Rank test with a Benjamini Hochberg correction in MapMan (Thimm *et al.*, 2004). Complete list of significant transcripts are given for each significant bin with the probe set ID, Poplar gene model, a brief description, and log2 expression ratio.

Table S8: Complete AgriGO analysis with all the significant GO groups per genotype, the description of the GO term, the number of transcripts, the Z-score and the False Discovery Rate (FDR).

Figure S1: Volumetric soil moisture at final day of harvest **(a)** and rate of soil drying ((final soil moisture – initial soil moisture)) x 100 **(b)**.

Figure S2: Volumetric soil moisture at DAD5 when stomatal conductance measurements were taken.

Figure S3: Expression ratio (log2) from real-time qPCR in response to drought per genotype for the candidate genes: *SPEECHLESS* **(a)**, *IP3* **(b)**, *ATHVA22A* **(c)** and *ERECTA* **(d)**. Same letter indicate no significant difference at 5% level, Student-Newman Keuls post-hoc testing. Each value with bars represents the average ± standard error.