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Toward improved drought tolerance in bioenergy crops: QTL for carbon isotope composition and stomatal conductance in *Populus*

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Abstract

Dedicated non-food bioenergy crops like poplar are needed as sustainable, low-input feedstocks for renewable energy in a future drier climate, where they can be grown on marginal soils. Such plants should have a low water, carbon, and chemical footprint. Capturing natural variation in traits associated with water use efficiency (WUE) is the first step to developing trees that require less water and may be adapted to drier environments. We have assessed stomatal conductance (g_s) and leaf carbon isotope composition ($\delta^{13}C$, an indirect indicator of leaf WUE) in two *Populus* species, *P. deltoides* and *P. trichocarpa* and their F_2 progeny, grown in the United Kingdom and in Italy. *Populus deltoides* leaves showed lower $\delta^{13}C$ than *P. trichocarpa*, suggesting a higher WUE in *P. trichocarpa*, although without drought preconditioning, g_s of *P. trichocarpa* was less responsive to dehydration and abscisic acid treatment than *P. deltoides*, suggesting that leaf anatomy may also contribute to $\delta^{13}C$ in *Populus*. Quantitative trait loci (QTL) were identified for $\delta^{13}C$ on eight linkage groups (LG) and two QTL for g_s. From these. QTL and differential gene expression in response to drought from microarray data, we focused on three hotspots and identified 23 novel candidate genes on LG VI, X, and XVI. We have begun to unravel the genetic basis of WUE in bioenergy *Populus* revealing important underpinning data for breeding and improvement in poplar genotypes for a future drier climate.

Introduction

There remains a pressing need to better understand the links between water supply and the genetic basis of plant drought tolerance – a complex trait, difficult to quantify and highly variable (Passioura 2012). For example, plant characteristics conferring positive drought tolerance in some circumstances may become negative, depending on the severity and duration of the drought (Tardieu 2012). At the same time, predicted changes in climate, suggest increased soil moisture deficits and evaporative demand are likely in many areas of the world (Kundzewicz et al. 2007). Against a background where land must be used more efficiently for food, fuel, and fiber production and in the face of population growth (Godfray et al. 2010), it seems likely that there will be a future requirement to cultivate new non-food crops, such a fast growing trees and grasses for energy purposes, on marginal agricultural land where water supply is likely to be limited (Oliver et al. 2009). Understanding the genetic basis of adaptation to drought therefore remains a priority, but limited breeding and improvement has been undertaken in such crops (Karp and Shield 2008) with few data available on which to test models. In contrast, genotypes better adapted to low precipitation regimes have already been identified in a wide range of arable crop species (recently reviewed by Morison et al. [2008]), with more limited data on trees such as pine (Guehl et al. 1995), oak (Brendel et al. 2008), and poplar (Monclus et al. 2005, 2006), but there is little understanding of the genetic basis of such traits. Poplar is a target tree for bioenergy, but some species of poplar are only moderately tolerant to drought (Somerville et al. 2010), as trees of this genus are usually adapted to a riparian, wet habitat (Aylott et al. 2008). However, there is also evidence to suggest that enough genetic diversity exists across the genus for targeted selection and breeding for drought tolerance, with wide variations reported in traits such as intrinsic water use efficiency, leaf carbon isotope discrimination, stomatal conductance, stomatal density (Monclus et al. 2006), and differences in gene expression and metabolic changes in response to drought in *Populus* identified (Street et al. 2006). The ability of certain *Populus* species to tolerate extremely droughted environments is also noted. For example, *Populus euphratica* was found in highly saline and arid environments such as the Negev desert (Brosché et al. 2005). Quantifying this genetic diversity and understanding the physiological traits associated with genetic variation provides the first step to identifying superior plants for drought tolerance that will underpin breeding efforts for these bioenergy species.

The question remaining, however, is what are the valuable traits that contribute to drought tolerance? Much of the research undertaken to date on arable crops, such as that on early vigor, flowering time, grain fill, and partitioning, is of limited relevance to bioenergy crops grown for biomass, but some traits, including root architecture, canopy temperature depression, water-use efficiency (WUE), stomatal conductance and carbon isotope composition and discrimination are likely to be of generic value (Tuberosa 2012). WUE can be defined either at the plant scale, WUE_p (the ratio between biomass production and water consumption over a period of time, usually weeks or months) or at short-term and smaller scale of leaves, WUE_t – the "instantaneous" ratio between the net CO_2 assimilation rate and the transpiration loss (Ponton et al. 2001; Bacon 2004; Seibt et al. 2008). High WUE_t can be achieved by lowering stomatal conductance, g_s (Leffler and Evans 2001) and/or increasing photosynthetic rates (Condon et al. 2002). Several studies have shown that WUE can be improved with stomatal closure at midday (Tenhunen et al. 1982) or through stomatal opening early in the morning (Bacon 2004). A positive correlation between WUE_t and leaf carbon isotope composition, $\delta^{13}C$ is now established (Farquhar et al. 1989; Condon et al. 2002; Brendel et al. 2008), enabling a rapid screen for WUE in plants in many environments and from a large number of genotypes (Farquhar et al. 1989; Jones et al. 1993; Condon et al. 2002; Bacon 2004; Rajabi et al. 2009), such as those in a mapping population enabling the elucidation of Quantitative trait loci (QTL) for this trait. A difficulty in this type of research, however, is the strong link between plant water consumption and yield, with consequent reductions in water use and higher WUE often associated with lower yield under most favorable conditions where there is no drought occurring (Collins et al. 2008). Although breaking this link is difficult, there have been recent successes with wheat adapted for highly arid environments in Australia, confirming this as a useful target trait for breeding, particularly for severely droughted climates, with the release of new cultivars as a result of carbon isotope research (Condon et al. 2004; Richards 2006; Hochman et al. 2009).

QTL analysis provides a framework that enables physiological and biochemical traits related to drought to be considered at the level of the genome and in several model and crop species. Allelic variation at QTL can affect either gene expression or protein function (Paran and Zamir 2003). QTL have been identified for drought traits including $\delta^{13}C$, WUE and stomatal behavior. For example, in *Arabidopsis*, Masle et al. (2005) mapped a transpiration efficiency QTL, linked to the *ERECTA* gene, while more recently using an *Arabidopsis* recombinant inbred population generated from plants selected from extremely arid or wet environments (McKay et al. 2008), $\delta^{13}C$ QTL have been mapped and related to flowering time (Tisné et al. 2010). Therefore, in *Arabidopsis*, it

appears that one strategy for drought resistance is escape by completing its life cycle early (Bray 2001). Clearly, this highlights a limitation in the use of *Arabidopsis* as an example for long-lived perennial tree species, where flowering may be only one part of the adaptive strategy deployed by these perennial organisms (Taylor 2002). Genotypic variation for WUE, $\delta^{13}C$ and g_s has, however, been described previously for a limited number of tree species (Ponton et al. 2001; Prasolova et al. 2003; Monclus et al. 2005; Voltas et al. 2006; Brendel et al. 2008). In *Populus*, Monclus et al. (2005, 2006) showed that productivity was not correlated consistently with $\delta^{13}C$, with genotypes found combining high WUE and productivity and so understanding the genetic basis of such a trait would be useful in the development of efficient crops growing under drought stress. Few data on QTL for $\delta^{13}C$ is published (Rae et al. 2009; Monclus et al. 2012) while no QTL data are available for g_s and the genes underlying QTL for WUE associated traits for *Populus*.

The aim of this study was to quantify genetic variation in traits related to WUE, in an F_2 mapping population of *Populus* (*P. deltoides* × *P. trichocarpa*), identifying the genetic basis of these traits. We investigated g_s and $\delta^{13}C$ and mapped QTL and candidate genes underlying QTL hotspots, identifying three areas of the genome for further study, enabling rapid future progress to be made in molecular tree breeding and, in addition, developing an improved understanding of adaptation of this tree genus to contrasting soil moisture environments, likely in future climates.

Materials and Methods

Plant material

To produce the mapping population, grandparents were selected from areas of the U.S.A. (Bradshaw and Stettler 1993) with highly contrasting rainfall – *P. deltoides* (*ILL-129)* selected from central Illinois with an average rainfall of 800–1000 mm and *P. trichocarpa* (93-968) selected from Western Washington with an average rainfall around 1800–2000 mm. A male *P. deltoides* and a female *P. trichocarpa* were crossed (Bradshaw and Stettler 1993) and two siblings from the resulting progeny (53-242 and 53-246) were crossed to obtain an F_2 population, Family 331, as explained previously (Wu et al. 1997; Rae et al. 2009).

Differences in stomatal behavior between *P. deltoides* and *P. trichocarpa* grandparents of the mapping population

In constant environmental conditions (radiation, temperature, wind speed, and humidity), changes in leaf temperature will indicate changes in g_s (Jones 1999; Grant et al. 2006; Leinonen et al. 2006). The dynamics of stomatal response to drying and abscisic acid (ABA) treatment for the grandparental leaves of the cross were investigated using thermal imaging, to understand if differences in this population for stomatal traits were likely to segregate in the F_2 population. Three replicates per treatment were used for both genotypes. Mature leaves (LPI 9) from 9-month-old greenhouse-grown plants were excised under water and placed in a controlled environment room to allow stabilization of stomatal conductance. LPI is the Leaf Plastochron Index (PI) measured using the PI which is equivalent to the interval in time between two successive leaves reaching 30 mm, as previously described (Taylor et al. 2003). The temperature of the room was 25°C and the relative humidity ~65–75%. The petioles were then recut under water (Sperry et al. 1988) and the leaves transferred to a tube with distilled water, exposed approximately horizontally and supported by a nylon net above a tray of cool water to provide a constant and thermally contrasting background. The temperature of the leaves was monitored at 30-sec intervals over 2 h using an infrared camera (NEC ThermoVision, TH7102MV; Metrum Information Storage, Finchampstead, Berkshire, U.K.) with a temperature resolution of 0.05°C and a spatial resolution of 0.5 mm^2. A grease spot was applied to the leaf as a dry reference and pieces of wet filter paper were used as a wet reference surface. After ~1 h of stabilization, one leaf of each genotype was transferred to a solution of ABA at 10^{-4} mol/L and a second leaf excised from the petiole, simulating acute dehydration. The remaining leaf was kept with the petiole in water as a control. Images were analyzed using the software ImageJ (Abramoff et al. 2004) to determine mean leaf temperature across the whole leaf and the temperatures of the grease spot and wet reference paper. From these temperatures, an index of conductance was calculated as $g' = ((T_{dry} - T_{leaf})/(T_{leaf} - T_{wet}))$, with T_{wet} (wet leaf temperature) taken from the filter paper and T_{dry} (dry temperature) taken from the grease spot, with a modification in the procedures of (Jones (1999).

Family 331 carbon isotope composition and stomatal conductance in contrasting wet and dry environments

Two hundred and fourteen genotypes of Family 331 were planted in two contrasting environments, in North Italy (Cavallermaggiore, 44°21′N, 8°17′E) and southeast U.K. (Headley, 51°07′, 0°50′W). The climates of each site and experimental design have been described previously (Rae et al. 2008), but briefly, annual average temperatures were 10.9°C in the United Kingdom and 12.9°C in Italy and annual rainfall were 470.9 mm in the United Kingdom

and 729.3 mm in Italy. Soil moisture was not recorded at the sites. Unrooted cuttings were planted in April 2003 at both sites. Each field was divided into six blocks which contained a single replicate of the grandparents, the F_1 parents (53-242 – male and 53-246 – female) and 210 genotypes of the F_2 population in a randomized complete block design (RCBD). A double row of commercial varieties were planted in order to reduce the edge effect (Rae et al. 2008). Plants in the United Kingdom received water three times per week during the night and in Italy the site was irrigated by flooding on four occasions (24 June, 16 July, 30 July, and 17 August 2003). Plants at both sites were grown as single stem trees.

Three biological replicates were used to determine $\delta^{13}C$ for each site with the first fully mature leaf per tree harvested at the end of August 2003 in the United Kingdom (205 genotypes) and the beginning of September 2003 in Italy (187 genotypes). Some individuals died at establishment at each site and could not been used in this analysis, explaining the lower number of genotypes at each site. Leaves were dried for 48 h in an oven at 80°C and then ground using a ball grinder (Glen Creston ball, Retsch MM300, Haan, Germany). Material was weighed (1 mg) and placed into a 6 × 4 mm tin capsule (Ultra-clean pressed tin capsules, Elemental Microanalysis, Devon, U.K.). Samples were analyzed using a Sercon 20-20 Stable Isotope Analyzer with ANCA-GSL Solid/Liquid Preparation Module (Sercon, Crewe, U.K.). The carbon isotope composition was determined by $\delta^{13}C$ (‰)=[($R_{sample}-R_{reference}$)/$R_{reference}$] × 1000, where Rsample and $R_{reference}$ are the $^{13}C/^{12}C$ ratios of the sample and the reference, respectively (methods fully described in Scrimgeour et al. 2004).

The $\delta^{13}C$ results from this experiment were compared to data from Rae et al. (2009) on Family 331 trees grown under short rotation coppice (SRC) in the same field in southeast U.K. at Headley.

Stomatal conductance of all F_2 trees at the U.K. site was measured in midsummer 2004 using a portable steady-state diffusion porometer (LI-1600; LI-COR Inc. Lincoln, Nebraska), with manual data recording. For each genotype, leaf 7 from the top of the main stem was removed. Leaf petioles were recut under water (Sperry et al. 1988), transferred to a tube with distilled water, and transported to a controlled environment with photosynthetic active radiation (PAR) ~300 μmol m^{-2} s^{-1}, air temperature of 25°C and relative humidity of 50% (±10%). Measurements were made on three replicates of each genotype in a period not exceeding 4 h after excision.

Leaf samples were also collected for leaf area (LA) measurements at each site. The first mature leaf (at insertion level 7–9) was selected for each genotype and was scanned into Corel Photopaint (Corell Inc., Mountain View, CA) and the area was calculated by MetaMorph Imaging System (Westchester, Philadelphia) Version 4.5, calibrated in μm.

Data analysis

Differences in stomatal behavior between *P. deltoides* and *P. trichocarpa*

Data analysis was performed using the statistical package SPSS statistical software package (SPSS 17.0, Chicago, IL). A test for normal distribution of data (Kolmogorov-Smirnov test) was performed for g' values measured by thermal imaging. Transformations ($\log_{10}$) were carried out in the case of non-normality and verified with a Kolmogorov-Smirnov test. A paired t-test was performed between the initial and final time for each genotype and treatment.

Family 331 carbon isotope composition and stomatal conductance

Statistical analyses for $\delta^{13}C$ and g_s of the mapping population were performed with R software (version 2.0.1, A Language and Environment Copyright, 2004). Microenvironmental effects within field sites were minimized for $\delta^{13}C$ and g_s using Papadakis spatial correction (Papadakis 1984), based on a 3 × 7 grid on individual data. Analysis of variance (ANOVA) was carried out for $\delta^{13}C$ and g_s before and after spatial correction using R-script and the following model of ANOVA:

$$Y_{ijl} = \mu + B_i + G_j + \epsilon_{ijl}$$

where μ is the general mean, B_i is the effect of block i considered as fixed, G_j is the effect of genotype j considered as random, and ε_{ij} is the error. The mean genotype data after spatial correction were used for QTL mapping if spatial correction was seen to reduce block effects.

Between sites comparison was tested using two-way ANOVA for $\delta^{13}C$:

$$Y'_{jkl} = \mu + G_j + S_k + G \times S_{jk} + \epsilon_{jkl}$$

where Y'_{jkl} are individual values adjusted for microenvironmental effects using Papadaki's spatial correction, μ is the general mean, G_j is the genotype effect (random), S_k is the site effect (random), $G \times S_{jk}$ is the genotype by site interaction (random), and ε_{jkl} is the error.

Correlation

A correlation matrix was performed in SPSS (SPSS 17.0, Chicago, IL) between phenotypic traits measured at the U.K. and Italian site: $\delta^{13}C$, g_s (only U.K. site) and LA,

measuring Pearson's correlation coefficients and two-tailed measures of significance.

Heritability

Variance components were calculated by equating observed mean squares to expected mean squares in a random model (Henderson 1953), and individual broad-sense heritability (h^2) was calculated for each trait (1) at each site using the equation

$$h^2 = \frac{V_G}{V_G + V_E},$$

and (2) across sites as

$$h^2 = \frac{V_G}{V_G + V_S + V_{G\times S} + V_E}$$

where V_G is the genetic variance, V_E is the residual variance, V_S is the site variance, and $V_{G \times S}$ is the genotype by site interaction variance.

QTL mapping and discovery of candidate genes

The genetic linkage map used to map QTL was produced by G. A. Tuskan et al. (pers. comm.), and consisted of 91 simple sequence repeat (SSR) markers genotyped on 350 of the full-sib progeny, and 92 fully informative amplified fragment length polymorphisms (AFLP) genotyped on 165 genotypes of the progeny (Yin et al. 2004). The total map distance was 1453.1 cM with an average of 8 cm between markers. The map resulted in 22 linkage groups (LG) and when two LGs were assigned to one chromosome, the LG was numbered with the chromosome number and a letter. The order of the letters indicated the order of LGs along the chromosome (Rae et al. 2006). Spatially corrected averages for δ^{13}C at the U.K. site and the Italian site were used to map the QTL. QTL mapping was carried out using the web-based software QTLexpress (Seaton et al. 2002), (http://www.gridqtl.org.uk/) with the function Large Single Full-Sib Family Analysis (Tree) which allows mapping of outbred pedigrees derived from heterozygous lines where more than two alleles may be segregating at each locus. Chromosome-wide permutation tests with 1000 iterations were performed and the identification of a QTL was achieved using the resulting F value where $P < 0.05$. Initially models incorporating the paternal, maternal, and interaction parameters were run. If a parameter did not differ significantly from zero, it was removed from the model and the analysis rerun. Confidence intervals were obtained by taking the distance in cM corresponding to an F drop-off of two from the maximum F value as described previously in Rae et al. (2006).

In addition to this, QTL analysis was carried out using multiQTL (http://www.multiqtl.com/) to enable multiple interval mapping (MIM). However, as this mapping software is for inbreeding pedigrees and does not allow for more than two alleles segregating at loci, QTL mapping was carried out using the pseudo testcross method (Grattapaglia and Sederoff 1994). QTL were identified for the parental maps separately. Single trait analysis was performed using first the interval mapping approach followed by MIM. The entire genome was scanned for QTL assuming a single model that is, one QTL per LG. Permutation tests comparing hypotheses H_1 (a single QTL present on the LG), and H_0 (no QTL on the LG) were run to obtain chromosome-wise statistical significance. In a second step, the genome was scanned for QTL assuming a two-linked QTL model. Permutation tests were carried out to compare the hypothesis H_2 (two-linked QTL on the LG) to H_0. Where the probability of H_2 versus H_0 was <0.05, further permutation tests were run to compare the hypotheses H_2 versus H_1 to ensure that the two-linked QTL model fitted the data better than a single QTL model. In all cases, chromosome-wide permutation tests with 1000 iterations were carried out to determine significance thresholds (Churchill and Doerge 1994).

The option "marker restoration" was used to reduce the effect of missing information. In a final step, MIM was performed, to ensure that all single and two-linked QTL identified in the single interval analysis were significant when multiple intervals were analyzed. For the remaining significant QTL again all necessary permutations were run and bootstrap analysis was performed to estimate the 95% confidence interval for the additive genetic effect.

QTL are reported here only when they were seen to be significant using MIM, however, for ease of interpretation, the parameters from QTLexpress are reported rather than the pseudo testcross parameters. The QTL were drawn using the software MapChart (Voorrips 2002).

"QTL hotspots" were defined where at least one QTL explained >5% of the variation and where multiple QTL were present in the same area of the LG. Using adjacent markers on the genetic and physical maps, genes within QTL were identified and studied in order to identify candidate genes. In order to reduce the number of genes from this list within the hotspots and to identify novel targets in this mapping pedigree, published microarray data (Supplementary documents, Street et al. [2006]) of gene expression in response to

drought, from the same genotypes of *P. deltoides* and *P. trichocarpa* reported here, were compared to the list of genes within the QTL hotspots. The set of genes where both species responded similarly to drought were labeled "commonly expressed" (either up- or downregulated) while genes that were differentially expressed between the two genotypes in response to drought were labeled "differentially expressed". Any gene listed both in the microarray analysis and within the QTL regions were investigated.

Results

Differences in stomatal behavior between *P. deltoides* and *P. trichocarpa*

Prior to treatments, leaves of both species under all conditions in the experiment had similar temperatures (Fig. 1A). Under well-watered conditions, mature leaves of both species showed similar values of temperature over time (Fig. 1). However, the responses to ABA and excision treatments differed considerably between the two species. The excised (stimulating acute dehydration) and ABA-treated leaves of *P. deltoides* first shortly decreased in temperature – the "Iwanoff effect" (Iwanoff 1928), which is due to a sudden loss in epidermal turgor (Kaiser and Grams 2006), then showed a rise in temperature indicating stomatal closure (Fig. 1B and see video available in the Video S1) stabilizing after ~10–15 min while those of *P. trichocarpa* did not change, even after 2 h (data not presented). Using the leaf and reference temperatures, relative conductance, g', was also calculated (Fig. S1). For *P. deltoides*, g' responded to acute dehydration and ABA solution by decreasing from 0.8 to 0.2 while g' of *P. trichocarpa* remained constant over time after stress (Fig. S1). Relative leaf conductance g' was also studied and compared between a steady period prior to treatment and the end of the experiment for each genotype and at each treatment. Only the excised treatment in *P. deltoides* showed a significant difference between the initial and the

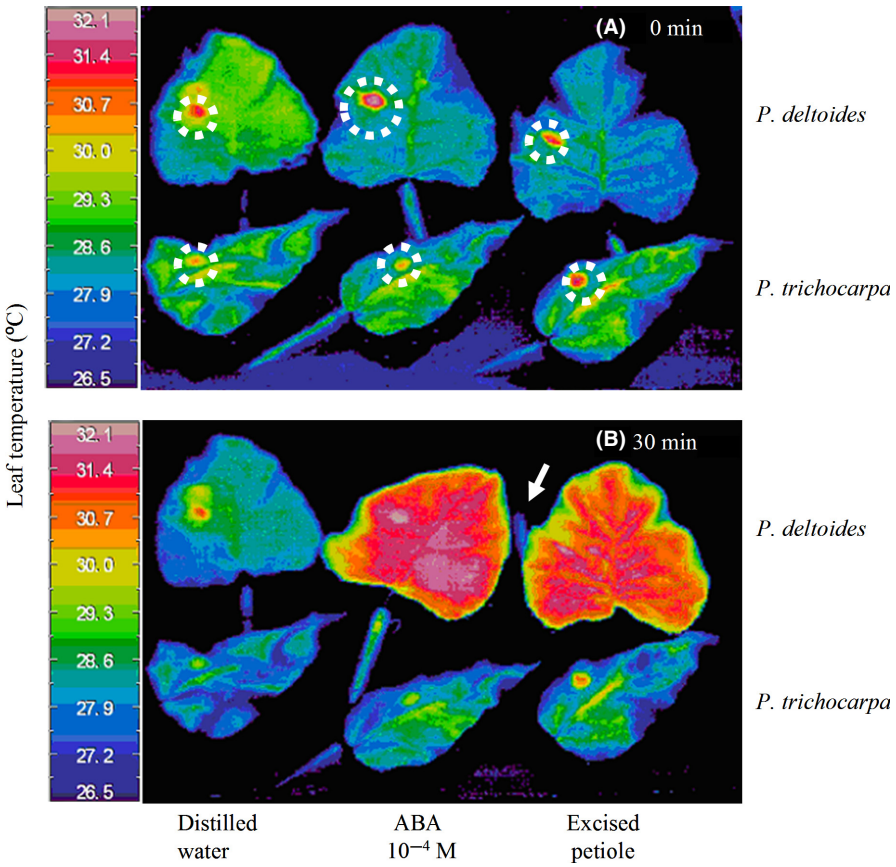

Figure 1. Infrared images of leaf temperature of mature leaves of *Populus deltoides* and *P. trichocarpa* under three different treatments: leaves in distilled water (left), in ABA 10^{-4} mol/L solution (middle), and with petiole excised simulating acute dehydration (right); (A) at the start of the treatments and (B) 30 min later. The left-hand scale shows leaf temperature in °C. A grease spot was applied to each leaf as a dry reference (white dashed circle) and pieces of wet filter paper (white arrow) were used as a wet reference surface. A video of this is given in Video S1.

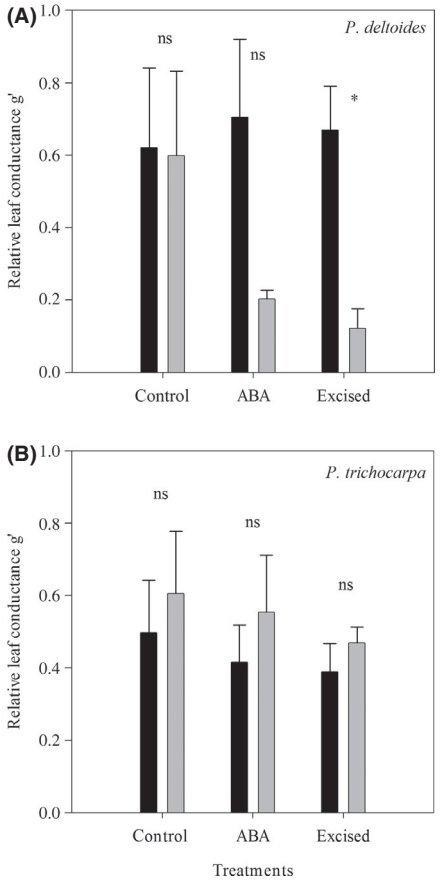

Figure 2. Relative conductance (g') calculated from leaf and reference temperatures in mature leaves of (A) *Populus deltoides* and (B) *P. trichocarpa* in control condition, ABA of 10^{-4} mol/L solution and excised condition at the initial (black column) and final time (gray column) of the experiment. $n = 3$. Each value with bars represents the average ± standard error. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, n.s., nonsignificant.

final time (Fig. 2). Although, ABA-treated leaves of *P. deltoides* showed a decrease over time, more replicates would be necessary to show a significant difference.

Family 331 carbon isotope composition and stomatal conductance

Leaf carbon isotope composition

For the two experimental datasets (United Kingdom and Italy), planted with an identical experimental design in contrasting climatic zones of Europe (Fig. 3A and B), leaf δ^{13}C values varied between the genotypes (Italy: $F_{186,217} = 1.7$, $P < 0.001$; United Kingdom: $F_{204,143} = 2.0$, $P < 0.001$). Leaves from the same population were also sampled by Rae et al. (2009) in July 2001 in a SRC condition in the United Kingdom and used to measure leaf δ^{13}C (Fig. 3C). Variation between genotypes was also significant (Rae et al. 2009). This dataset was compared with the data grown in single stem condition in Italy and in the United Kingdom There was a consistent pattern across all three datasets in that *P. deltoides* showed a lower δ^{13}C value than *P. trichocarpa*. The absolute magnitude of this differed in each environment, suggesting an important environment as well as genotype effect determining this trait (Fig. 3A–C). The spread of the F_2 data for δ^{13}C also changed with the environment. Two-way ANOVA showed that there was a significant difference between sites ($F_{1,350} = 772.4$, $P < 0.001$), but no genotype by site interaction ($F_{172,350} = 1.03$, $P = 0.29$). In the U.K.–Italy study, δ^{13}C was lower on average at the U.K. site (−27.4‰) than the Italian site (−25.3‰). The spread of isotopic composition was increased in Italy, where trees were subjected to periods of soil drying, varying between −23‰ and −29‰, while in the U.K. data for the F_2 only varied between −25‰ and −29‰, with many more genotypes falling within the mean frequency categories. These data suggest that the more stressful environment in Italy resulted in a wider expression of phenotypic plasticity but in contrast to this, the SRC-grown *Populus* revealed a similar spread of data (Rae et al. 2009) to that in Italy and may reflect the intensive management practice with trees grown with multi-stems or more water demanding trees. *P. trichocarpa* had higher δ^{13}C values compared to *P. deltoides* and the F_1 parents at each site and considerable transgressive segregation was apparent for this trait with extreme F_2 phenotypes in the population (Rieseberg et al. 1999). Moderate heritability for this trait was seen at each site (United Kingdom = 0.49, Italy = 0.36) but was low when calculated across the two sites (0.01) presumably due to the large environmental differences between the two sites. A correlation matrix was performed between the phenotypic traits measured in Italy and in the United Kingdom: δ^{13}C, LA and g_s (measured only in the United Kingdom). Leaf δ^{13}C values were positively correlated between the two sites ($r = 0.28$, $P < 0.001$). No correlation was observed between leaf δ^{13}C in Italy with LA in Italy or between LA in the United Kingdom with δ^{13}C in the United Kingdom or g_s (Table S1).

Stomatal conductance

After logarithmic transformation, the data for g_s in Family 331 were normally distributed with significant differences between the genotypes ($F_{215,372} = 1.9$, $P < 0.001$). *P. trichocarpa* (240 mmol m^{-2} s^{-1}) had higher values on average than *P. deltoides* (135 mmol m^{-2} s^{-1}), and the F_1 parents showed higher g_s than the grandparents. The progeny had a large range of g_s values from 10 to 470 mmol m^{-2} s^{-1}, again with considerable transgressive

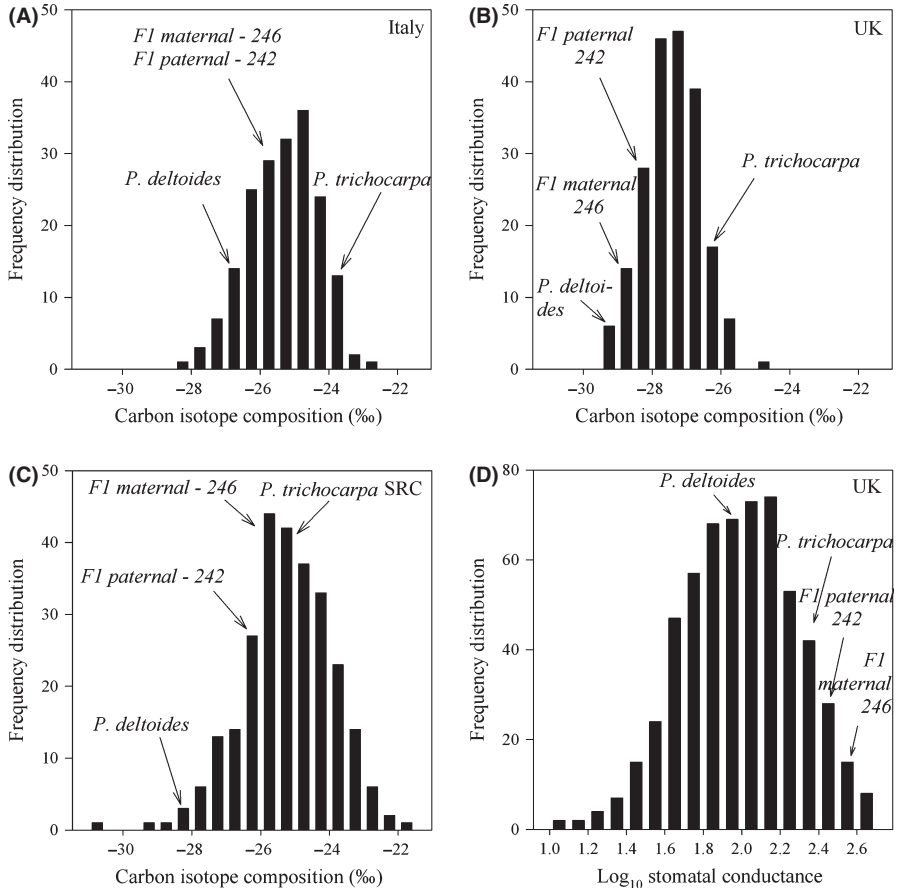

Figure 3. Frequency distribution of mean leaf carbon isotope composition, $\delta^{13}C$ (‰), in F_2 Family 331 of *Populus* for different environmental conditions and sites: single stem trees grown a fully replicated randomized and identical experiment in Italy (A) or in the United Kingdom (B) or grown in the United Kingdom as Short Rotation Coppice SRC (C) from Rae et al. (2009) and mean $\log_{10}$ stomatal conductance from detached leaves of Family 331 trees from the single stem trees in the United Kingdom (D).

segregation observed (Fig. 3D). Moderate heritability was seen for this trait (0.35).

QTL discovery and candidate genes

QTL were identified on eight LGs for $\delta^{13}C$ (Fig. 4). Total phenotypic variance for $\delta^{13}C$ explained by mapped QTL was 37.1% at the Italian site and 26.6% at the U.K. site. At the Italian site five QTL were mapped for $\delta^{13}C$, on LG III, V, X, XII, and XVII. Individual QTL explained between 4.5% and 12.2% of the total variation, while at the U.K. sites five QTL for $\delta^{13}C$ mapped on LG IV, V, VI, X, and XVI, and explained between 3.5% and 9.0% of the total variation. QTL for $\delta^{13}C$ were also discovered in Rae et al. (2009) using the SRC dataset in LG III, V, VI, VIII, and X (Fig. 4). Two QTL for g_s measured at the U.K. site were mapped on LG VIII and XVI (Table 1), explaining 2.6% and 6.9% of the total variation, respectively. The relatively small amount of variation accounted for by the QTL (total of 9.5%) reflects the difficulty of making measurements on large populations for physiologically based traits such as stomatal conductance, although it is also the case that many QTL identified are likely to be the largest effect QTL (Jacobsson et al. 2005; Yang et al. 2009; Ravi et al. 2011).

Hot spots defined with at least one QTL explaining >5% of the variation and with multiple QTL present in the same area of the LG were on LG III, V, VI, X, and XVI. Hot spots on LG VI, X, and XVI were particularly interesting and warrant further study (Table S2). LG VI contained one QTL for $\delta^{13}C$ in the United Kingdom, for both single stem and SRC trees (Fig. 5), as well as other QTL found in the literature, for example osmotic potential at full turgor (Tschaplinski et al. 2006) close to the QTL hotspot on LG VI and several QTL for leaf development traits already published (Rae et al. 2006; Street et al. 2006; Street et al. 2011). QTL for $\delta^{13}C$ were mapped consistently on LG X, in all three contrasting environ-

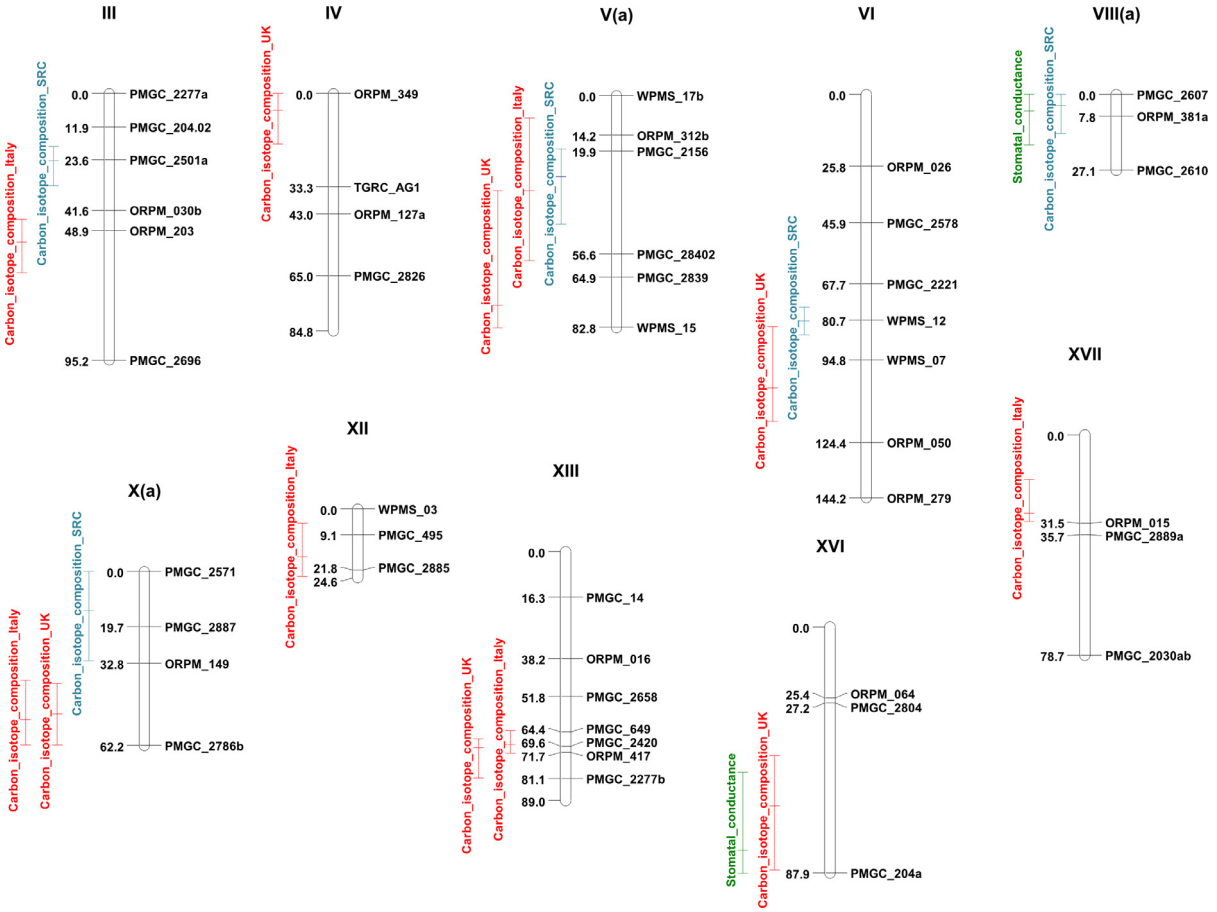

Figure 4. Quantitative traits loci (QTL) for $\delta^{13}C$ and g_s across the *Populus* genome. QTL mapped with the web-based software QTLexpress, with confidence intervals in cM and drawn in the software MapChart (Voorrips 2002). Confidence intervals were corresponding to an *F* drop-off of two from the maximum *F*.

Table 1. The quantitative trait loci (QTL) discovered for $\delta^{13}C$ and g_s found in the United Kingdom and Italy as single stem trees.

Trait	Site	LG	Position	Confidence interval	*P*-value	Paternal effect	Maternal effect	Interaction effect	% Variance explained
$\delta^{13}C$	Italy	III	53	45–64	<0.001	0.401	–	–	12.23
$\delta^{13}C$	Italy	Va	34	8–59	0.022	−0.200	−0.325	−0.285	6.33
$\delta^{13}C$	Italy	X	53	39–62	0.009	0.2700	–	–	4.47
$\delta^{13}C$	Italy	XII	17	10–24	0.001	–	0.294	–	6.31
$\delta^{13}C$	Italy	XVII	28	16–31	0.001	−0.220	0.235	–	7.73
$\delta^{13}C$	UK	IV	6	0–18	0.032	–	0.195	–	3.73
$\delta^{13}C$	UK	Va	75	34–83	0.025	–	−0.186	–	3.52
$\delta^{13}C$	UK	VI	105	83–117	0.001	0.248	–	–	6.77
$\delta^{13}C$	UK	X	51	40–62	0.001	0.241	0.233	–	9.03
$\delta^{13}C$	UK	XVI	64	46–87	0.023	–	0.245	–	3.54
g_s	UK	VIII(a)	6	0–18	0.022	8.983	–	–	2.55
g_s	UK	XVI	80	52–88	0.005	7.945	−10.597	−15.981	6.90

ments, collocating for both single stem results in Italy and United Kingdom (Fig. 5). On LG XVI, one QTL for g_s, one QTL $\delta^{13}C$ measured at the U.K. site collocated with a leaf width to length ratio from a previous study (Rae et al. 2006). Estimation of parental parameters suggests a difference in genetic control in this region as there

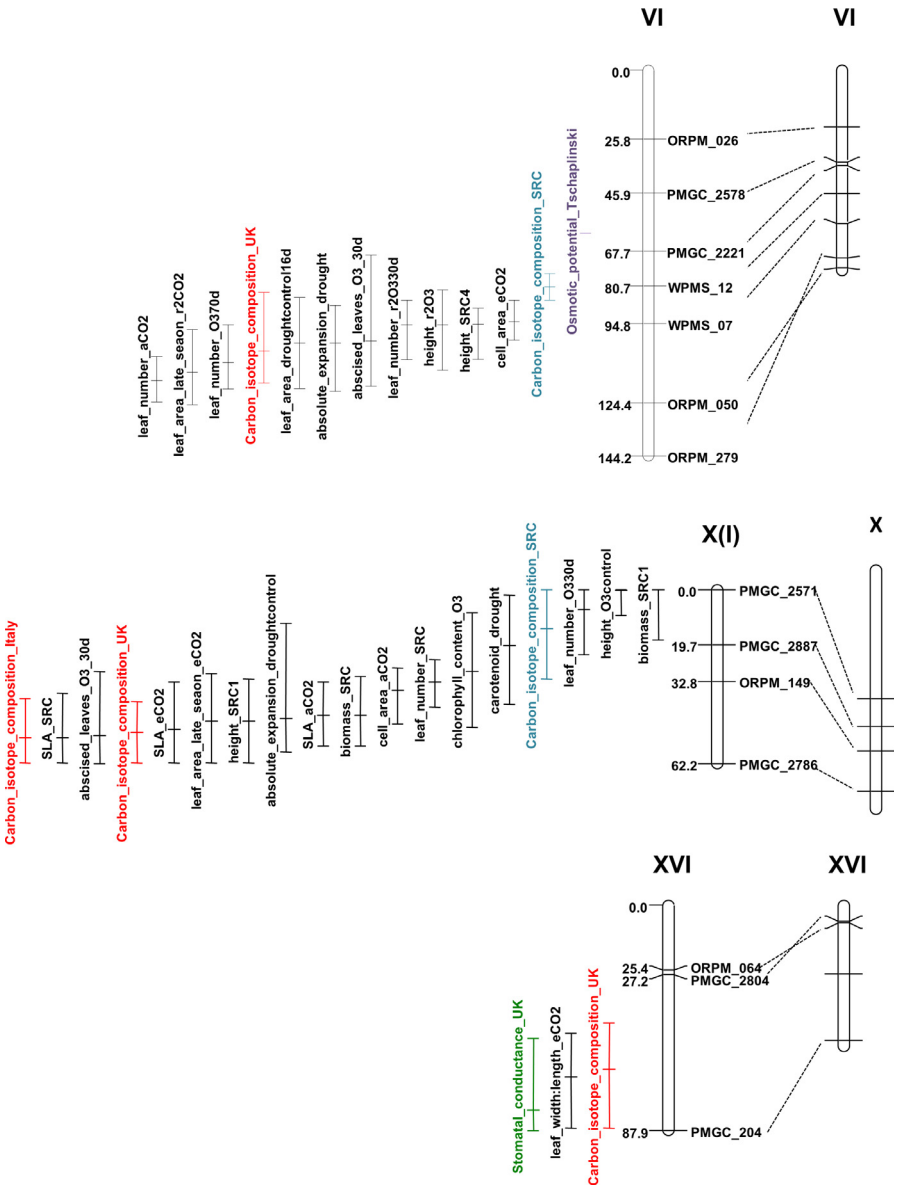

Figure 5. QTL "hotspots" on linkage group VI, X, and XVI. The traits are $\delta^{13}C$ and g_s (United Kingdom and Italy). QTL mapped with the web-based software QTLexpress, with confidence interval in cM and drawn in the software MapChart (Voorrips 2002). Other QTL were collected from the literature: osmotic potential at full tugor (Tschaplinski et al. 2006), response to ozone stress (Street et al. 2011), response to elevated CO_2 (Rae et al. 2006), response to drought stress (Street et al. 2006), and trees grown as SRC (Rae et al. 2009). Detailed explanations of published traits are given in Table S2.

was no significant effect from the maternal parent when plants were grown in Italy, but both maternal and paternal effects are seen when grown in the United Kingdom 950, 758, and 979 genes were found within the QTL hotspots on LG VI, X, and XVI using the nearest markers from the QTL 95% confidence intervals. A complete list of these genes is given in Table S3. Several candidate genes with a putative role in determining tolerance to drought stress were apparent in this list. Indeed from 15 gene models contained in the whole genome with a dehydration description such as dehydration-responsive family protein, two were on LG X, and one on LG XVI. The hotspots also contained one universal stress protein (USP) on LG VI and two USPs on LG XVI out of 59 in the genome. One gene model related to response to other stress such as low temperature and salt were present on LG VI and X. Other examples on LG VI was *ERECTA*, a gene known to be related to the regulation of plant

transpiration efficiency as well as leaf organogenesis, cell expansion, and cell division (Masle et al. 2005). *ERECTA* only has two copies, one on each of chromosomes 6 and 18. A late embryogenesis abundant (LEA) group 1 was present within the hotspot on LG XVI. LEA proteins respond to abiotic stress, such as dehydration, osmotic stress, and ABA (Bartels and Sunkar 2005; Hong-Bo et al. 2005) and their main role is to protect the cells from dehydration damage. Twenty-five genes models with a description of LEA or LEA-like proteins are present in the whole genome.

Microarray data from Street et al. (2006) that quantified the differential expression of genes in response to drought for the grandparental trees of this cross – of *P. deltoides* (ILL-129) and *P. trichocarpa* (93-968) were combined with the list of genes within the QTL to define the candidate genes. These genes represent a set of genes that are the target for a future research. Overall, 46 differentially expressed genes revealed by the microarray data in response to drought, were found within the QTL (Table S4) and 23 genes that were differentially expressed between grandparental species in response to drought were found within the three focused QTL hotspots: seven on LG VI, six on LG X, and nine on LG XVI. Examples included a universal stress protein (UspA), ubiquitin, and chaperone proteins (e.g., C3HC4-type RING finger), a translation initiation factor (elF-5A), serine/threonine protein kinases, an ankyrin repeat family protein, and a LEA group 1.

Discussion

The aim of this study was to gain a better understanding of the genetic basis of stomatal conductance and carbon isotope composition within *Populus*, providing a starting point to unravel the genetic nature of these traits and their contribution to drought tolerance in *Populus*. Identifying three QTL "hotspots" and their underlying genes provide valuable new information to underpin future breeding efforts in this genus.

Stomatal behavior was first observed in the two grandparental *Populus* species showing variation in stomatal conductance in response to dehydration and ABA application that gave a strong indication that stomatal traits would segregate in the F_2 mapping population. Decreased stomatal conductance following leaf excision and exposure to ABA was observed for *P. deltoides* and our results confirm other observations that *P. trichocarpa* and its hybrids show limited ability for rapid stomatal closure in response to water deficit (Schulte and Hinckley 1987a,b; Schulte et al. 1987; Hinckley et al. 1989; Kim et al. 2008). This limited stomatal response can partly be explained by stomatal insensitivity to ABA. Although increase in ABA concentration has been observed for both species under stress conditions (Schulte and Hinckley 1987a), mature leaves of *P. trichocarpa* appear unable to respond to this ABA produced and here we have shown that exogenous ABA application was ineffective in inducing stomatal closure in *P. trichocarpa* compared to *P. deltoides*. However, stomatal behavior was modified when trees of *P. trichocarpa* were preconditioned with water stress, followed by rewatering (Schulte and Hinckley 1987b; Schulte et al. 1987). If *P. trichocarpa* is exposed to drought, ABA production during the period of stress will influence developing leaves and enable stomata to function correctly (Schulte and Hinckley 1987a).

Measurements of leaf $\delta^{13}C$ in the field in Italy and in the United Kingdom revealed consistently higher values of leaf $\delta^{13}C$ for *P. trichocarpa* than for *P. deltoides*. Our data are in agreement with the lower values in $\Delta^{13}C$ also observed in *P. trichocarpa,* reflecting higher $\delta^{13}C$ than *P. deltoides* in a well-watered site in France in 2005 and 2006 (Dillen et al. 2011). The implication from all three of our datasets and those in the earlier studies is a higher WUE for *P. trichocarpa,* in both sites (Italy and United Kingdom). Dillen et al. (2011) suggested that this was consistent with the higher stomatal numbers in this species and yet our findings are counterintuitive from our and other data on stomatal behavior in these two species, that suggests a poor control of water loss in *P. trichocarpa* and thus potentially poor WUE. $\delta^{13}C$ is controlled by both water loss and photosynthetic rate, suggesting high rates of photosynthesis in *P. trichocarpa*. However, in previous studies, net photosynthesis was lower in *P. trichocarpa* compared to *P. deltoides* (Bassman and Zwier 1991). Stomatal conductance was certainly higher for *P. trichocarpa* in this study, which could explain a better WUE for this genotype. Nevertheless, although rainfall between January and August 2003 in the United Kingdom was a total of 386 mm compared to 152 mm in Italy, leaf $\delta^{13}C$ was also higher in Italy for *P. trichocarpa*. It has been suggested by Warren and Adams (2006), that caution should be used in the interpretation of data on carbon isotopic discrimination, in particular, when cross-species comparisons are made, where differences between leaf anatomy and structure are likely, such as exists between the grandparental species here. We know that leaves of the *P. trichocarpa* grandparent have large cells, with very large intercellular spaces, and fewer stomata (Ferris et al. 2002; Rae et al. 2004; Street et al. 2006), where large airspaces in particular could influence the internal conductance to CO_2. This is because the relationship between WUE and carbon isotope discrimination is influenced by the leaf internal conductance, a term rarely quantified but which may be problematic if differences in leaf anatomy, cell size, and intercellular spaces as well as leaf thickness are known as in our population (Ferris

et al. 2002; Rae et al. 2004; Street et al. 2006). Internal conductance ($g_i = A/(C_i - C_c)$) is the diffusion of carbon from the substomatal cavities (C_i) to the sites of carbon fixation (C_c) (Warren 2008) and may vary between species which would influence the relationship between $\Delta^{13}C$ and WUE (Warren and Adams 2006). Although anatomical differences may contribute to our findings, it seems likely, given the evidence, that once *P. trichocarpa* is preconditioned to drought, the WUE of emerging leaves may indeed be higher than that of *P. deltoides* as Schulte et al. (1987) demonstrated, developing stomata of *P. trichocarpa* with precondition by water stress responded very well to drought. Our own observations confirm this because *P. trichocarpa* showed a better WUE compared to *P. deltoides* in Italy in dry conditions after preconditioning was applied. Indeed compared to the site in the United Kingdom which was watered three times a week during the night, the Italian site experienced periods of drought and rewatering in the summer with flooding at four occasions between the end of June and mid-August 2003.

We also quantified the phenotypic variation in WUE-related traits in an F_2 mapping population using the measurement of carbon isotope composition, for which there is extensive theoretical and empirical data to suggest that this composition is mostly positively correlated with WUE, for a wide range of crops, such as wheat (Farquhar and Richard 1984; Condon et al. 2002), coffee (DaMatta et al. 2003), rice (Impa et al. 2005; Xu et al. 2009), sugar beet (Rajabi et al. 2009), oak (Ferrio et al. 2003; Brendel et al. 2008) and poplar (Ripullone et al. 2004; Marron et al. 2005; Monclus et al. 2005; Dillen et al. 2008). Here we were able to quantify variation in the F_2 population for leaf $\delta^{13}C$, revealing considerable differences, dependent upon both genotype and the varying environments and management conditions to which the trees were subjected. On average, $\delta^{13}C$ was higher in Italy than that in the United Kingdom A weak positive correlation between the dataset for the United Kingdom and Italy grown as single stem trees was also observed (Table S1, $r = 0.28$, $P < 0.001$). Although few genotypes expressed high leaf $\delta^{13}C$ in Italy and low leaf $\delta^{13}C$ in the United Kingdom or vice versa, genotypes with extreme values of leaf $\delta^{13}C$ (high or low) were generally the same for both sites and no significant genotype by site interaction was observed. No correlation was observed when comparing LA with $\delta^{13}C$ at each site (Table S1). LA is a good indicator of yield (Rae et al. 2004; Monclus et al. 2005), which means genotypes combining high yield potential and high WUE can be found in our population. Carbon isotope discrimination ($\Delta^{13}C$) was also studied in two F_1 families of poplar (*P.* × *P. nigra* and *P. deltoides* × *P. trichocarpa)*. Similar to our results, Dillen et al. (2011) showed lower $\Delta^{13}C$ in Italy than that in France in 2005, thus higher $\delta^{13}C$ in Italy. However, there was no significant Spearman rank correlation between sites in 2005 and significant G × E interactions for both families. This study goes further, however, in that we have gone on to elucidate the underlying genetic basis of these traits, identifying QTL for $\delta^{13}C$ and g_s that accounted for a moderate amount of genetic variation. Carbon isotope measurements have also been mapped for *Arabidopsis* (Masle et al. 2005), rice (Xu et al. 2009), chestnut (Casasoli et al. 2004), maritime pine (Brendel et al. 2002), and oak (Brendel et al. 2008), but few published data are available for poplar or willow (Monclus et al. 2012), two important lignocellulosic feedstocks for temperate climates (Sims et al. 2006). Few QTL exist for stomatal conductance for any species in the published literature (Ulloa et al. 2000; Hervé et al. 2001; Fracheboud et al. 2002), reflecting the difficulty of measuring this trait on many hundreds of individuals in replicated QTL mapping population experiments. For several regions of the genome "QTL hotspots" were defined where at least one QTL explained >5% of the variation and where multiple QTL were present. Three QTL hotspots were focused on and mapped to LG VI, X, and XVI (Fig. 5) and contained two and three $\delta^{13}C$ QTL on LG VI and X, respectively, and one QTL for $\delta^{13}C$ and one for g_s on LG XVI. The data for $\delta^{13}C$ were measured for 2 years in the United Kingdom with the same genotypes as a SRC (Rae et al. 2009) and single stem trees in this study, increasing our confidence in the QTL discovered, particularly those consistent between environments in the "hotspots". More QTL were found within these three areas of the genome from the literature for different traits that may be related to WUE: cell area (Rae et al. 2006), LA (Rae et al. 2006, 2009; Street et al. 2006), stomatal density (Rae et al. 2006), biomass (Rae et al. 2009; Street et al. 2011), and osmotic potential at full tugor (Tschaplinski et al. 2006). QTL were also discovered recently for carbon isotope discrimination in a F_1 population *P. deltoides* × *P. trichocarpa* mapped on LG VIb, VII, X, and XVIIb (Monclus et al. 2012), confirming our results with three out of four of their QTL colocating to those reported here. This provides strong evidence that these are robust areas of the *Populus* genome underpinning WUE and worthy of further exploration. Markers linking the genetic and physical map of the F_2 pedigree enabled us to determine gene models localized within these QTL "hotspots" in the *Populus* genome (Tuskan et al. 2006). 950, 758, and 979 genes were found within the 95% confidence intervals to which the QTL on LG VI, X, and XVI were mapped, respectively (Table S3). Several candidate genes with a putative role in determining tolerance to drought stress are apparent in this list, such as dehydration-responsive family protein, universal stress proteins (USP) or gene models stress such as low temperature and salt.

Combining microarray data (Street et al. 2006) and gene lists within QTL hotspots, 46 gene models were found to be significantly expressed in response to drought and also present within the QTL and 23 were on the hotspots on LG VI, X, and XVI (Table S4): ten were upregulated in response to drought in *P. deltoides* and *P. trichocarpa* (two on LG VI, three on LG X, and five on LG XVI), seven were downregulated in both species (three on LG VI, three on LG X, and one on LG XVI) and six were differentially regulated by the two species in response to drought (three on LG VI and three on LG XVI). Many of those gene models were stress- or water use -related: C3HC4-type RING finger genes, elF-5A (a translation initiation factor which has been observed in *Arabidopsis* mutants to improve growth under osmotic stress [Ma et al. 2010]), genes involved in signaling (serine/threonine protein kinases) and protein turnover such as chaperones and ubiquitins. On LG X, a model gene commonly expressed in response to drought for *P. deltoides* and *P. trichocarpa* was an ankyrin repeat family protein involved in salt tolerance (Table S4). A universal stress protein (UspA) was also on LG XVI and upregulated in both species. Candidate genes within hotspots should be viewed with caution, but combined with microarray data this gene list provides a valuable resource for further analysis, either through developing markers for these genes and testing them in different genetic backgrounds, such as, for example, poplar or willow bioenergy breeding programs (Karp et al. 2011) or reverse genetic approaches in *Arabidopsis* and *Populus* to test proof of concept and identify how manipulation of these genes may lead to an altered phenotype (Du et al. 2009; Behnke et al. 2010; Mohamed et al. 2010).

In conclusion, we have revealed wide variation in traits related to WUE and response to drought within a *Populus* F_2 pedigree, enabling three "hotspots" within the genome linked to WUE traits to be identified. Our preliminary analysis has already found a small number of candidate genes in these hotspots that provide targets for future molecular breeding and improved drought tolerance in *Populus*. Although many forward genetic studies are now moving away from mapping pedigrees such as the one described here, in favor of Genome Wide Association Studies (GWAS), these are not without their difficulties in trees, where population structure may limit usefulness (Ingvarsson and Street 2010). There remains considerable potential with the availability of RNA-Seq and other next generation genotyping technologies, to fine map QTL, enabling gene discovery linked to proof of concept in using RNA_i, particularly in *Populus* (Zhu et al. 2013). Wider applications of these data are also possible throughout the Salicaceae to willow as recent papers have shown linkage maps of *Salix* aligned with the *Populus* genome (Hanley et al. 2006; Berlin et al. 2010).

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Video S1. Video of leaf temperature of mature leaves of *P. deltoides* and *P. trichocarpa* under three different treatments: leaves in distilled water (left), in ABA 10^{-4} mol/L solution (middle) and with petiole excised simulating acute dehydration (right). The right-hand scale shows leaf temperature in °C. A grease spot was applied to each leaf as a dry reference and pieces of wet filter paper were used as a wet reference surface.

Figure S1. Relative conductance (g') calculated from leaf and reference temperatures in mature leaves of (A) *P. deltoides* and (B) *P. trichocarpa* in control condition (solid lines), ABA of 10^{-4} mol/L solution (broken lines) and excised condition (dotted lines). A representative example is shown for each treatment and species.

Table S1. Pearson's correlation coefficients and two-tailed measures of significance between phenotypic traits measured in Italy and the U.K.

Table S2. Details of QTL list from Figure 5 with the name of the traits, the name of the QTL used in the figure, the LG, the treatment or the location for each QTL and the reference from the literature.

Table S3. A complete list of genes contained within the QTL hotspots with the protein ID, the gene model name, the position on the linkage group, the description, the EuKaryotic Orthologous Groups (KOG ID, class and description), and Gene Ontology (GO) categories.

Table S4. List of genes from the microarray analysis (Street et al. 2006) highly expressed in response to drought which are also contained in the QTL hotspots on LG VI and X. The genes were either commonly expressed by *P. deltoides* and *P. trichocarpa* (up or down) or were differentially expressed by the two species in response to drought.