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## RESEARCH PAPER

# The diversity of $^{13}\text{C}$ isotope discrimination in a *Quercus robur* full-sib family is associated with differences in intrinsic water use efficiency, transpiration efficiency, and stomatal conductance

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## Abstract

$^{13}\text{C}$  discrimination in organic matter with respect to atmospheric  $\text{CO}_2$  ( $\Delta^{13}\text{C}$ ) is under tight genetic control in many plant species, including the pedunculate oak (*Quercus robur* L.) full-sib progeny used in this study.  $\Delta^{13}\text{C}$  is expected to reflect intrinsic water use efficiency, but this assumption requires confirmation due to potential interferences with mesophyll conductance to  $\text{CO}_2$ , or post-photosynthetic discrimination. In order to dissect the observed  $\Delta^{13}\text{C}$  variability in this progeny, six genotypes that have previously been found to display extreme phenotypic values of  $\Delta^{13}\text{C}$  [either very high ('high  $\Delta$ ') or low ('low  $\Delta$ ') phenotype] were selected, and transpiration efficiency (TE; accumulated biomass/transpired water), net  $\text{CO}_2$  assimilation rate ( $A$ ), stomatal conductance for water vapour ( $g_s$ ), and intrinsic water use efficiency ( $W_i=A/g_s$ ) were compared with  $\Delta^{13}\text{C}$  in bulk leaf matter, wood, and cellulose in wood. As expected, 'high  $\Delta$ ' displayed higher values of  $\Delta^{13}\text{C}$  not only in bulk leaf matter, but also in wood and cellulose. This confirmed the stability of the genotypic differences in  $\Delta^{13}\text{C}$  recorded earlier. 'High  $\Delta$ ' also displayed lower TE, lower  $W_i$ , and higher  $g_s$ . A small difference was detected in photosynthetic capacity but none in mesophyll conductance to  $\text{CO}_2$ . 'High  $\Delta$ ' and 'low  $\Delta$ ' displayed very similar leaf anatomy, except for higher stomatal density in 'high  $\Delta$ '. Finally, diurnal courses of leaf gas exchange revealed a higher  $g_s$  in 'high  $\Delta$ ' in the morning than in the afternoon when the difference decreased. The gene *ERECTA*, involved in the control of water use efficiency, leaf differentiation, and stomatal density, displayed higher expression levels in 'low  $\Delta$ '. In this progeny, the variability of  $\Delta^{13}\text{C}$  correlated closely with that of  $W_i$  and TE. Genetic differences of  $\Delta^{13}\text{C}$  and  $W_i$  can be ascribed to differences in stomatal conductance and stomatal density but not in photosynthetic capacity.

**Key words:** Carbon isotope discrimination, pedunculate oak, stomatal conductance, stomatal density, transpiration efficiency, water use efficiency.

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Abbreviations:  $A$ , net  $\text{CO}_2$  assimilation rate;  $A_{\text{max}}$ , maximum  $A$  measured during daily course of gas exchange ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ );  $A_{\text{mean}}$ , average  $A$  calculated from daily course of gas exchange ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ );  $A_{\text{sat}}$ , light-saturated  $A$  at ambient  $\text{CO}_2$  concentration ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ );  $C_a$ , atmospheric  $\text{CO}_2$  mole fraction ( $\mu\text{mol mol}^{-1}$ ); Chl, chlorophyll content ( $\text{g m}^{-2}$ );  $C_i$ ,  $\text{CO}_2$  mole fraction in intercellular air spaces ( $\mu\text{mol mol}^{-1}$ ); DM, dry biomass;  $g_i$ , mesophyll conductance for  $\text{CO}_2$  ( $\text{mol m}^{-2} \text{s}^{-1}$ );  $g_s$ , stomatal conductance for water vapour;  $g_{\text{max}}$ , maximum  $g_s$  measured during daily course of gas exchange ( $\text{mol m}^{-2} \text{s}^{-1}$ );  $g_{\text{mean}}$ , average  $g_s$  calculated from daily course of gas exchange ( $\text{mol m}^{-2} \text{s}^{-1}$ );  $g_{\text{sat}}$ ,  $g_s$  at ambient  $\text{CO}_2$  concentration and light saturation ( $\text{mol m}^{-2} \text{s}^{-1}$ );  $J_{\text{max}}$ , maximal electron transport rate ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ); LET, lower epidermis thickness ( $\mu\text{m}$ ); LMA, leaf mass-to-area ratio ( $\text{g m}^{-2}$ ); LA, leaf area estimated from dry biomass ( $\text{m}^2$ );  $N_{\text{area}}$ , nitrogen content on an area basis ( $\text{g m}^{-2}$ );  $N_{\text{mass}}$ , nitrogen content on a mass basis ( $\text{mg g}^{-1}$ ); PMT, palisade mesophyll thickness ( $\mu\text{m}$ ); PT, plant transpiration ( $\text{kg H}_2\text{O m}^{-2}$  leaf area); qPCR, quantitative polymerase chain reaction; QTL, quantitative trait loci; SD, stomatal density ( $\text{mm}^{-2}$ ); SMT, spongy mesophyll thickness ( $\mu\text{m}$ ); TE, transpiration efficiency ( $\text{mg DM g}^{-1} \text{H}_2\text{O}$ ); TLT, total leaf thickness ( $\mu\text{m}$ ); UET, upper epidermis thickness ( $\mu\text{m}$ );  $V_{\text{cmax}}$ , maximum carboxylation rate of Rubisco ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ );  $W_i$ , intrinsic water use efficiency;  $W_{\text{sat}}$ ,  $W_i$  at ambient  $\text{CO}_2$  concentration and light saturation ( $\mu\text{mol mol}^{-1}$ );  $W_{\text{mean}}$ , average  $W_i$  calculated from daily course of gas exchange ( $\mu\text{mol mol}^{-1}$ );  $\alpha$ , slope of the response of  $g_s$  to light determined with daily course of gas exchange ( $10^4 \text{ mol m}^{-2} \text{s}^{-1}/\text{unit of global irradiance}$ );  $\Delta\text{BA}$ , basal area increment during the experiment ( $\text{mm}^2$ );  $\Delta\text{BM}$ , biomass accumulation (g);  $\Delta^{13}\text{C}$ ,  $^{13}\text{C}$  isotope discrimination;  $\Delta^{13}\text{C}_b$ ,  $\Delta^{13}\text{C}$  of bulk leaf material ( $\text{‰}$ );  $\Delta^{13}\text{C}_c$ ,  $\Delta^{13}\text{C}$  of cellulose in the wood ( $\text{‰}$ );  $\Delta^{13}\text{C}_w$ ,  $\Delta^{13}\text{C}$  of wood ( $\text{‰}$ );  $\delta^{13}\text{C}_{\text{air}}$ ,  $\delta^{13}\text{C}$  of atmospheric  $\text{CO}_2$  in the greenhouse ( $\text{‰}$ ).

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## Introduction

Water use efficiency is a composite and complex trait that currently receives much attention from agronomists, ecophysiologicalists, and geneticists [see the review by Condon *et al.* (2004) among others]. Water use efficiency is usually defined as the amount of biomass (or harvestable organs) produced for a given amount of transpired water, i.e. as transpiration efficiency (TE, g DM g<sup>-1</sup> H<sub>2</sub>O). At an instantaneous time scale, it is defined as the ratio between net CO<sub>2</sub> assimilation ( $A$ ) and (i) leaf transpiration, i.e. instantaneous water use efficiency or (ii) stomatal conductance ( $g_s$ ), i.e. intrinsic water use efficiency ( $W_i$ ,  $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ ). Based on the modelled relationship between carbon isotope discrimination during photosynthesis ( $\Delta^{13}\text{C}$ ) and  $C_i/C_a$  (the ratio of CO<sub>2</sub> mole fractions in sub-stomatal air spaces and in the atmosphere),  $W_i$  can be estimated using  $\Delta^{13}\text{C}$  as an indicator (Farquhar and Richards, 1984). Depending on plant compartments and compounds (e.g. soluble leaf sugars, bulk leaf material, wood-extracted cellulose),  $\Delta^{13}\text{C}$  represents a time-integrated estimate of  $C_i/C_a$ , and of  $W_i$  weighted by net CO<sub>2</sub> assimilation rate over different temporal scales. At the two extremes of the time-scale,  $\Delta^{13}\text{CO}_2$  of air between inlet and outlet of a leaf gas exchange chamber would yield an instantaneous estimation of  $W_i$ , whereas  $\Delta^{13}\text{C}$  of wood represents a long-term integration of  $W_i$  over a whole growing season with possible contributions from storage compounds assimilated even earlier.

$\Delta^{13}\text{C}$  has been widely used as an index for changes in the micro-environment, as it proved very plastic in response to environmental changes such as water availability, vapour pressure deficit or temperature. The most striking application of this plasticity is the use of tree ring  $\Delta^{13}\text{C}$  over years and decades to give evidence of year-to-year variability, as well as long term drifts in climate (Bert *et al.*, 1997; Duquesnay *et al.*, 1998; Saurer *et al.*, 2004). In addition to the large environmental influence on  $\Delta^{13}\text{C}$ , intra-specific genetic variability of  $\Delta^{13}\text{C}$  was detected in crops (Farquhar and Richards, 1984; Condon *et al.*, 2004; Rebetzke *et al.*, 2006) and in trees (Guehl *et al.*, 1996; Lauteri *et al.*, 1997; Roupsard *et al.*, 1998). This genetic variability of  $\Delta^{13}\text{C}$  was used as an index for genetic variability of  $W_i$  and hence of TE. This may not be as straightforward as expected due to interference of processes leading to  $^{13}\text{C}$  discrimination independently of  $C_i/C_a$  (e.g. changes in mesophyll conductance or post-photosynthetic discrimination; Evans *et al.*, 1986; Warren and Adams, 2006; Seibt *et al.*, 2008). The simple model relating  $\Delta^{13}\text{C}$  and  $W_i$  (Farquhar and Richards, 1984) makes the assumption that mesophyll conductance to CO<sub>2</sub> is infinite, while it has been proposed that it is of similar order of magnitude as stomatal conductance (see reviews by Evans and von Caemmerer, 1996; Flexas *et al.*, 2008; Warren, 2008).

Therefore, when  $\Delta^{13}\text{C}$  is used as an indicator of  $W_i$  in studies involving a high number of individuals (Brendel *et al.*, 2002; Casasoli *et al.*, 2004; Brendel *et al.*, 2008) there is a need to check for the tightness of the relationship between the two traits. Measuring  $W_i$  and TE is much more

challenging and time-consuming than recording  $\Delta^{13}\text{C}$  in dry matter.  $W_i$  estimated from leaf gas exchange is subject to a large temporal variability and to measurement uncertainties (lack of precision of the gas exchange techniques; Flexas *et al.*, 2007), while TE requires a careful record of transpiration with weighing or sap flow measurement techniques and estimation of biomass accumulation using allometric relationships and destructive harvesting.

There is only a rather small number of reports confirming that intra-specific genetic differences in  $\Delta^{13}\text{C}$  were matched by differences in TE in trees (Zhang and Marshall, 1994; Guehl *et al.*, 1996; Roupsard *et al.*, 1998; Cernusak *et al.*, 2007), whereas comparisons with gas-exchange-based estimates of  $W_i$  have been published for a number of species [e.g. Cregg *et al.* (2000) for *Pinus ponderosa* provenances; Livingston *et al.* (1999) for *Picea glauca* crosses]. The published results usually displayed some degree of correlation, with nevertheless significant deviation from the expected model (Ponton *et al.*, 2002).

Variation in  $W_i$  ( $=A/g_s$ ), can be controlled by either of the two traits (Farquhar *et al.*, 1989). Genetic variation of  $W_i$  has been studied for different tree species using gas exchange techniques, but rarely relates clearly to one of the two traits (Zhang *et al.*, 1993; Lauteri *et al.*, 1997; Olivas-Garcia *et al.*, 2000). Very few investigators have attempted a more detailed study of genetic variability of  $W_i$  of tree species and related leaf level traits (Lauteri *et al.*, 1997; Olivas-Garcia *et al.*, 2000; Grossnickle *et al.*, 2005). Further, few gene products have yet been found that can be related directly to variation in  $W_i$ . Masle *et al.* (2005) have shown that the expression of the gene *ERECTA* influences water use efficiency in *Arabidopsis thaliana*. This gene affects gas exchange coordination through its impact on leaf anatomy, such as mesophyll cell proliferation and stomatal density. It is therefore a likely candidate gene to take into account in case of studying the natural diversity of  $W_i$ .

Oak, and in particular pedunculate oak (*Quercus robur* L.), is a perfect candidate for addressing such questions. A large inter- and intra-specific variability of  $\Delta^{13}\text{C}$  was detected in oaks. The two species, *Q. robur* L. and *Q. petraea* Matt. Liebl., have been demonstrated to consistently differ by 1‰ when growing in mixed populations as well as in common garden plantations (Ponton *et al.*, 2001, 2002). Ponton *et al.* (2002) found that the correlation between  $\Delta^{13}\text{C}$  and  $W_i$  was identical in the two species, providing an argument in support of the hypothesis that the inter-specific difference of  $\Delta^{13}\text{C}$  actually reflected differences in  $W_i$ . A large within-species variability was also detected in a common garden plantation of *Q. robur*; extreme values differed by as much as 4‰. In order to clarify the genetic determinism and identify potential genomic regions involved in the control of this variability, Brendel *et al.* (2008) used a full-sib family of pedunculate oak comprising 278 siblings. A small number of highly significant quantitative trait loci (QTL) was detected for  $\Delta^{13}\text{C}$ , i.e. there were a few well-defined regions in the genome that controlled

$\Delta^{13}\text{C}$ . Among these regions, a major QTL was detected using data from three successive years, explaining repeatedly over 20% of the variability of  $\Delta^{13}\text{C}$  in this family. However, none of the QTL detected for  $A$ ,  $g_s$ , or  $W_i$  was in close vicinity to this major QTL for  $\Delta^{13}\text{C}$ . Therefore, little information was provided on the physiological causes of the  $\Delta^{13}\text{C}$  variability. Given the numerous factors other than  $W_i$  that might contribute to the variability of  $\Delta^{13}\text{C}$  (see above), it was of high importance to document whether the observed variability within this family was correlated with differences in intrinsic water use efficiency ( $W_i$ ). Moreover the variability of TE remains unknown in this family.

The present study aimed at investigating the physiological and anatomical causes of the  $\Delta^{13}\text{C}$  variability. The approach used was to concentrate on a small number of genotypes (siblings) displaying large differences in  $\Delta^{13}\text{C}$  recorded from bulk leaf organic matter by Brendel *et al.* (2008), in order to multiply the number of complementary measurements. In the present study, three genotypes that displayed very high values of  $\Delta^{13}\text{C}$  during the QTL study in the field were selected as a 'high  $\Delta$ ' phenotype, and the same procedure was used for a 'low  $\Delta$ ' phenotype. The two 'phenotypes' were grown together in a greenhouse and compared in order to test the following working hypotheses:

- (i) the 'high  $\Delta$ ' phenotype also displays larger values of  $\Delta^{13}\text{C}$  in wood and cellulose extracted from the wood;
- (ii) the 'high  $\Delta$ ' phenotype displays a lower intrinsic water use efficiency ( $W_i$ ) and a similar mesophyll conductance to  $\text{CO}_2$  compared with the 'low  $\Delta$ ' phenotype;
- (iii) it also displays a lower whole plant TE;
- (iv) lower values of  $W_i$  correlate with higher stomatal conductance and density rather than with lower photosynthetic capacity;
- (v) the differences in  $W_i$  are paralleled by differences in the expression of the *ERECTA* gene.

## Materials and methods

### Plant material and growth conditions

Clonal copies of several genotypes were sampled within the full-sib progeny of pedunculate oak (*Quercus robur* L.) created at INRA Bordeaux (Scotti-Saintagne *et al.*, 2004; Brendel *et al.*, 2008). The procedure described by Roussel *et al.* (2009) was used. Briefly, genotypes were selected on the basis of  $\Delta^{13}\text{C}$  measurements made during three consecutive years (2000–2002) in the nursery at Bourran (south-west France). Three genotypes displaying the highest values of  $\Delta^{13}\text{C}$  (N101, 171, 287) were selected and propagated via rooted cuttings, as well as three genotypes with the lowest values of  $\Delta^{13}\text{C}$  (N118, 136, 170). Four vegetative copies were used per genotype. This procedure allowed two phenotypes called 'high  $\Delta$ ' and 'low  $\Delta$ ', respectively, each comprising four copies of three different genotypes, to be sampled.

The complete experimental design consisted of 24 individuals overall that were placed in a greenhouse with a randomized complete-block design. One vegetative copy of each genotype was placed in each of the four blocks.

The vegetative copies of the selected genotypes were produced during 2002 by UMR BioGeCo, INRA Pierroton (south-west France) and then transferred to Champenoux (north-east France) during January 2005, where they were transplanted to 10 l containers with a peat and sand mixture (1/1, v/v). A complete fertilization (4.5 g l<sup>-1</sup> of slow-release fertilizer Nutricote T100; N, P, K, Mg; 13, 13, 13, 2 +trace elements and 0.2 g l<sup>-1</sup> of lime) was provided. All individuals were watered daily to field capacity with deionized water. During the whole experimental period, 97% of all watering in the evening was at a relative extractable soil water content above 60%. Variation in air temperature was limited to the range 15–25 °C during the experiment. Relative humidity (66±8%, average ±standard deviation),  $\text{CO}_2$  concentration (401±23  $\mu\text{mol mol}^{-1}$ ), and daily global irradiance were continuously recorded in the greenhouse. The plants were grown under a natural photoperiod. The experiments were run from the end of February 2005 to the middle of October 2005.

### Carbon isotope composition: total organic matter, wood, and cellulose

Carbon isotope analyses were conducted on three different carbon pools: bulk leaf organic matter, wood, and cellulose extracted from wood. Sun-exposed leaves were harvested from the second and third growth-flush after measuring diurnal cycles of leaf gas exchange (see below). The harvested leaves were stored at -80 °C before being oven-dried for 72 h at 50 °C and ball-milled prior to mass spectrometer analysis.

A segment of the main stem was harvested at the level of the second growth-flush. The bark was removed and wood was oven-dried for 1 week at 50 °C and ball-milled. Fifty milligrams of this powder was used for cellulose extraction according to the method of Leavitt and Danzer (1993), modified by Loader *et al.* (1997).

Carbon isotope composition of 1 mg bulk leaf material, wood, and cellulose was measured using a continuous flow isotope ratio mass spectrometer (Delta S; Thermo Finnigan, Bremen, Germany). The relative abundance of  $^{13}\text{C}$  (‰) was expressed as:

$$\delta^{13}\text{C} = (R_s - R_b) / R_b \cdot 1000$$

where  $R_s$  and  $R_b$  refer to the  $^{13}\text{C}/^{12}\text{C}$  ratio in the sample and in the Vienna Pee Dee Belemnite standard, respectively.

Carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) was calculated as:

$$\Delta^{13}\text{C} \approx (\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{sample}}) / (1 + \delta^{13}\text{C}_{\text{sample}}/1000)$$

where  $\delta^{13}\text{C}_{\text{air}}$  is the carbon isotope composition of atmospheric  $\text{CO}_2$ .  $\delta^{13}\text{C}_{\text{air}}$  was assessed in the greenhouse by growing *Zea mays* plants and recording the  $\delta^{13}\text{C}$  of leaf

organic matter according to Marino and McElroy (1991).  $\delta^{13}\text{C}_{\text{air}}$  was found to be close to  $-10.05\text{‰}$ .

#### *Plant growth traits and determination of TE*

During the experimental period, the diameter at root collar was recorded weekly on each individual. The difference between initial and final basal area ( $\Delta\text{BA}$ ,  $\text{mm}^2$ ) was calculated as the difference between final and initial stem cross-section, using the diameter data. At the end of the experiment, the height (i.e. length of the longest stem) of each individual was recorded. Then plants were harvested, divided into stems, leaves, and roots and oven-dried ( $50\text{ °C}$ ) for 1–2 weeks before being weighed. Final biomass was calculated as the sum of shoot and root biomass. From leaf dry biomass, leaf area (LA,  $\text{m}^2$ ) was estimated using a relationship calibrated on 10 supplementary pedunculate oaks:  $\text{LA} (\text{m}^2) = 0.0131 + 0.0143 \times \text{leaf dry weight (g)}$  ( $R^2 = 0.97$ ;  $P < 0.001$ ).

Biomass accumulation ( $\Delta\text{BM}$ ) was calculated for each of the 24 individuals as the difference between the final and initial biomass. Initial biomass was estimated from allometric relationships using data recorded on additional vegetative copies from the same family harvested during April. These plants were cut into individual branch segments. Diameter and length were recorded to compute the cylinder volume of each segment. Then segments were oven dried for 3 d ( $50\text{ °C}$ ) and weighed. The resulting relationship between volume and dry mass [ $\text{dry mass (g)} = 0.013 + 7.10^{-4} \times \text{volume (mm}^3)$ ;  $R^2 = 0.99$ ;  $P < 0.001$ ] allowed the initial above-ground biomass to be estimated. The initial root biomass was estimated using the estimated initial shoot biomass and the final root/shoot biomass ratio, assuming it remained stable during the course of the experiment. Initial root biomass represented only about 4% of the final root biomass and errors in its estimation could have only minor consequences for the estimate of accumulated biomass.

Cumulated water use was estimated from daily weighing two potted individuals of each genotype (12 individuals overall) from April 2005 to October 2005. Pots were covered with plastic to limit direct evaporation from the soil. Leaf-specific plant transpiration (PT,  $\text{kg H}_2\text{O m}^{-2}$ ) was estimated as the ratio between cumulated water loss and leaf area. Transpiration efficiency (TE,  $\text{g DM g}^{-1} \text{H}_2\text{O}$ ) was computed as the dry biomass accumulated during the experiment relative to cumulated water loss.

#### *Leaf traits*

Measurements were made on fully expanded leaves of the second and the third growth-flushes. The second growth-flush started at the end of April and the third growth-flush 1 month later.

#### *Photosynthesis and stomatal conductance*

Leaf gas exchange was recorded under saturating light on four sun-exposed leaves per individual (two leaves for each growth-flush) during August and September 2005, with an

open-flow gas exchange system (Li-6400; Li-Cor, Lincoln, NE, USA). Leaves were inserted into the chamber and photosynthesis was induced for about 40 min at  $25\text{ °C}$ , at a  $\text{CO}_2$  mole fraction of  $370\text{ }\mu\text{mol mol}^{-1}$ , a photosynthetic photon flux density of  $1200\text{ }\mu\text{mol m}^{-2} \text{s}^{-1}$ , and an air flux of  $300\text{ }\mu\text{mol s}^{-1}$ . Light-saturated net  $\text{CO}_2$  assimilation rate ( $A_{\text{sat}}$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) and stomatal conductance for water vapour ( $g_{\text{sat}}$ ,  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) were recorded and used to compute intrinsic water use efficiency under saturating irradiance ( $W_{\text{sat}}$ ,  $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ ). Afterwards, for one leaf per growth-flush, a response curve of  $A$  to  $\text{CO}_2$  mole fraction in intercellular air spaces ( $C_i$ ,  $\mu\text{mol mol}^{-1}$ ) was recorded using the following procedure:  $\text{CO}_2$  mole fraction was gradually decreased from  $370\text{ }\mu\text{mol mol}^{-1}$  to  $50\text{ }\mu\text{mol mol}^{-1}$  in five steps, and afterwards increased from  $370\text{ }\mu\text{mol mol}^{-1}$  to  $2000\text{ }\mu\text{mol mol}^{-1}$  in five steps. During each step, 4 min were allowed for gas exchange stabilization. Environmental parameters, as well as  $A$ ,  $g_s$ , and  $C_i$  were recorded three times during each step. These  $A-C_i$  curves were used to adjust the photosynthesis model of Farquhar *et al.* (2001), producing estimates of maximal RuBP carboxylation rate ( $V_{\text{cmax}}$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) and maximal electron transport rate ( $J_{\text{max}}$ ,  $\mu\text{mol e}^- \text{ m}^{-2} \text{ s}^{-1}$ ) as described by Dreyer *et al.* (2001). The adjusted model did not explicitly include mesophyll conductance to  $\text{CO}_2$  ( $g_i$ ,  $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), the computed values represent therefore apparent values of  $V_{\text{cmax}}$  and  $J_{\text{max}}$  under the hypothesis of infinite  $g_i$ . With the same  $A-C_i$  curves, the procedure developed by Ethier and Livingston (2004) was used to compute  $g_i$  and to derive unbiased estimates of  $V_{\text{cmax}}$  and  $J_{\text{max}}$ . Unfortunately, the fitting procedure with the three parameters  $g_i$ ,  $V_{\text{cmax}}$ , and  $J_{\text{max}}$  did not always produce reliable data and therefore unbiased estimates were computed for 27 out of the 36 curves.

#### *Daily courses of net $\text{CO}_2$ assimilation rate, stomatal conductance, and intrinsic water use efficiency*

Eight daily time courses of leaf gas exchange ( $A$ ,  $g_s$ ) were recorded during September and October from 08:00 to 19:00 with a portable photosynthesis chamber (LiCor 6200). During each time course, gas exchange of sun-exposed leaves was measured on the six individuals of a block, either from the second or the third growth-flush, resulting in 14–24 points per day, leaf and growth-flush.

During each time course (1 d of measurement), one growth-flush of the six individuals of one block was analysed, yielding therefore 8 d of measurement (two growth-flushes  $\times$  four blocks). To test the repeatability of the data, one time course was repeated on two consecutive days. No significant day effect was detected using a pairwise  $t$ -test for  $A$  and  $g_s$  data. Means were therefore used for the block and growth-flush measured during these two time courses.

Length and width of the leaves were measured and their product was used to estimate leaf area using a relationship previously obtained on 30 leaves representing a large range of areas:

$$\text{leaf area} = 0.39 + 0.55 \times \text{length} \times \text{width}$$

$$(R^2 = 0.98; P < 0.001)$$

Mean values of  $A$ ,  $g_s$ , and resulting  $W_i$  ( $A_{\text{mean}}$ ,  $g_{\text{mean}}$ , and  $W_{\text{mean}}$ , respectively) as well as the maximum stomatal conductance ( $g_{\text{max}}$ ) and maximal net assimilation rate ( $A_{\text{max}}$ ) were recorded on each growth-flush of each plant.

The response of stomata to the increase of light in the morning was estimated using daily time-course data: minimum  $g_s$  ( $g_{s1}$  before 10:00) and maximum  $g_s$  ( $g_{s2}$  before 15:00) as well as corresponding values of global irradiance (respectively,  $I_1$  and  $I_2$ ) were used to calculate a slope [ $\alpha = (g_{s2} - g_{s1}) / (I_2 - I_1)$ ]. This estimator (thereafter called ' $\alpha$ ') was expressed in  $\text{mol H}_2\text{O W}^{-1} \text{s}^{-1}$ .

#### Chlorophyll and nitrogen content and leaf mass-to-area ratio

Chlorophyll content per unit leaf area was estimated using a CCM-200 chlorophyll content meter (Opti-Sciences, Hudson, NH, USA). Total chlorophyll content per leaf area (Chl,  $\text{g m}^{-2}$ ) was derived from CCM readings (CCI) using the following relationship calibrated for oak using biochemical chlorophyll extraction according to Barnes *et al.* (1992):

$$\text{Chl} (\text{g m}^{-2}) = 0.021 (\text{CCI unit}) + 0.05$$

Chlorophyll content was recorded weekly on 10 leaves per individual with three replicates on each leaf for the second growth-flush between April and July. A least-square mean, generated from an analysis of variance model taking into account the date effect, was computed for each growth-flush and individual.

Leaf mass-to-area ratio (LMA,  $\text{g m}^{-2}$ ) was obtained from 3 to 10 leaves per growth-flush and individual. The area of fresh leaves was measured with a leaf area meter (Delta-T Devices Ltd, Cambridge, UK). LMA was estimated after drying the leaves at  $50^\circ\text{C}$  for 72 h. Leaf nitrogen content on a mass basis ( $N_{\text{mass}}$ ,  $\text{mg N g}^{-1}$ ) was measured with an elemental analyser (Carlo Erba NA 1500 NC, Rodano, Italy) on the leaves used for  $\Delta^{13}\text{C}$  measurements, and leaf nitrogen content on an area basis ( $N_{\text{area}}$ ,  $\text{g N m}^{-2}$ ) was calculated from  $N_{\text{mass}}$  and LMA.

#### Leaf anatomy and stomatal density

Overall, 14 discs ( $1 \text{ cm}^2$ ) were punched from three leaves per individual for measuring stomatal density and leaf anatomy and were immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Microphotographs were made with a scanning electron microscope (LEO 1450 VP, Cambridge, UK; 20–30 Pa inside chamber, accelerating voltage 15 KV, working distance 12 mm). One microphotograph of the abaxial epidermis ( $1.7 \text{ mm}^2$ ) of nine discs from three different leaves was used to estimate stomatal density (SD) using Scion software (Scion Corporation, Frederick, MA, USA).

On the five other discs ( $1 \text{ cm}^2$ ), four semi-thin cryosections were photographed per disc to record anatomical features using the Visilog 6.3 software (Noesis, France): total thickness (TLT,  $\mu\text{m}$ ), and thickness of palisadic and spongy mesophylls (PMT and SMT, respectively), upper and lower epidermis (UET and LET, respectively). Leaf density ( $\text{Dens}$ ,  $\text{g cm}^{-3}$ ) was computed as the ratio between LMA and leaf thickness.

#### ERECTA expression

The oak orthologous sequence for the *ERECTA* gene was identified using *Arabidopsis thaliana* protein sequence data and tBlastn analysis against our oak EST database (for more details, see Supplementary data available at JXB online). Alignment of protein sequences between *Arabidopsis thaliana* and *Quercus robur* showed that the sequence used in this study is highly similar to *ERECTA*, whereas the similarity with *ERECTAlike1* and *ERECTAlike2* genes is less.

Five leaves were harvested from the top of each individual during a sunny day in October 2005 between 08:00 and 10:30. Leaves were immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  before analysis. Total RNA of each individual from each block (i.e. the five leaves of each of the three genotypes) was extracted in triplicate following the method described by Le Provost *et al.* (2007). For each block, genotypes belonging to the same extraction and phenotype (low  $\Delta$  and high  $\Delta$ ) were bulked together in order to obtain three technical replications per block. Bulks were obtained by mixing equal amounts ( $1 \mu\text{g}$ ) of total RNA. RNA quality and quantity were estimated by spectrophotometry and visual inspection on 2% agarose gel. To eliminate the residual genomic DNA present in the samples, RNA was treated with RNase-free DNase RQ1 (Promega<sup>®</sup>, Madison, WI, USA). One microgram of total RNA was then reverse transcribed using the Improm-IITM reverse transcription system (Promega<sup>®</sup>) according to the manufacturer's instructions. After reverse transcription, the cDNA solution was diluted 10-fold before quantitative polymerase chain reaction (qPCR) analysis. qPCR reaction and quantification were performed on a Chromo4<sup>™</sup> multi-colour real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following the procedure described by Paiva *et al.* (2008). PCR primer pairs were designed using the primer2 software (Rozen and Skaletsky, 2000) for the *ERECTA* gene and a control gene corresponding to an oak actin protein. Primers were designed to have an optimal size of 20 bp (18–22 bp), a GC content of 40–60%, and a  $T_m$  of  $60^\circ\text{C}$ . Other criteria such as primer self-annealing were also taken into account. Oligonucleotides were synthesized by Eurogentec (Liege, Belgium). Primer pairs are listed below:

*ERECTA* forward: 5'-TGCAAGCCAGTGCTATCAAG-3'

*ERECTA* reverse: 5'-CCATGAAGCAGATCCCAAAG-3'

qPCR and data analysis were performed following the procedure described by Paiva *et al.* (2008). Briefly, data were analysed using the Excel (Microsoft) macro GENEX

v1.10 (gene expression analysis for iCycle iQ<sup>®</sup> real-time PCR detection system, v1.10, 2004; Bio-Rad Laboratories), using the methods derived from the algorithms of Vandesompele *et al.* (2002).

Differences in *ERECTA* expression between 'low  $\Delta$ ' and 'high  $\Delta$ ' were analysed simultaneously for blocks 1, 2, and 4, whereas a different qPCR was performed for the third block. These data are not comparable and thus significant differences were tested using the *t*-test for each block separately. A one-sided *t*-test was used as *a priori* there was the hypothesis that the 'high  $\Delta$ ' phenotype should have the lower *ERECTA* expression level.

### Statistical analyses

Response variables were analysed with a linear mixed model to deal with the covariance structure of the data. For analysing whole plant responses, only 'phenotype' was treated as fixed whereas 'block' and 'genotype' were treated as crossed random effects. For leaf responses, 'growth-flush number' was added as fixed effect as well as its interaction

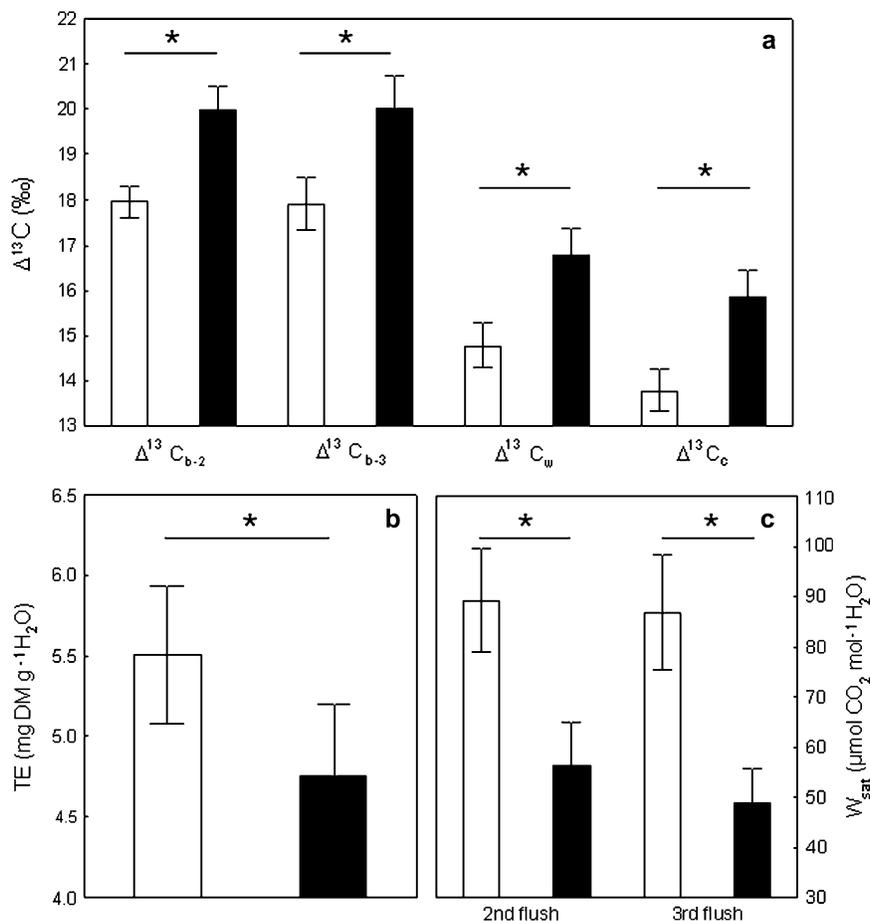
with 'phenotype' whereas 'block' effect was treated as random crossed with both random effects 'genotype' and 'individual' nested in 'genotype'. In the latter case when the interaction was found to be non-significant, the analyses were rerun after removing the interaction in the fixed part of the model.

All models were fitted with R 2.7.2 (R Development Core Team, 2008) software, and namely the 'lmer' function from the library 'lme4' (Bates *et al.*, 2008). For testing fixed effects, an MCMC sample was drawn from the output of the fitted model, and an effect was declared significant when its 95% highest posterior density interval did not contain zero.

## Results

### $\Delta^{13}\text{C}$ in different compartments

Carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) recorded in the three carbon pools: bulk leaf organic matter, wood, and cellulose extracted from wood ( $\Delta^{13}\text{C}_b$ ,  $\Delta^{13}\text{C}_w$ , and  $\Delta^{13}\text{C}_c$ , respectively) differed significantly between the two phenotypes,



**Fig. 1.** Means ( $\pm 95\%$  confidence intervals, based on *N*) in the two phenotypes 'low  $\Delta$ ' (open columns) and 'high  $\Delta$ ' (closed columns) of: (a) carbon isotope discrimination ( $N=12$ ) recorded in bulk matter of leaves from the second ( $\Delta^{13}\text{C}_{b,2}$ ) and the third ( $\Delta^{13}\text{C}_{b,3}$ ) growth-flush, in wood ( $\Delta^{13}\text{C}_w$ ), and in the cellulose extracted from the wood ( $\Delta^{13}\text{C}_c$ ); (b) transpiration efficiency (TE;  $N=6$ ); (c) intrinsic water use efficiency ( $N=9$ ) calculated as net  $\text{CO}_2$  assimilation over stomatal conductance from light-saturated leaf gas exchange ( $W_{\text{sat}}$ ) for leaves from the second and the third growth-flush. Asterisks indicate significant differences between phenotypes ( $P < 0.05$ ). No difference was recorded for  $\Delta^{13}\text{C}_b$  and  $W_{\text{sat}}$  between the two growth-flushes.

$\Delta^{13}\text{C}$  being higher in the ‘high  $\Delta$ ’ phenotype independently of the carbon pool measured (Fig. 1). The difference was as high as 2‰ in all compartments, which would translate, according to the model (Farquhar and Richards, 1984), into a 30% difference of intrinsic water use efficiency ( $W_i$ ). Leaves from the two growth-flushes displayed very close values of  $\Delta^{13}\text{C}$  (no growth-flush effect was observed for  $\Delta^{13}\text{C}_b$ ). Wood  $\Delta^{13}\text{C}$  values were lower by 3.5‰ and cellulose by almost 4.5‰, compared with leaf material.

### Transpiration efficiency (TE)

TE, recorded as the ratio of accumulated biomass on cumulated water use, was significantly higher in the ‘low  $\Delta$ ’ compared with the ‘high  $\Delta$ ’ phenotype (5.5 versus 4.8 mg g $^{-1}$ , i.e. a 16% difference; Fig. 1). Table 1 displays water use and biomass accumulation in the two phenotypes during the experimental period. Biomass production and growth were large and no block effect was detected (homogeneity over the whole experimental design). Biomass accumulation, basal area increase, height, and leaf area were not significantly different in the two phenotypes with a large intra-phenotype variability. The cumulated water use was stable among blocks ( $P=0.53$ ) and was much larger in ‘high  $\Delta$ ’ despite some intra-phenotype variability. When expressed relative to total leaf area, cumulated transpiration (PT) was still significantly higher in ‘high  $\Delta$ ’.

### Leaf gas exchange under saturating irradiance

Under saturating irradiance and ambient  $\text{CO}_2$ , large differences were recorded between the two phenotypes, ‘low  $\Delta$ ’ displaying consistently smaller values of  $A_{\text{sat}}$  and  $g_{\text{sat}}$  whether on leaves from the second or the third growth-flush (Table 2). This resulted nevertheless in the very clear difference in  $W_{\text{sat}}$  shown in Fig. 1.  $W_{\text{sat}}$  was 58% and 78% higher in the ‘low  $\Delta$ ’ phenotype for the second and the third growth-flush, respectively (Fig. 1). No growth-flush effect

**Table 1.** Means  $\pm$  standard deviations and P-level values at 95% level for the difference between ‘low  $\Delta$ ’ and ‘high  $\Delta$ ’ phenotypes (indicated by ‘\*\*’) for: biomass accumulation ( $\Delta\text{BM}$ , g), basal area increment during the experiment ( $\Delta\text{BA}$ , mm $^2$ ), length of the main stem (Height, m), total leaf area (LA, m $^2$ ), and cumulated transpiration on a leaf area basis (PT, kg m $^{-2}$ )

The number in parenthesis refers to the number of plants in each phenotype used for computing average values and testing ‘low  $\Delta$ ’ versus ‘high  $\Delta$ ’ effect.

	Mean $\pm$ standard deviation		‘Low $\Delta$ ’ versus ‘High $\Delta$ ’ effect
	‘Low $\Delta$ ’	‘High $\Delta$ ’	
$\Delta\text{BM}$	353 $\pm$ 124 (12)	360 $\pm$ 97 (12)	–
$\Delta\text{BA}$	11.5 $\pm$ 2.3 (12)	14.2 $\pm$ 2.2 (12)	–
Height	2.0 $\pm$ 0.6 (10)	1.7 $\pm$ 0.5 (10)	–
LA	0.77 $\pm$ 0.22 (12)	0.73 $\pm$ 0.18 (12)	–
PT	82.6 $\pm$ 9.5 (6)	109.7 $\pm$ 14.2 (6)	*

was observed for  $W_{\text{sat}}$ . The data for both growth-flushes fit the same  $A_{\text{sat}}$  ( $g_{\text{sat}}$ ) trendline in Fig. 2. This trendline displayed a severe curvature and saturation at the highest values of  $g_{\text{sat}}$ , yielding significantly smaller values of  $W_{\text{sat}}$  for the individuals with the ‘high  $\Delta$ ’ phenotype.

No differences were found between the phenotypes for maximal carboxylation velocity ( $V_{\text{cmax}}$ ), neither under the hypothesis of infinite mesophyll conductance to  $\text{CO}_2$  (apparent  $V_{\text{cmax}}$ ) nor for  $g_i$ -corrected values (Table 2). Light-driven electron flux ( $J_{\text{max}}$ ) was significantly higher for ‘high  $\Delta$ ’ for both apparent and corrected values. This is in agreement with the lack of a significant difference for mesophyll conductance for  $\text{CO}_2$  ( $g_i$ ) between the phenotypes.

### Diurnal courses of leaf gas exchange

The two phenotypes displayed significantly different diurnal courses of leaf gas exchange ( $A$ ,  $g_s$  and  $W_i$ ; Fig. 3). The differences were obvious in the morning when the ‘high  $\Delta$ ’ phenotype displayed much higher  $g_s$  than ‘low  $\Delta$ ’. In the afternoon,  $g_s$  decreased for ‘high  $\Delta$ ’, whereas it remained almost stable throughout the day for ‘low  $\Delta$ ’ (Fig. 3a). Net  $\text{CO}_2$  assimilation was slightly larger in ‘high  $\Delta$ ’ but the diurnal course of  $A$  was quite similar for the two phenotypes (Fig. 3b). As a result,  $W_i$  was lower in ‘high  $\Delta$ ’ throughout the day (Fig. 3c).

Mean and maximal values for  $A$ ,  $g_s$ , and  $W_i$  (Table 2) were calculated from the diurnal time courses. Maximum as well as mean  $g_s$  and  $A$  were significantly higher for ‘high  $\Delta$ ’ compared with ‘low  $\Delta$ ’. For  $A_{\text{mean}}$  and  $g_{\text{mean}}$  the difference was significantly higher for the third compared with the second growth-flush. The difference in  $A$  was smaller compared with  $g_s$  and thus a significantly lower  $W_{\text{mean}}$  was detected for ‘high  $\Delta$ ’ compared with ‘low  $\Delta$ ’. Relative differences between the two phenotypes, calculated from  $W_{\text{mean}}$ , were 37% and 49% for the 2nd and the 3rd growth-flushes, respectively. The response of  $g_s$  to the increase in irradiance in the morning ( $\alpha$ ) was significantly higher for ‘high  $\Delta$ ’ compared with ‘low  $\Delta$ ’.

### Leaf structure

A significant difference between phenotypes was detected for  $N_{\text{mass}}$ , Chl, and palisade mesophyll thickness (PMT), with higher values for ‘high  $\Delta$ ’ compared with ‘low  $\Delta$ ’ for all three traits (Table 2). With the exception of chlorophyll content (Chl) and upper epidermis thickness (UET), all structural leaf traits showed a significant growth-flush effect, which was similar for both phenotypes (Table 2). Only for lower epidermis thickness (LET) was the difference between phenotypes inverted between second and third growth-flush.

No significant difference was detected between phenotypes for stomatal density. This was mainly due to one outlier datapoint (growth-flush 3 of genotype 101 in block C) with very high SD (712 mm $^{-2}$ ; see also the high standard deviation for the ‘low  $\Delta$ ’ mean for growth-flush 3 in

**Table 2.** Statistical analyses for traits measured at the leaf level

Significant differences at the 95% level between the two phenotypes 'low  $\Delta$ ' and 'high  $\Delta$ ', growth-flushes, and phenotype $\times$ growth-flush interaction are indicated by '\*'. Values for traits with a significant phenotype effect are in bold. The number in parenthesis refers to the number of plants in each phenotype used for computing average values and testing 'low  $\Delta$ ' versus 'high  $\Delta$ ' effect.  $A_{\text{sat}}$  ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), net  $\text{CO}_2$  assimilation rate; and  $g_{\text{sat}}$  ( $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ), stomatal conductance for water vapour (both measured under ambient  $\text{CO}_2$  and light saturation);  $V_{\text{cmax}}$  ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), maximum carboxylation rate of Rubisco;  $J_{\text{max}}$  ( $\mu\text{mol e}^- \text{ m}^{-2} \text{ s}^{-1}$ ), maximal electron transport rate;  $g_i$  ( $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), mesophyll conductance to  $\text{CO}_2$ ;  $W_{\text{mean}}$  ( $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ ), average  $W_i$ ;  $A_{\text{mean}}$  ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), average  $A_i$ ;  $g_{\text{mean}}$  ( $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ), average  $g_s$  as computed from daily courses of leaf gas exchange;  $A_{\text{max}}$  ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), maximum  $A_i$ ; and  $g_{\text{max}}$  ( $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ), maximum  $g_s$  (both measured during daily courses of gas exchange);  $\alpha$  ( $10^4 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ /unit of global irradiance), slope of the response of  $g_s$  to light determined with daily course of gas exchange;  $N_{\text{mass}}$  ( $\text{mg g}^{-1}$ ), nitrogen content on a mass basis;  $N_{\text{area}}$  ( $\text{g m}^{-2}$ ), nitrogen content on an area basis; Chl ( $\text{g m}^{-2}$ ), chlorophyll content; TLT ( $\mu\text{m}$ ), total leaf thickness; UET ( $\mu\text{m}$ ), upper epidermis thickness; PMT ( $\mu\text{m}$ ), palisade mesophyll thickness; SMT ( $\mu\text{m}$ ), spongy mesophyll thickness; LET ( $\mu\text{m}$ ), lower epidermis thickness; Dens ( $\text{g cm}^{-3}$ ), foliar density; LMA ( $\text{g m}^{-2}$ ), leaf mass-to-area ratio; SD ( $\text{mm}^{-2}$ ), stomatal density; SD\*, without outlier data point.

	Mean ( $\pm$ standard deviation) for the 2nd flush		Mean ( $\pm$ standard deviation) for the 3rd flush		Statistics		
	'Low $\Delta$ '	'High $\Delta$ '	'Low $\Delta$ '	'High $\Delta$ '	Low versus high	Flush	Int.
<b><math>A_{\text{sat}}</math></b>	<b>10.3<math>\pm</math>1.1 (9)</b>	<b>14.2<math>\pm</math>2.0 (8)</b>	<b>10.4<math>\pm</math>2.2 (9)</b>	<b>15.5<math>\pm</math>2.4 (9)</b>	*	–	–
<b><math>g_{\text{sat}}</math></b>	<b>0.12<math>\pm</math>0.02 (9)</b>	<b>0.27<math>\pm</math>0.06 (8)</b>	<b>0.13<math>\pm</math>0.05 (9)</b>	<b>0.33<math>\pm</math>0.09 (9)</b>	*	–	–
Apparent $V_{\text{cmax}}$	66.0 $\pm$ 15.0 (9)	71.7 $\pm$ 11.8 (8)	65.6 $\pm$ 8.8 (9)	74.4 $\pm$ 10.1 (9)	–	–	–
<b>Apparent <math>J_{\text{max}}</math></b>	<b>161.2<math>\pm</math>26.1 (8)</b>	<b>174.6<math>\pm</math>31.3 (8)</b>	<b>155.6<math>\pm</math>17.3 (9)</b>	<b>187.2<math>\pm</math>26.2 (9)</b>	*	–	–
Corrected $V_{\text{cmax}}$	110.3 $\pm$ 18.9 (2)	116.6 $\pm$ 22.7 (8)	105.1 $\pm$ 26.8 (6)	144.8 $\pm$ 29.7 (9)	–	–	–
<b>Corrected <math>J_{\text{max}}</math></b>	<b>169.4<math>\pm</math>38.7 (2)</b>	<b>183.6<math>\pm</math>34.0 (8)</b>	<b>164.4<math>\pm</math>16.2 (6)</b>	<b>204.3<math>\pm</math>32.4 (9)</b>	*	–	–
$g_i$	0.17 $\pm$ 0.01 (2)	0.19 $\pm$ 0.07 (8)	0.18 $\pm$ 0.06 (6)	0.18 $\pm$ 0.06 (9)	–	–	–
Daily course							
<b><math>W_{\text{mean}}</math></b>	<b>114.8<math>\pm</math>32.8 (12)</b>	<b>83.5<math>\pm</math>29.5 (12)</b>	<b>111.3<math>\pm</math>24.3 (12)</b>	<b>74.6<math>\pm</math>25.7 (12)</b>	*	–	–
<b><math>A_{\text{mean}}</math></b>	<b>9.8<math>\pm</math>1.7 (12)</b>	<b>10.2<math>\pm</math>2.0 (12)</b>	<b>9.7<math>\pm</math>3.4 (12)</b>	<b>13.9<math>\pm</math>2.6 (12)</b>	*	*	*
<b><math>g_{\text{mean}}</math></b>	<b>0.09<math>\pm</math>0.03 (12)</b>	<b>0.14<math>\pm</math>0.06 (12)</b>	<b>0.10<math>\pm</math>0.05 (12)</b>	<b>0.22<math>\pm</math>0.10 (12)</b>	*	*	*
<b><math>A_{\text{max}}</math></b>	<b>13.5<math>\pm</math>2.7 (12)</b>	<b>15.1<math>\pm</math>3.7 (12)</b>	<b>13.0<math>\pm</math>4.1 (12)</b>	<b>18.1<math>\pm</math>3.3 (12)</b>	*	–	–
<b><math>g_{\text{max}}</math></b>	<b>0.16<math>\pm</math>0.05 (12)</b>	<b>0.24<math>\pm</math>0.09 (12)</b>	<b>0.16<math>\pm</math>0.06 (12)</b>	<b>0.35<math>\pm</math>0.15 (12)</b>	*	*	–
<b><math>\alpha</math></b>	<b>4.4<math>\pm</math>2.5 (12)</b>	<b>11.5<math>\pm</math>6.0 (10)</b>	<b>5.0<math>\pm</math>2.5 (12)</b>	<b>11.1<math>\pm</math>4.3 (11)</b>	*	–	–
Structural traits							
<b><math>N_{\text{mass}}</math></b>	<b>34.1<math>\pm</math>3.4 (12)</b>	<b>29.8<math>\pm</math>4.8 (10)</b>	<b>36.6<math>\pm</math>3.4 (12)</b>	<b>31.4<math>\pm</math>3.1 (12)</b>	*	*	–
$N_{\text{area}}$	3.0 $\pm$ 0.5 (12)	2.8 $\pm$ 0.5 (10)	3.5 $\pm$ 0.6 (12)	3.6 $\pm$ 0.6 (12)	–	*	–
<b>Chl</b>	<b>0.89<math>\pm</math>0.13 (12)</b>	<b>0.78<math>\pm</math>0.16 (12)</b>	<b>0.87<math>\pm</math>0.19 (12)</b>	<b>0.77<math>\pm</math>0.14 (12)</b>	*	–	–
TLT	140 $\pm$ 7 (12)	147 $\pm$ 10 (12)	148 $\pm$ 17 (12)	155 $\pm$ 12 (12)	–	*	–
UET	11.8 $\pm$ 0.6 (12)	11.6 $\pm$ 1.0 (12)	12.2 $\pm$ 1.2 (12)	12.1 $\pm$ 1.0 (12)	–	–	–
<b>PMT</b>	<b>55.7<math>\pm</math>3.3 (12)</b>	<b>60.9<math>\pm</math>5.0 (12)</b>	<b>51.1<math>\pm</math>5.1 (12)</b>	<b>56.1<math>\pm</math>5.8 (12)</b>	*	*	–
SMT	62.9 $\pm$ 4.9 (12)	65.4 $\pm$ 6.6 (12)	74.7 $\pm$ 10.6 (12)	76.6 $\pm$ 6.3 (12)	–	*	–
LET	9.5 $\pm$ 0.6 (12)	8.9 $\pm$ 0.4 (12)	9.8 $\pm$ 0.9 (12)	10.0 $\pm$ 0.6 (12)	–	*	*
Dens	0.57 $\pm$ 0.08 (12)	0.57 $\pm$ 0.07 (12)	0.63 $\pm$ 0.07 (12)	0.63 $\pm$ 0.04 (12)	–	*	–
LMA	79.6 $\pm$ 11.4 (12)	83.6 $\pm$ 9.2 (12)	92.2 $\pm$ 10.7 (12)	97.1 $\pm$ 7.4 (12)	–	*	–
SD	412 $\pm$ 46 (12)	461 $\pm$ 48 (12)	483 $\pm$ 97 (12)	531 $\pm$ 63 (12)	–	*	–
<b>SD*</b>	<b>412<math>\pm</math>46 (12)</b>	<b>461<math>\pm</math>48 (12)</b>	<b>462<math>\pm</math>67 (11)</b>	<b>531<math>\pm</math>63 (12)</b>	*	*	–

Table 2). When this outlier was removed, a significant difference was observed between phenotypes (SD\* in Table 2), with a higher stomatal density for 'high  $\Delta$ '. Further, a significant difference was also observed between phenotypes for SD when growth-flush 2 was analysed separately (analysis not presented).

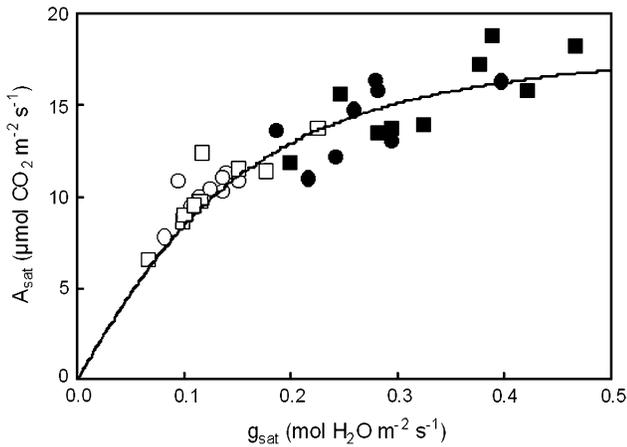
#### Expression levels of the *ERECTA* gene

The expression of the *ERECTA* gene, relative to the control gene, was analysed for both phenotypes for all blocks separately. A significant trend was observed for three blocks out of the four (Fig. 4) with a 1.78–4 times higher relative expression level of the *ERECTA* gene in 'low  $\Delta$ ' compared with 'high  $\Delta$ '.

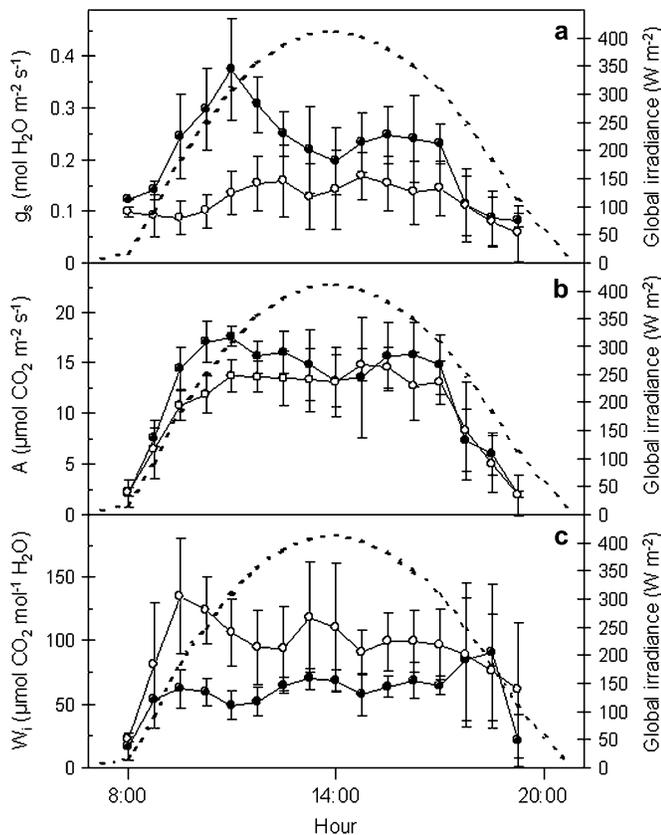
## Discussion

In this study, the complex trait 'water use efficiency' was broken down into its main components, and it was checked whether the  $^{13}\text{C}$  isotope discrimination ( $\Delta^{13}\text{C}$ ) recorded from the  $^{13}\text{C}$  content in dry matter was a valid estimator for the intra-specific genetic variability of this trait. The sampling of two phenotypes with high or low  $\Delta^{13}\text{C}$ , using previously recorded data in a *Quercus robur* full-sib progeny (Brendel *et al.*, 2008), allowed the variability within this progeny to be represented by a small number of individuals and therefore enabled a large number of measurements to be made to document the phenotypic differences.

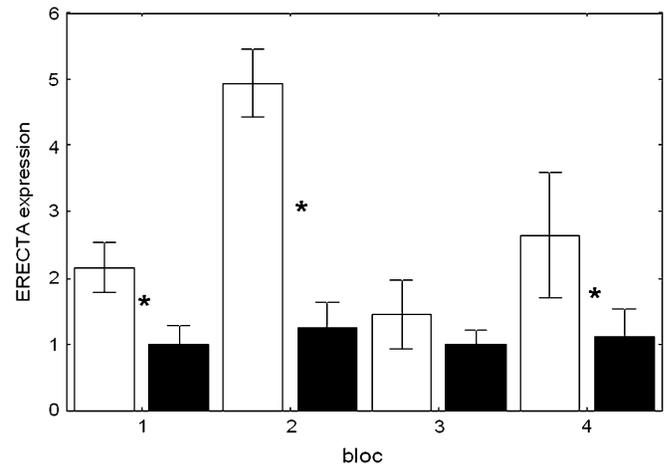
$\Delta^{13}\text{C}$  recorded in the different carbon pools (organic matter, wood, and wood cellulose) differed significantly (2‰) between 'low  $\Delta$ ' and 'high  $\Delta$ ' phenotypes. This



**Fig. 2.** Net CO<sub>2</sub> assimilation rate ( $A_{\text{sat}}$ ) versus stomatal conductance for water vapour ( $g_{\text{sat}}$ ) under ambient CO<sub>2</sub> and saturating irradiance. Open and closed symbols: 'low  $\Delta$ ' and 'high  $\Delta$ ' phenotype, respectively. Values from 2nd and the 3rd growth-flushes are represented with circles and squares, respectively. Each point represents an individual value of  $A_{\text{sat}}$  and  $g_{\text{sat}}$ . A non-rectangular hyperbola:  $A_{\text{sat}} = \{19 + 104 \times g_{\text{sat}} - [(19 + 104 \times g_{\text{sat}})^2 - 2.8 \times 19 \times 104 \times g_{\text{sat}}]^{1/2}\} / 1.4$ ,  $R^2 = 0.78$  was adjusted.



**Fig. 3.** Example of a daily course of (a) stomatal conductance for water vapour ( $g_s$ ), (b) net CO<sub>2</sub> assimilation rate ( $A$ ), and (c) intrinsic water use efficiency ( $W_i$ ) computed as the ratio  $A/g_s$ , in 'low  $\Delta$ ' (open symbols) and 'high  $\Delta$ ' individuals (closed symbols). Measurements were made during 31 July 2005 on leaves of the 3rd growth-flush. Each point is the average for each phenotype ( $N=3$ )  $\pm$  standard deviation. The dashed line represents the daily course of global irradiance in the greenhouse.



**Fig. 4.** Leaf *ERECTA* transcript levels, expressed relative to expression of the control gene, for 'low  $\Delta$ ' (open columns) and 'high  $\Delta$ ' (closed columns) phenotypes. For each phenotype of each block, *ERECTA* expression was estimated from the extraction of a pool of the three genotypes and of several leaves per genotype. Error bars represent standard deviations ( $N = 3$ ).

difference was stable across the carbon pools used and matched the results of a preliminary study with the same genotypes, but based on soluble sugars and starch in leaves, i.e. on pools with a faster turnover rate (Roussel *et al.*, 2009). These results taken together confirm the stability of the isotopic signature of the tested genotypes, and the tight genetic control over this signature. They confirm also that the signature is not blurred by post-photosynthetic discrimination that is known to occur during the synthesis of lignins or other compounds from photosynthates (Bowling *et al.*, 2008). Furthermore they support the idea that the discrimination processes yielding such differences do not just display a transient expression during a given stage of development, but that they probably act over the whole life cycle of leaves as shown by the stability of the inter-phenotype differences among compounds with short (soluble sugars) and long (wood, starch, cellulose) turn-over times.

The data also showed that these differences in  $\Delta^{13}\text{C}$  were closely matched by differences in  $W_i$  estimated from leaf gas exchange measurements under ambient CO<sub>2</sub> and saturating light, or from mean values (weighted by net CO<sub>2</sub> assimilation) recorded during diurnal cycles of leaf gas exchange. The two methods yielded rather close values for the 'low  $\Delta$ ' phenotype, independently of the growth-flush from which the leaves were sampled, and divergent values for the 'high  $\Delta$ ' phenotype. The difference between phenotypes was around 50% for the first method, and 30–40% for the second one. This lies within the range of values expected from the  $\Delta^{13}\text{C}$  values, and strongly supports the hypothesis that the variability of  $\Delta^{13}\text{C}$  recorded in the full-sib family by Brendel *et al.* (2008) was to a large extent due to the variability of  $W_i$  among the full-sibs. Nevertheless, at this stage it cannot be excluded that other processes, like mesophyll conductance to CO<sub>2</sub> ( $g_i$ ), could also contribute

to the observed variability of  $\Delta^{13}\text{C}$ . The overall  $\Delta^{13}\text{C}$  during carbon assimilation is dependent on the  $\text{CO}_2$  concentration at the sites of carboxylation ( $C_c$ ) and  $C_c$  is strongly dependent on  $g_i$  (Farquhar and Richards, 1984). However, no difference was found for  $g_i$ , as estimated from adjusting  $g_i$  to  $A-C_i$  curves concurrently to  $V_{\text{cmax}}$  and  $J_{\text{max}}$  (Ethier and Livingston, 2004; Warren and Dreyer, 2006). The adjusted values were around  $0.18 \text{ mol m}^{-2} \text{ s}^{-1}$ , i.e. in the same order of magnitude as stomatal conductance. Such values are in broad agreement with published values (Evans and von Caemmerer, 1996) and match those produced for oaks with a fluorescence-gas exchange technique (Roupsard *et al.*, 1996). The lack of difference between phenotypes, although one cannot conclude to a definitive dismissal of  $g_i$  as a contributor to diversity in  $\Delta^{13}\text{C}$ , nevertheless shows that this contribution is probably small. Additional measurements might be required to definitely support this point.

A clear confirmation that the differences observed in  $\Delta^{13}\text{C}$  and in  $W_i$  were related to differences in TE was also provided. TE is an unambiguous estimate of water use efficiency integrated over a full growing season at the whole-plant level. Such direct and unambiguous confirmations are rare, especially for forest tree species, and were produced for *Larix occidentalis* (Zhang and Marshall, 1994), *Populus davidiana* (Zhang *et al.*, 2005), maritime pine (Guehl *et al.*, 1996), an African acacia (Roupsard *et al.*, 1998), and a tropical pioneer tree (Cernusak *et al.*, 2007). In the present study, the inter-phenotype difference in TE reached 16%, which is smaller than the estimates using  $\Delta^{13}\text{C}$  and  $W_i$ . This might be due to the fact that the instantaneous measurements concentrated on sun leaves as well as clear, sunny days, whereas TE also integrates the gas exchange of shade leaves and of overcast or rainy days. Moreover, TE depends not only on  $W_i$  but also on the fraction of carbon lost by respiration, the fraction of water lost during non-photosynthetic events (nocturnal stomata opening, cuticular transpiration or soil evaporation), and vapour pressure deficit (Farquhar and Richards, 1984). In the present study, the environmental effect was limited by the experimental design, limiting variation in vapour pressure deficit, as well as the use of several copies to calculate genotype means. However, the difference in  $g_s$  in the morning could have led to differences in leaf temperature and thus in leaf-to-air vapour pressure difference (LVPD). Accordingly, 'high  $\Delta$ ' with higher  $g_s$  would have resulted in lower LVPD and consequently in lower transpiration and higher TE. This would decrease the difference in TE between the phenotypes and could thus explain the discrepancy with the differences observed for  $W_i$ , in addition to potential inter-phenotype variations in the fraction of carbon lost by respiration, water losses during night-time, and cuticular or stem transpiration.

Gas exchange measurements provided additional information about the processes involved in the intra-specific variability of  $W_i$ . Net assimilation rate ( $A$ ) was higher in 'high  $\Delta$ ', which is counter intuitive with lower  $W_i$ . Indeed, higher stomatal conductance resulted in slightly increased  $A$ , solely due to smaller stomatal limitations of photosyn-

thesis, and not to higher photosynthetic capacity *per se*. Indeed  $V_{\text{cmax}}$  and  $g_i$  did not exhibit any difference, while maximum light-driven electron flow ( $J_{\text{max}}$ ) was smaller in 'low  $\Delta$ ', possibly as a result of a down-regulation of the investment into thylacoid components due to permanent stomatal limitation of photosynthesis. Nevertheless, the present data allowed a firm conclusion to be reached: the two phenotypes did not differ in photosynthetic capacity, but in stomatal control of photosynthesis and  $W_i$ .

The difference found for maximum stomatal conductance under controlled conditions was confirmed by *in situ* diurnal time courses: 'low  $\Delta$ ' showed a lower maximum  $g_s$ , compared with 'high  $\Delta$ '. Further, the daily time courses of stomatal opening and closing were different between phenotypes, suggesting not only a genetic variation in maximal stomatal opening but also of stomatal kinetics. Within-species genetic differences in diurnal time courses of gas exchange have already been shown for *Pseudotsuga menziesii* (Zhang *et al.*, 1993), where differences between varieties in  $W_i$  were largest in the middle of the afternoon. In the present study, however, the difference in  $W_i$  was largest in the morning, which, other than being related to the difference in  $g_{\text{max}}$ , could probably be due to the difference observed in stomatal behaviour during stomatal opening in the morning, which could indicate a different stomatal reactivity to irradiance. However, differences in stomatal sensitivity to relative humidity or temperature cannot be excluded. Independent data from other genotypes from the same pedunculate oak family have shown some differences in sensitivity to both irradiance and humidity (M Roussel, unpublished data). Differences at population or provenance level have been found by some authors [Dang *et al.* (1994) for *Alnus rubra* Bong.; Grossnickle *et al.* (2005) for *Thuja plicata*; see also references therein]; however, no examples were found for genetic variation in reactivity to irradiance. Few genes have been related to daily variation in stomatal conductance; however, results such as variation in guard cell aquaporin expression (Sarda *et al.*, 1997) suggest possible candidates. Considering recent developments in cell-specific expression studies, further gene candidates will probably be available in the near future.

The important role of stomatal control of water use was confirmed by differences in TE: the two phenotypes did not differ in biomass allocation or in transpiring leaf area (both were similar in the two phenotypes), but in leaf-specific water use. This result at the whole plant scale strongly supports a major role for stomata in determining the differences in  $W_i$  and TE.

Variation in maximum stomatal conductance is related to differences in stomatal density (Nobel, 1999), which has been observed for different poplar species (Reich, 1984), but this is not necessarily a common observation (Olivas-Garcia *et al.*, 2000). Here, when one single individual with very high values of stomatal density is excluded from the analysis, both growth-flushes showed a significantly higher stomatal density for the phenotype with higher stomatal conductances (maximum or mean values). Variation in stomatal density might be compensated by variation in

stomatal size. An earlier study with genotypes from the pedunculate oak family showed that genotypes with a higher stomatal density also had smaller stomata, but that this did not compensate completely for differences in total stomatal aperture (Roussel *et al.*, 2009).

Masle *et al.* (2005) showed that the gene product of *ERECTA* influences significantly carbon isotope discrimination and water use efficiency in *Arabidopsis thaliana*, with lower transcript levels relating to higher carbon isotope discrimination and lower water use efficiency. Here, it was found that the 'high  $\Delta$ ' phenotype showed lower transcript levels, as was expected from the study on *A. thaliana*. Further, Masle *et al.* (2005) measured higher stomatal conductance and higher stomatal density for mutants with reduced *ERECTA* expression and low water use efficiency. This is also paralleled by the present results, suggesting that *ERECTA* could play a similar role in oaks as in *Arabidopsis*. Other than the role of *ERECTA* in proliferation and growth of cells in different tissues (Shpak *et al.*, 2003), leaf adaxial–abaxial symmetry (Xu *et al.*, 2003), and inflorescence architecture (Douglas *et al.*, 2002), this receptor-like protein kinase, first described by Torii *et al.* (1996), has mainly been linked recently to the regulation of stomatal density by inhibiting the differentiation of epidermal cells into guard cells (Bergmann, 2006). Most of the studies on expression of *ERECTA* done on mutants of *A. thaliana* did not provide any evidence that allelic or expressional diversity of this gene plays a role in the diversity for water use efficiency observed in natural populations. Here, an intraspecific diversity in the expression of this gene has been shown in mature leaves of a widespread forest tree species to be related to genetic differences in water use efficiency.

## Conclusions

The phenotypes, which had been selected for leaf  $\Delta^{13}\text{C}$ , effectively showed the expected differences in wood and cellulose  $\Delta^{13}\text{C}$ , suggesting little perturbation of the signal recorded during carboxylation by post-photosynthetic discrimination. The study also showed that the diversity in  $\Delta^{13}\text{C}$  recorded within this pedunculate oak family closely matched two independent estimates of water use efficiency: intrinsic water use efficiency ( $W_i$ ) and TE. This strongly supports the use of  $\Delta^{13}\text{C}$  as an efficient indicator of  $W_i$  for screening the genetic variability in this species. Moreover, the present results suggest that there was no perturbation caused by a potential intra-specific variability of the parameters involved in the theoretical relationships among these three estimates of water use efficiency, such as, for example, mesophyll conductance to  $\text{CO}_2$ . At the whole plant level, diversity in water use efficiency was related to consumption of water and not to biomass production. This finding was corroborated at the leaf level, since the observed diversity in  $\Delta^{13}\text{C}$  and  $W_i$  was linked to differences in stomatal conductance rather than in photosynthetic capacity. Differences in stomatal density as well as in diurnal stomatal dynamics might be causes of the observed diversity

in stomatal conductance and need to be investigated in more detail. The diversity in the expression of the gene *ERECTA* has given the first indication of a supposed mechanism causing diversity in stomatal density.

## Supplementary data

Supplementary data giving the bioinformatic strategy applied to identify the oak orthologous sequence for the *ERECTA* gene are available at *JXB* online.

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