

Elucidating genomic regions determining enhanced leaf growth and delayed senescence in elevated CO₂

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ABSTRACT

Limited information is available on the genetic variation and control for plant growth response to elevated CO₂ (e[CO₂]). Such information is necessary to understand plant adaptation and evolution in future rising CO₂. Here, quantitative trait loci (QTL) for leaf growth, development, quality and leaf senescence were determined in a tree pedigree – an F₂ hybrid of *Populus trichocarpa* T. & G and *Populus deltoides* Marsh, following season-long exposure to either current day ambient carbon dioxide (a[CO₂]) or e[CO₂] at 600 µL L⁻¹. Leaf growth and development differed between the grandparents such that *P. trichocarpa* showed greater response to e[CO₂]. In the F₂ generation, leaf development and quality traits including leaf area, leaf shape, epidermal cell area, and stomatal number, specific leaf area (SLA), and the phenology trait, canopy senescence index, were sensitive to e[CO₂]. Sixty-nine QTL were mapped for the 19 traits of plants in a[CO₂] while 60 QTL were mapped for plants in e[CO₂]. The results suggest that although many QTL mapped to common positions in a[CO₂] and e[CO₂], confirming their importance in determining growth, there was also differential genetic control for a number of traits including leaf senescence. Candidate genes were shown to collocate to regions where response QTL mapped. This study is the first to identify candidate genes that may be important in determining plant adaptation to future high-CO₂ world.

Key-words: adaptation; poplar; QTL.

INTRODUCTION

The concentration of atmospheric CO₂ is rising at an unprecedented rate as a consequence of fossil fuel combustion and by 2050, a concentration of 550 µmol mol⁻¹ is likely (Grace 2004). Exposure to elevated CO₂ (e[CO₂]) usually results in increased plant growth. Leaves grow faster and reach a larger final size in e[CO₂], an observation made for a wide range of species and growing conditions (Taylor *et al.* 1994, 2001; Pritchard *et al.* 1999), several developmental changes are also now known to occur in response to

e[CO₂], including altered leaf cell development (cell size and number, Taylor *et al.* 2003), stomatal patterning (reduced stomatal numbers, Hetherington & Woodward 2003) and leaf quality (often calculated as SLA, specific leaf area, Tricker *et al.* 2004). The consequences of such responses for altered plant fitness and long-term adaptation remain the subject of speculation because most current research has been focused on physiological changes.

We need to understand more about long-term adaptation and genetic changes in future e[CO₂], particularly for adaptive traits that are relevant to plant productivity and ecological characteristics that determine survival, fitness and interaction with pests and pathogens (Ward & Kelly 2004). In an ideal world, we would wish to identify the genes that determine ecological success in future CO₂ environments (Feder & Mitchell-Olds 2003) and for trees a good start point would be to identify aspects of leaf development and growth that show phenotypic plasticity. Such traits are likely to be linked to reproductive fitness (Kramer 1995; Wu, Bradshaw & Stettler 1997), which can be identified and these should be studied further. Fast-growing trees in the genus *Populus* are characterized by rapid leaf extension rates and the production of large leaves (Ridge *et al.* 1986; Ferris *et al.* 2001; Rae *et al.* 2004), with leaf area development sensitive to and stimulated by e[CO₂] (Taylor *et al.* 2003; Walter *et al.* 2005). It remains unclear how such stimulatory effects will lead to long-term adaptation (associated with genetic changes), although for physiological processes such as photosynthesis, it is already known that gene expression changes occur, for example, for small subunits of the Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) protein (Moore *et al.* 1999; Taylor *et al.* 2005) and gene expression profiling using microarrays has begun to identify candidate genes that are sensitive to e[CO₂] (Taylor *et al.* 2005). It is likely that phenotypic plasticity will provide a clue to future plant adaptation to e[CO₂], where CO₂ may be viewed as a selection pressure, inducing long-term ecological and evolutionary change. Leaf development, longevity, petiole orientation and stomatal number all determine the amount of intercepted radiation, carbon gain and water loss in a forest canopy which, together with photosynthetic efficiency, determines net primary productivity (Monteith 1977; Lambers & Poorter 1993). We focus this study on such leaf characteristics, because this is of considerable significance to commercial tree growth, as well as to ecology and evolution of trees in future conditions.

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Elucidation of quantitative trait loci (QTL) can be used to understand fundamental aspects of genetic control in plants, particularly model species, grown under differing conditions. It can provide evidence that a plant characteristic of interest has a genetic component and is a good starting point for future studies on individual genes and genomic regions, or in focusing on the inheritance and evolution of specific traits of interest. To our knowledge, few studies of QTL identification in e[CO₂] have been published (Ferris *et al.* 2002). This is surprising because the approach has yielded valuable insight into plant response to a range of environmental changes including salinity (Koyama *et al.* 2001), drought (Lanaceras *et al.* 2004), nitrogen supply (Loudet *et al.* 2003) and soil aluminium concentration (Hoekenga *et al.* 2003), and has prompted gene-cloning strategies and identified *Arabidopsis* as a valuable model to understand genetic variation in an ecological context (Alonso-Blanco *et al.* 1998).

Here we have studied the effect of e[CO₂] using an interspecific inbred F₂ pedigree (family 331) from the two species, *Populus trichocarpa* T. & G and *Populus deltoides* Marsh (Bradshaw *et al.* 1994; Bradshaw & Stettler 1995). These species contrast greatly in leaf morphology, and the F₂ generation segregates for a wide variety of leaf traits making this an ideal pedigree in which to study quantitative traits. In addition, poplar is now recognized as the 'model' forest tree (Taylor 2002; Wullschlegel, Jansson & Taylor 2002), and has several advantages as a model system, including the availability of mapping pedigrees with linkage maps, transformation systems and a large genomic resource including expressed sequence tag collections and poplar microarrays (Andersson *et al.* 2004; Taylor *et al.* 2005), and most importantly the release of the complete genome sequence, the first for a tree, in 2004 (Brunner, Busov & Strauss 2004; <http://genome.jgi-psf.org/Poptr1/Poptr1.home.html>). This resource will enable significant advances to be made in our understanding of both economically important traits that are unique to perennial woody plants, as well as a considerable increase in our ability to answer fundamental questions in forest ecology and evolution. However, the difficulties of QTL discovery in trees can never be underestimated because they are outbreeding and dioecious. The quantitative genetics of outbreeding species is complicated by their heterozygosity so that up to four alleles may be segregating at each locus. Few QTL mapping programmes allow for the segregation of more than two alleles, and although they can map QTL in outbreeding pedigrees using a pseudo-test cross model (Grattapaglia *et al.* 1996), some mapping resolution may be lost. Methods for mapping in outbreeding populations are being developed (Knott *et al.* 1997; Lin & Wu 2005) and software is becoming available (Seaton *et al.* 2002). For the pedigree under study here, an ideal mapping method is the linear regression approach put forward by Haley & Knott (1992). This method has been modified for use with outbred populations and is available in a user-friendly format QTL Express (Seaton *et al.* 2002).

The study described here aims to identify the genetic basis of tree response to future CO₂ concentrations through

the elucidation of QTL. Our ultimate goal is to identify the underlying genes for response and to determine whether they are good candidates for future adaptive selection as CO₂ continues to rise.

MATERIALS AND METHODS

Plant material and exposure conditions

A three-generation *Populus* mapping pedigree was generated by the hybridization of *P. trichocarpa* Clone 93-968 from western Washington and *P. deltoides* Clone ILL-129 from central Illinois in 1981. Two full-siblings, 53-246 and 53-242, from the resulting F₁ family (family 53) were crossed to form an F₂ family (family 331; Bradshaw & Stettler 1993; Bradshaw *et al.* 1994). Two hundred and eighty-five members of this family were used in this study.

This experiment was conducted in 16 open-top chambers (OTC) at the Forestry Commission field site, Headley, UK. (51°07'N, 0°50'W). On 13 and 14 May 1999, the *P. trichocarpa* and *P. deltoides* grandparents, the F₁ parents and F₂ genotypes were established from unrooted hardwood cuttings derived from a stool bed at the University of Washington, Seattle, WA, USA. Cuttings were grown in John Innes No. 2 compost (lime-free) in plastic tubes (91 cm in height, 15 cm in diameter), in a randomized block design. For each treatment, the 289 genotypes were placed randomly into one of eight chambers (c. 36 genotypes/chamber). The plastic tubes were placed 25 cm apart in a circular pattern. The pots were buried to a depth of 10 cm for stability. Eight of these chambers received a[CO₂] while eight chambers received e[CO₂] at a target concentration of 600 μmol mol⁻¹ CO₂. Details of chamber design and monitoring of CO₂ have been reported previously (Ferris *et al.* 2002). Measurements were conducted throughout the growing season.

Leaf growth

During July (68–69 d after planting, DAP), a very young growing leaf from each genotype was labelled with coloured cotton and photographed flat against a white background (with marked scale) using a digital camera (Nikon Coolpix 950; Nikon UK Ltd, Kingston upon Thames, London, UK) described fully in Taylor *et al.* (2003). These leaves were rephotographed at 75–76 DAP. Images were imported into an image processing and analysis programme for resizing and format conversion. The programme 'scion image' (Scion Corporation, Rederick, MD, USA) was used to measure leaf area, length, width and leaf width to length ratio on the two separate dates. Leaf length extension and area expansion rates were calculated from this data. The number of leaves on the main stem was counted at 125 DAP.

In September (130–134 DAP), one single fully mature leaf was excised from each tree. Leaves were photocopied and leaf areas (mm²) were measured with an Image Analyser (Delta T Devices, Cambridge, UK). A template of 1.5 cm² was placed on the base of the abaxial and adaxial

surface of the leaf and the area sprayed with clear lacquer (Halfords, Reddith, UK) and left to dry for 20–25 min. A leaf imprint was obtained with tape (Ferris & Taylor 1994), then placed on a microscope slide. Digital images of the epidermal impressions were captured with a light microscope (Axiophot 2 Universal Microscope; Carl Zeiss, Jena, Germany) and a digital imaging software (Metamorph Imaging System, West Chester, PA, USA), was used to obtain one digital image per slide of mature adaxial epidermal cells from between the midrib and the major veins. The areas of 10 adaxial epidermal cells per slide were obtained randomly from the image. An estimation of cell number was calculated for each poplar genotype from the mean cell area and the leaf area.

The number of stomata per half field of view was converted to number of stomata per mm². These data were used to calculate the stomatal index (SI), which relates stomatal density (SD) to the number of epidermal cells per unit area (ECD), where $SI = [SD / (SD + ECD)] \times 100$.

Individual leaf dry mass was recorded following oven drying at 80 °C for 48 h and SLA (mm² g⁻¹) measured.

Leaf plasticity and elasticity

One expanding leaf was taken from each tree in September (130 DAP) to measure the plasticity and elasticity of the leaf cells using a home-made Instron-type apparatus (Gardner, Taylor & Bosac 1995), using the technique described by Van Volkenburgh, Hunt & Davies (1983). Leaf samples were stored in 70% methanol then rehydrated in distilled water for 20 min. Sections of leaf tissue were stretched between two small brass clamps to a known weight load. Results were expressed as percentage plasticity (the percentage irreversible extension per 10 g load) and percentage elasticity (percentage reversible extension per 10 g load).

Leaf senescence

Leaf senescence was measured as 'non-leaf greenness' in November (181 DAP) in both a[CO₂] and e[CO₂]. Trees were scored using a visual index expressed as a percentage. The colour change was estimated as the relationship between the number of brown turned leaves and the total leaves on the stem. The index ranged from 0 to 10, where 0 was no leaf senescence (i.e. still green) and 10 = 100% complete senescence. The remaining indices were 1 ≤ 10%; 2 ≤ 20%; 3 ≤ 30%; 4 ≤ 40%; 5 ≤ 50%; 6 ≤ 60%; 7 ≤ 70%; 8 ≤ 80%; and 9 ≤ 90%.

Petiole length

Petiole length was measured using a paper ruler for leaves two below the labelled leaves at 68–69 (DAP).

Data analyses

Differences between plants grown in a[CO₂] and e[CO₂] were tested using a paired *t*-test in Minitab statistical package (version 13; Minitab Inc., Philadelphia, USA).

QTL mapping

The genetic linkage map for this pedigree was produced at Oak Ridge National Laboratory consisting of 91 simple sequence repeats (SSRs) genotyped on 350 individuals and 92 fully informative amplified fragment length polymorphisms (AFLPs) genotyped on 165 individuals (Yin *et al.* 2004). The primer sequence of SSR markers were blasted against the poplar genome sequence and linkage groups (LGs) orientated based on the physical sequence (i.e. 3' to 5').

Trait data were tested for normal distribution using Andersson–Darling test. In cases where data were non-normally distributed Box–Cox transformations were carried out.

One-way analysis of variance (ANOVA) was carried out for each trait to test for variation between genotypes. The error mean square (MS_E) from this was taken as environmental variance, V_E , and the genetic variance, V_G , was calculated as

$$V_G = \frac{(MS_G - MS_E)}{r}$$

where, MS_G is the mean square between genotypes and r is the number of replicates. Within family broad-sense heritability was calculated by dividing the genetic variance by the total or phenotypic variance ($V_P = V_G + V_E$) for each trait (Falconer & Mackay 1996).

The data were analysed for QTL using the linear regression approach put forward by Haley & Knott (1992). The analysis was carried out using the option for outbred large, single full-sib families with the software QTL Express (Seaton *et al.* 2002). This method determines the identity-by-descent (IBD) probabilities at specific chromosomal locations from multiple marker data, then fits statistical models to the observations and IBD coefficients. Chamber effect was included as a fixed effect in the QTL analysis. Chromosome-wide permutation tests with 1000 iterations were carried out to determine *P*-values and a significance threshold of 0.05 was taken as evidence for presence of a QTL (Churchill & Doerge 1994). Confidence intervals for the position of a QTL were defined as the interval in which the *F*-statistic of the presence of a QTL was at least twofold of its maximum value. After identifying QTL by an initial genome scan, two-way ANOVA was carried out, using R-script, for each molecular marker flanking a QTL, so that QTL by CO₂ interactions could be identified. The model used was $Y'_{jkl} = \mu + M_j + T_k + M \times T_{jk} + \varepsilon_{jkl}$, where Y'_{jkl} are individual trait values for each marker genotype at both CO₂ levels, μ is the general mean, M_j is the marker genotype effect (fixed), T_k is the CO₂ treatment (fixed), $M \times T_{jk}$ is the marker by treatment interaction (fixed), and ε_{jkl} is the error.

Percentage differences between trees grown in a[CO₂] and e[CO₂] were calculated for each tree for each trait as $(e[CO_2] - a[CO_2]) / a[CO_2] \times 100\%$. The results were then treated as traits and QTL mapped for 'response traits' using QTL Express in the same way as described earlier.

The genotype data for SSRs were converted into the format for use in MAPMAKER-EXP and links between these SSRs and RFLPs previously mapped by Bradshaw

et al. (1994) for this population were found so that comparisons with QTL cited in literature could be compared with those mapped in this study.

The positions of candidate genes previously shown to be up- or down-regulated in response to $e[\text{CO}_2]$ in a free air CO_2 enrichment (FACE) microarray experiment (Taylor *et al.* 2005) were found from the physical sequence of poplar and their positions on the linkage map estimated to identify candidate genes that collocated to regions where QTL mapped.

RESULTS

Grandparental characteristics

Leaf and cell characteristics showed similar results to those previously described for the grandparental species under similar ambient conditions (Wu *et al.* 1997; Ferris *et al.* 2002; Rae *et al.* 2004), but the differential response of the two grandparents to $e[\text{CO}_2]$ is of interest here. The *P. trichocarpa* grandparent showed increased leaf growth in $e[\text{CO}_2]$ with more than a fourfold increase in leaf expansion rate, whereas *P. deltoides* showed little response to $e[\text{CO}_2]$, with even a slight decrease in early-season leaf growth and cell number (Fig. 1). For some traits, consistent responses in the two grandparents were observed, for example for senescence index, which was reduced in $e[\text{CO}_2]$, suggesting delayed senescence in both *P. deltoides* and *P. trichocarpa*, in $e[\text{CO}_2]$.

Population response to $e[\text{CO}_2]$

Paired *t*-tests carried out to identify population response to $e[\text{CO}_2]$ confirm many previous studies that have quantified the effects of $e[\text{CO}_2]$ on leaf growth, development and productivity (Fig. 2), and provide strong evidence that the

QTL mapped from these data will be relevant and of value for further detailed molecular analysis. SLA was reduced, as observed previously in *Populus* (Tricker *et al.* 2004) and leaf growth, in general, was stimulated (Taylor *et al.* 2003). Leaf production, as assessed from main-stem leaf number, was not sensitive to $e[\text{CO}_2]$. SD but not SI was reduced in $e[\text{CO}_2]$ suggesting that the primary effect on stomatal numbers was through increased leaf cell area and not the initiation of guard cells, again in accordance with previous studies (Tricker *et al.* 2005), although this point is controversial (Hetherington & Woodward 2003). The population distributions for selected traits are shown (Fig. 3) with the grandparental and parental values in $a[\text{CO}_2]$ and $e[\text{CO}_2]$ marked. A full data set for all frequency distributions is given in the Supplementary Materials (Fig. S1).

QTL mapped

A total of 69 QTL were mapped in the pedigree in $a[\text{CO}_2]$, 60 QTL in $e[\text{CO}_2]$ and 28 response QTL for the 19 traits (Fig. 4). There was evidence for QTL for all traits grown in both $a[\text{CO}_2]$ and $e[\text{CO}_2]$. QTL for response to CO_2 were found for all traits with the exception of leaf expansion rate, plasticity, cell number and SLA. QTL mapped to all LGs with the exception of LGXVIII. The PU numbers of candidate genes reported to be differentially regulated under FACE in a previous study by Taylor *et al.* (2005) are marked on the linkage map, and QTL previously mapped in this population have been indicated where it was possible to identify the LG from previously published work using a different linkage map (Fig. 4). All details of these QTL are given in Tables S1, S2 and S3 (see Supplementary Materials), including maternal and paternal effects, confidence interval for each QTL and percentage variance accounted for.

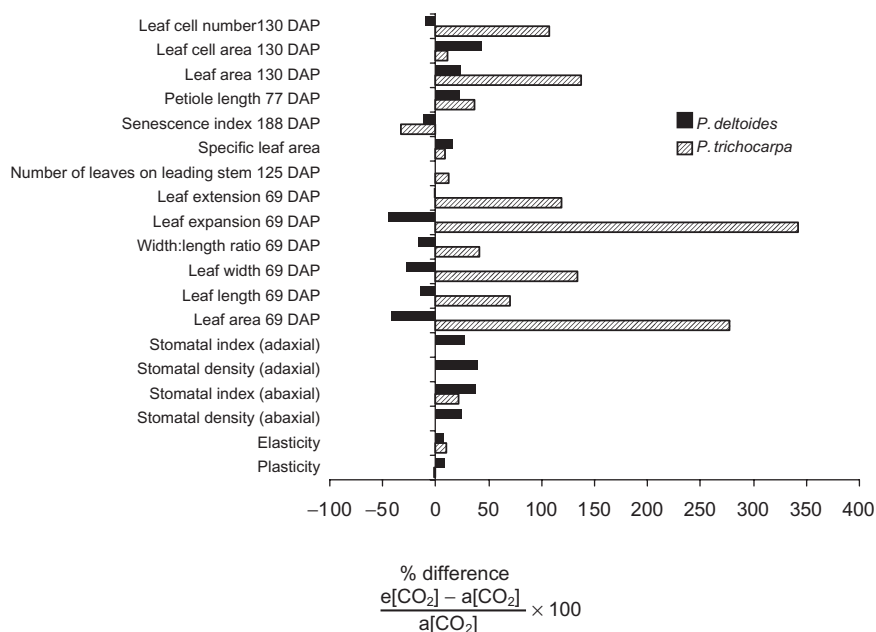


Figure 1. Effect of elevated CO_2 on the grandparents of an F_2 pedigree of *Populus*. Response shown for leaf traits for the *Populus trichocarpa* (hatched symbols) and *Populus deltoides* (closed symbols) grandparents. DAP, days after planting.

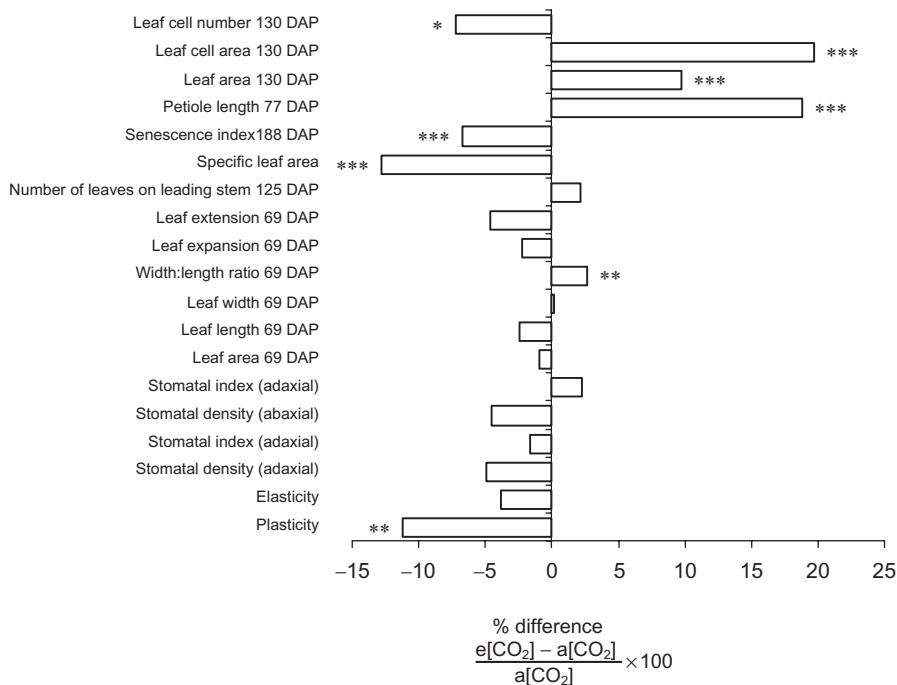


Figure 2. Effect of elevated CO₂ in an F₂ *Populus* population from a cross between *Populus trichocarpa* and *Populus deltoides*. Statistical significance is given as results from a paired-test where '*' indicates 0.05 > P > 0.01, '**' indicates 0.01 > P > 0.001 and '***' indicates P < 0.001. DAP, days after planting.

Many QTL collocated for correlated traits across the two CO₂ treatments, for example several leaf growth traits have QTL which mapped between 0 and 12 cM on LGI both in a[CO₂] and e[CO₂]. In addition, QTL for leaf length and width were mapped to this position (data not shown). Furthermore, no response QTL mapped to this region suggesting that genes governing leaf growth traits were not differentially controlled in the two CO₂ concentrations. Two-way ANOVA for molecular markers in this region agree with this conclusion in that significant variation was seen between genotypes but not between CO₂ or genotype by environment (*G* × *E*) interaction. QTL for leaf length to width ratio have been reported previously on this LG (Wu *et al.* 1997).

However, only 21 QTL collocated for the same traits across both a[CO₂] and e[CO₂], suggesting that there was much differential control of these traits across the two CO₂ environments. Of particular interest is the collocation of response QTL (shown in green in Fig. 4). For example, on LGXIII, QTL for stomatal traits in a[CO₂] mapped close to response QTL for stomatal traits. There is no evidence for the presence of QTL mapped in e[CO₂] for these traits, so this suggests that this trait is differentially controlled across the two CO₂ environments.

Senescence index proved to be a trait of moderate to high heritability (0.68, Table 1) and showed a highly significant decrease in e[CO₂], indicating that leaves remained green on the trees later in the season (Fig. 3). Six senescence QTL were mapped to similar positions on LGIII, LGIV, LGV, LGVI, LGVII and LGXII, under both a[CO₂] and e[CO₂]. A response QTL mapped to a similar position as an e[CO₂] QTL on LGI and the a[CO₂] and e[CO₂] QTL on LGV. This implies that the QTL on LGI represents a gene that

was switched on or of greater importance in explaining the senescence response to e[CO₂], but not a[CO₂]. The presence of the three QTL on LGV imply that this region controls senescence in both a[CO₂] and e[CO₂], but the presence of the response QTL suggests that the QTL act differently in the two treatments. This is confirmed by the two-way ANOVA that showed there to be significant genotype and CO₂ effect at markers in this region, but no significant *G* × *E* interaction.

Also of interest, and worthy of further study, are the cluster of collocating response QTL on LGXII for leaf area and cell traits, and the presence of two candidate genes. The PU numbers of candidate genes reported to be differentially expressed in e[CO₂] in a FACE experiment, *Populus* free air carbon enrichment (Taylor *et al.* 2005) are marked on the linkage map. Collocation of QTL to these genes is of particular interest suggesting that they may be involved in the genetic determination of the traits studied here.

The summed percentage variance explained by all QTL per trait ranged from 6.1 to 28.1 for a[CO₂], 6.6 to 38.1 for e[CO₂] and 2.0 to 99.0 for response QTL (Table 1). The confidence intervals for the position of QTL, defined as the interval in which the *F*-statistic of the presence of a QTL was at least twofold of its maximum value, varied between 4 and 54 cM, with an average of 22 cM.

The heritability values and the suggested presence of *G* × *E* interactions based on non-homogenous variances for traits scored in a[CO₂] and e[CO₂], and the number of markers which showed significant genotype by CO₂ interaction from a two-way ANOVA are shown in Table 1. It can be seen that most traits showed a low to moderate heritability, although senescence index, number of leaves and leaf area showed moderate to high heritability.

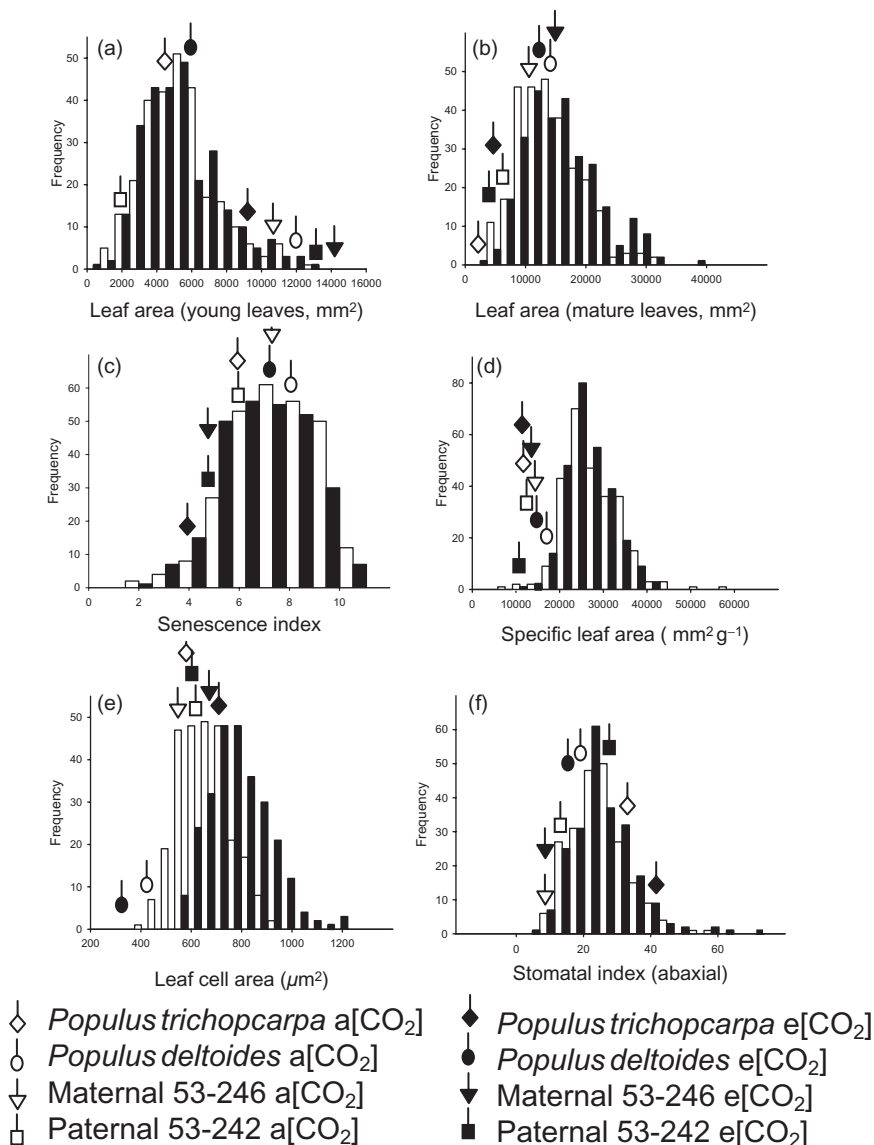


Figure 3. Phenotypic responses to elevated CO₂ in a *Populus* population. Distribution of the phenotypes for leaf traits in ambient (open bars) and elevated (closed bars) CO₂. (a) Leaf area (young leaves), (b) leaf area (mature leaves), (c) senescence index, (d) specific leaf area, (e) leaf cell area and (f) stomatal index.

DISCUSSION

Plant growth and developmental changes in e[CO₂]

Few studies have considered the genetic basis of plant response to e[CO₂], and yet there is considerable evidence that past changes in atmospheric CO₂ have acted as a selection pressure, leading to altered plant development and adaptation. For example, stomatal numbers have been shown to have declined since pre-industrial and across geological timescales (Beerling & Woodward 1997; Hetherington & Woodward 2003) – an effect attributed to rising atmospheric CO₂. Such correlative studies are however, controversial and here we have taken a different approach, for the first time to our knowledge, using genetic variation to link developmental responses to their underlying QTL. Altered plant growth and development in e[CO₂] often includes important changes in leaf development and morphology. These changes are of adaptive significance because

they are likely to have an effect on plant competitive ability and fitness. By exposing a mapping population to e[CO₂], we have revealed these responses as well as detected the underlying QTL determining leaf growth and development.

The data for the pedigree response to e[CO₂] confirm many previous studies that have quantified the effects of e[CO₂] on leaf growth, development and productivity, and provide strong evidence that the QTL mapped from these data will be relevant and of value for further detailed molecular analysis. Leaf area was stimulated as the season progressed and leaf shape altered, with the production of larger and wider leaves in e[CO₂], as observed previously in this genus (Ferris *et al.* 2001; Taylor *et al.* 2003). This is an important finding because biomass gain in poplar is known to be tightly linked to the production of large leaves (Rae *et al.* 2004) and because increased leaf area is likely to be a trait associated with increased competitive ability. This relationship suggests that leaf size and shape may be valuable indicator traits for both carbon gain and long-term

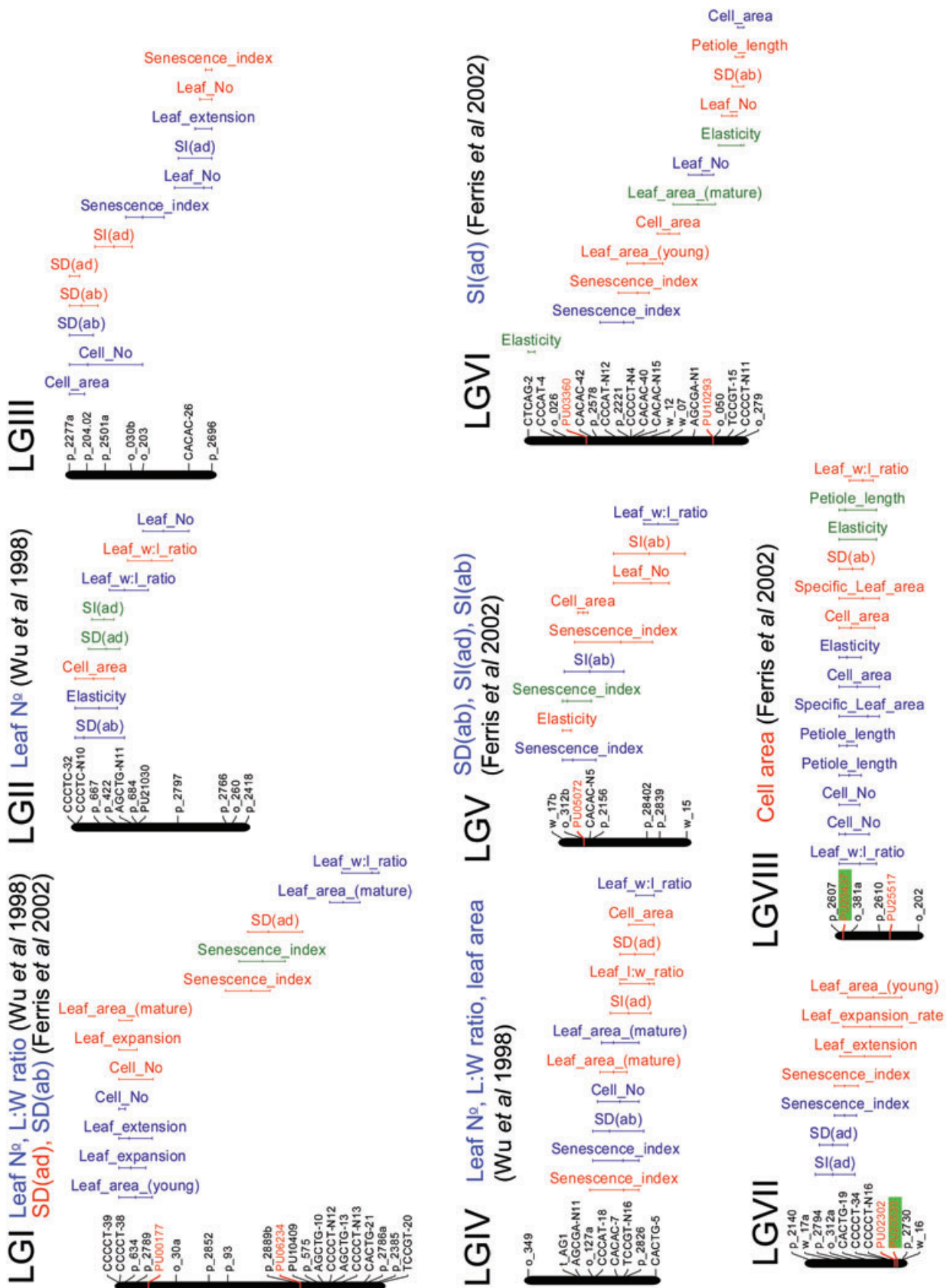


Figure 4. Quantitative trait loci (QTL) for leaf growth and senescence. QTL mapped to each linkage group (LG) for traits scored in ambient (blue), or elevated CO₂ (red) and response QTL (green) are shown. Genes shown to be altered in expression in semi-mature leaves grown in elevated CO₂ (Taylor *et al.* 2005) are marked in red on the linkage map and those altered in expression in young leaves highlighted in pale green. The full annotation for these PU numbers is given as S4, LG, which have been previously shown to contain QTL from the literature, are indicated. L, length; SD, stomatal density; SI, stomatal index; W, width.

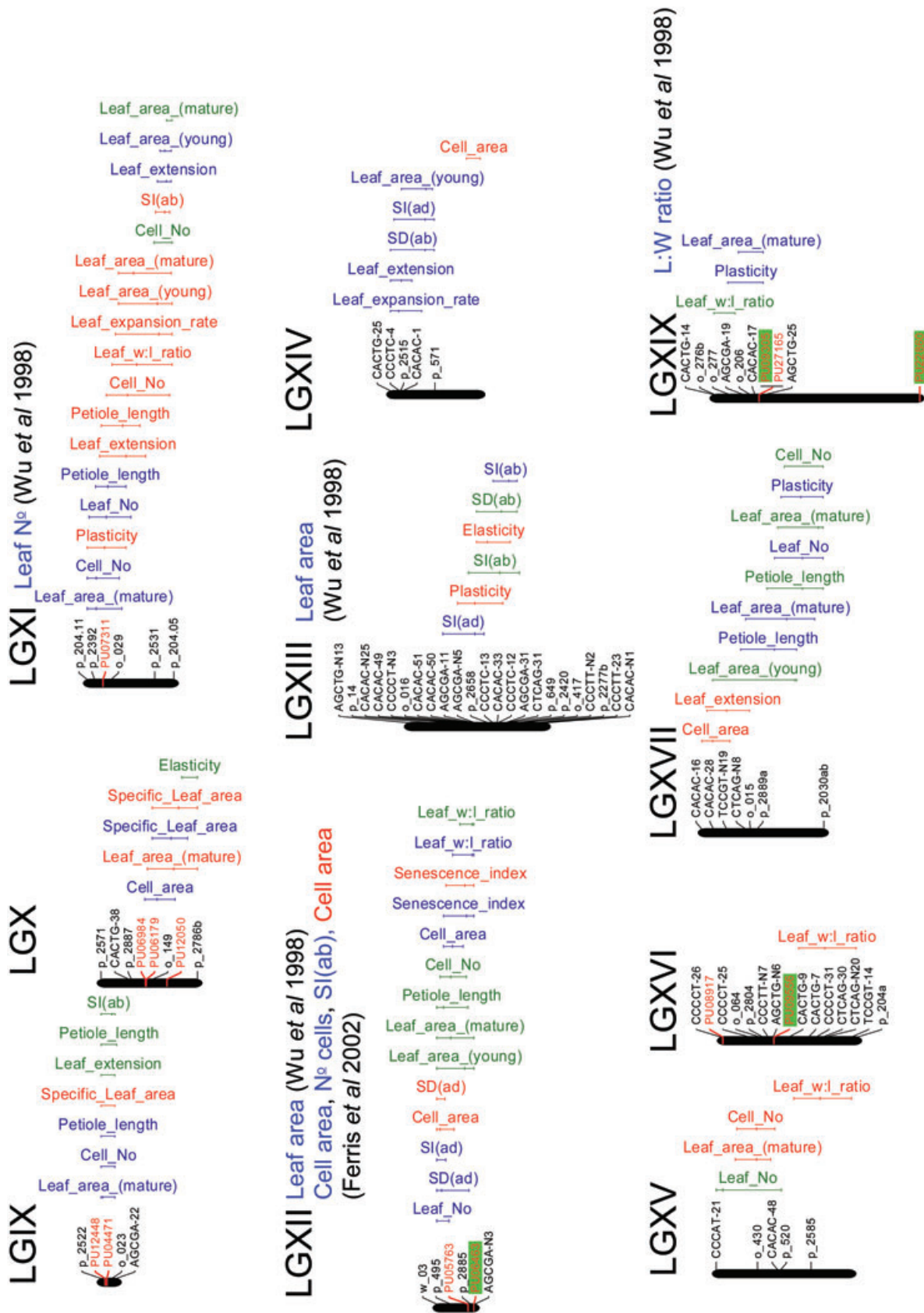


Figure 4. Continued

Table 1. The total genetic variation explained by the mapped quantitative trait loci (QTL) in ambient and elevated CO₂ (a[CO₂], e[CO₂], respectively) and for response QTL (rCO₂), heritability values across a[CO₂] and e[CO₂] and the genotype by environment interaction denoted as $G \times E$ for significant differences between variances for the pedigree grown in a[CO₂] and e[CO₂] and denoted as number (n) of $G \times E$ effects for the number of markers that showed significant genotype by CO₂ effect

Trait	Total variance explained				$G \times E$	n° of $G \times E$ effects
	a[CO ₂]	e[CO ₂]	rCO ₂	Heritability		
Leaf area (mm ²) 69 DAP	15.4	12.8	5.2	0.15		3
Leaf width to length ratio 69 DAP	23.5	17.8	5.3	0.55		1
Leaf expansion (mm ² d ⁻¹) 69 DAP	6.1	13.1	–	0.10	*	6
Leaf extension (mm d ⁻¹) 69 DAP	14.6	14.5	2.0	0.09	*	7
Senescence index (188 DAP)	21.9	36.4	99.0	0.68		0
Petiole length (mm) 77 DAP	20.9	8.3	73.0	0.42	*	0
Number of leaves on leading stem 125 DAP	24.2	19.8	3.1	0.51		0
Specific leaf area (mm ² g ⁻¹)	10.4	9.4	–	0.44	***	0
Stomatal density (adaxial)	9.3	22.1	4.2	0.25		5
Stomatal index (adaxial)	14.4	8.0	6.4	0.13		3
Stomatal density (abaxial)	19.7	19.5	3.1	0.35	*	1
Stomatal index (abaxial)	8.7	7.8	8.3	0.04	*	11
Plasticity	9.5	6.6	–	0.92	***	0
Elasticity	7.4	8.0	14.3	0.74	*	2
Leaf cell area (μm ²) 130 DAP	25.5	38.1	–	0.03	**	0
Leaf area (mm ²) 130 DAP	28.1	19.0	12.7	0.50	**	1
Leaf cell number × 10 ⁶ 130 DAP	26.9	11.2	10.6	0.42		4

DAP, days after planting.

*Statistical significance at $0.05 > p > 0.01$.

**Statistical significance at $0.01 > p > 0.001$.

***Statistical significance at $p < 0.001$.

evolutionary responses to e[CO₂] (Ward & Kelly 2004), particularly because leaf area was shown to have a high heritability (Table 1). There have been several studies that have shown that both cell size and number are important for leaf area development of poplar (Taylor *et al.* 1994) which suggest that cell production is likely to be important for spatial patterns of leaf development and cell expansion important in determining final leaf size (Taylor *et al.* 2003). Cell expansion may be more responsive to changes in the environment including altered atmospheric CO₂, as shown here (Fig. 3), confirming earlier findings in this genus (Taylor *et al.* 1994). Leaf quality, particularly leaf thickness (often inferred from SLA) and leaf C and N are also sensitive to e[CO₂] (Tricker *et al.* 2004) and here we found a significant decline in e[CO₂].

Senescence was delayed in e[CO₂] – a controversial finding because only limited evidence on the effects of e[CO₂] on autumnal senescence in trees is available and this provides no clear picture, with conflicting effects depending on species, growth conditions and length of experiment. Delayed autumnal senescence was found for *Quercus* in e[CO₂] (Li, Dijkstra & Hymus 2000) and increased leaf longevity in *Populus* in e[CO₂] (Tricker *et al.* 2004), while Sigurdsson (2001) reported advanced senescence in *Populus* following exposure to e[CO₂] and Herrick & Thomas (2003) and Norby, Hartz-Rubin & Verbrugge (2003) found no effect for *Liquidambar* and *Acer*, respectively. We can hypothesize that these differences are related to different physiological responses of leaves to e[CO₂]. Altered leaf quality, in particular reduced SLA is known to be

associated with increased leaf longevity and it may be that such leaves show delayed autumnal senescence, staying photosynthetically active longer. If nitrogen is limiting however, this response may be absent, as suggested by Sigurdsson (2001). Some evidence from model systems that the balance between C and N is critical in determining senescence response has also been provided by Wingler, Mares & Pourtau (2004).

QTL analyses – identifying the genetic basis of plant response to e[CO₂]

The results of this study suggested that although some QTL from the ambient and elevated CO₂ treatments collocated on the genetic map, there were many QTL that were solely identified in the different growing conditions, and furthermore these were collocated to a number of genes discovered from previous experiments. This was confirmed by the presence of response QTL and two-way ANOVA.

A number of interesting regions worthy of future study can be seen in Fig. 4. Several leaf traits have QTL that mapped between 0 and 12 cM on LG1 both in a[CO₂] and e[CO₂]. In addition, QTL for leaf length and width were mapped to this position (data not shown). Furthermore, no response QTL mapped to this region suggesting that genes governing leaf traits were not differentially controlled in the two CO₂ concentrations. Two-way ANOVA for molecular markers agree with this conclusion in that significant variation was seen between genotypes but not between CO₂ concentration or $G \times E$ interaction. Previous

literature also located QTL for leaf length to width ratio on this LG (Wu *et al.* 1997). QTL on LGI and III for the number of leaves on the main stem were also found to map to the same linkage group in a previous study (Wu *et al.* 1997).

Many traits showed differential control under the two CO₂ treatments. An example of this is senescence index that showed a highly significant decrease in e[CO₂]. Delayed autumn senescence in trees is of considerable interest, firstly because rather little is known about the genetic mechanisms controlling this type of senescence and whether or not SAGs (senescence associated genes) are similar to those identified in herbaceous plants (Navabpour *et al.* 2003), and secondly because delayed senescence can result in considerable enhancement of seasonal forest carbon gain, estimated as an extra 5.7 g carbon m⁻² d⁻¹ (Baldocchi *et al.* 2001). Here we have identified several genomic regions in *Populus* that may be used to elucidate the genetic control of autumnal senescence in trees.

QTL mapped to all LGs in either a[CO₂] or e[CO₂] with the exception of LGXVIII. From regression analysis, there was some evidence that the number of QTL mapped per LG was related to length of the LG ($P = 0.062$ for a[CO₂] and $P = 0.014$ in e[CO₂]). The number of QTL detected per trait ranged from two to eight with an average of four per trait in a[CO₂] and three and a half in e[CO₂]. This is in keeping with similar studies (Kearsey & Farquhar 1998). Individual QTL were shown to explain relatively little of the total phenotypic variance, and summing the effects for all QTL mapped for each trait explained less than half the total variation with the exception for response QTL for senescence index for which 99% of the variation may be explained by the QTL mapped. Previous QTL mapping studies in trees have reported results suggesting that the traits measured were controlled by several loci with relatively large effect (Bradshaw & Stettler 1995; Grattapaglia *et al.* 1996). These studies for growth and wood traits were however, carried out with considerably smaller sample sizes, and so were less likely to detect QTL of small effect. The fact that much of the variation is left unexplained in this study suggests that there may be additional QTL with smaller effects that cannot be detected. Other studies using QTL Express in outbred tree pedigrees have also reported low values for the variance explained (Sewell *et al.* 2002; Brown *et al.* 2003). The genome scan using QTL Express was supported by carrying out two-way ANOVA for molecular markers flanking QTL (full data not shown). The majority of markers closest to the QTL mapped using QTL Express showed significant effects for the traits studied, backing up the presence of QTL detected using this software. This suggests linear regression may be a superior method for detecting QTL with small effects. The low variation explained and the presence of other significant effects observed by carrying out ANOVA at markers suggests that there are likely to be more undetected QTL segregating in this pedigree.

The confidence intervals for QTL mapped ranged from 4 to 54 cM. QTL Express allows confidence intervals to be

calculated by the bootstrap method, but these are often highly overestimated especially when the contribution of the QTL is weak (Visscher, Thompson & Haley 1996; Walling, Visscher & Haley 1998). The method used in this study is comparable to the one-LOD drop off interval to calculate the 95% confidence interval used by maximum likelihood mapping methods. However, it may be of more interest to study the regions in which QTL overlap for correlated traits.

$G \times E$ interactions were tested for each trait by testing for homogeneity of variances between CO₂ treatments. Differences in the population variances suggest that the genotypes are acting differently in the two environments. Highly significant $G \times E$ interactions were seen for SLA and leaf plasticity; moderate interactions were seen for young and mature leaf growth, plasticity, cell area, petiole length and stomatal traits. In addition to this, CO₂ response effects were mapped using the idea that the percentage difference between plants grown in a[CO₂] and e[CO₂] could be used as a trait score and response QTL mapped. Two-way ANOVA for markers were used to test for QTL \times CO₂ effects. Few interactions proved to be significant. Interactions appeared to be evenly distributed across LGs, and all traits, with the exception of number of leaves, SLA, plasticity and cell area, showed genotype by CO₂ interactions at least one marker position.

Of the 28 QTL identified for percentage change between a[CO₂] and e[CO₂], five mapped to LGXII. Of interest here is the presence of two candidate genes shown to be differentially expressed in a FACE experiment (Taylor *et al.* 2005). Response QTL for both young and mature leaf traits collocate with Polcalcin, putative-calcium-binding pollen allergen, PU05763, which was up-regulated in young leaves, and 60S ribosomal protein related, PU06463, which was down-regulated in semi-mature leaves. This LG is relatively small at 24.8 cM, but would be worth considering in more detail in future research.

The annotated full sequence of *P. trichocarpa* (Brunner *et al.* 2004; <http://genome.jgi-psf.org/Poptr1/Poptr1.home.html>), will be central to further study and should prove invaluable in elucidating the nature of the genetic control of these and other traits, combined with genomic studies. The use of microarrays has allowed the first glimpse of gene expression changes following long-term acclimation to e[CO₂] (Taylor *et al.* 2005), allowing a combined QTL-microarray approach (genetical genomics), to identify patterns of gene expression that co-occur with QTL, providing candidate genes for further mapping and positional cloning (Kirst *et al.* 2004). Elevated CO₂ revealed a number of new QTL and these traits are of prime importance for future analysis and may provide the initial links between altered development and evolution in future CO₂ environments (Kohut 2003).

In summary, we have identified a large number of QTL for leaf developmental and growth traits in a[CO₂] and e[CO₂]. This natural genetic variation provides the first clues to long-term adaptation that may occur in response to future rising atmospheric CO₂. Particular traits of interest include leaf size and shape, SLA, leaf senescence and

stomatal initiation. The availability of the full poplar sequence and associated genomic resources, in particular microarrays, is enabling research to be focused on understanding these developmental and potentially evolutionary responses to e[CO₂], at the level of the gene. We already have suggested candidate genes and future research should further explore the role of these genes in determining growth response to rising CO₂.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online:

Figure S1. Distribution of the phenotypes for young leaf traits (68 DAPs).

Figure S2. Distribution of the phenotypes for (a) number of leaves on leading stem, (b) senescence index and mature leaf traits, (c) specific leaf area, (d) leaf area (September), (e) number of cells per leaf, (f) leaf cell area, (g) stomatal density on adaxial surface, (h) stomatal index on adaxial surface, (i) stomatal density of abaxial surface, (j) stomatal index on abaxial surface, for leaves measured of the family 331 pedigree of poplar grown in open top chambers in either ambient CO₂ (open bar) or elevated CO₂ (closed bar).

Table S1. QTL mapped for plants grown in ambient CO₂.

Table S2. QTL mapped for plants grown in elevated CO₂.

Table S3. Response QTL calculated as (e[CO₂] – a[CO₂])/a[CO₂].

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