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Quantitative trait loci of tolerance to waterlogging in a European oak (*Quercus robur* L.): physiological relevance and temporal effect patterns

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ABSTRACT

***Quercus robur* L. is a mid-European broadleaved tree species that grows readily on temporary waterlogged soils. An experiment aiming to identify potential markers of tolerance to waterlogging in this species and to assess the degree of genetic control over the corresponding traits was conducted. Quantitative trait loci (QTL) were assessed in an F_1 progeny for responses to waterlogging, and the relevance of the observed traits as markers of tolerance was investigated using a precise description of the time course of their expression. Five significant QTL involved in the response to waterlogging were identified. In particular, QTL were detected for the development of hypertrophied lenticels and for the degree of leaf epinasty, but not for the formation of adventitious roots. A multi-environment QTL model allowed a detailed description of the time course (7 weeks) of the allelic substitution effect of some of these QTL. Correlation clustering identified significant clusters of QTL, at inter-trait as well as at intra-trait level. These clusters suggest the occurrence of a genetically controlled response cascade to waterlogging.**

Key-words: allelic substitution effect; correlation clustering; epinasty; hypertrophied lenticel; multi-environment model; root hypoxia.

INTRODUCTION

Quercus robur L. is a mid-European broadleaved tree species that favours deep and well-watered soils and is able to grow readily even on temporary waterlogged soils (Lévy, Becker & Duhamel 1992). This contrasts with the closely related *Quercus petraea* (Matt.) Liebl that requires well-drained soils. Ecological divergence likely plays a role in the maintenance of the separation of these two species despite the absence of a complete reproductive barrier (Petit *et al*. 2004). Natural selection may enable speciation even in the presence of gene flow (Doebeli *et al*. 2005; Lexer *et al*. 2005). In such a model, disruptive selection favours in each species the fixation of alleles with opposite effects on the phenotype (Schluter 2001). In oaks, disruptive selection for the response to waterlogging may partly explain the divergence between *Q. robur* and *Q. petraea*. Natural selection may have favoured alleles involved in tolerance to root hypoxia in the *Q. robur* genome. In consequence, keeping species integrity against a potentially large interspecific gene flow is an important issue to maintain individuals adapted to waterlogged stations.

The identification of traits related to waterlogging tolerance that are under genetic control is a step towards the detection of traits that (1) are relevant for understanding the physiological basis of waterlogging tolerance in young *Q. robur* trees, and (2) identify genomic regions that differ between the two species. No information is yet available about the degree of genetic determinism of waterlogging tolerance in trees. To detect the degree of genetic control on traits and the genomic regions involved, a quantitative trait loci (QTL) approach is required. The identification of QTL for tolerance-related traits is also a prerequisite for a future search of putative candidate genes for waterlogging tolerance. Furthermore, some alleles of these genes could be specific to the species *Q. robur* and therefore used for species identification.

Traits related to waterlogging tolerance in trees are expected to differ from those reported in annual plants like rice (Setter *et al*. 1997; Sripongpangkul *et al*. 2000; Jackson & Ram 2003) in terms of underlying key mechanisms for survival. The physiological processes involved in tolerance to waterlogging and to the resulting root hypoxia are still poorly known in the case of tree species (Kozlowski & Pallardy 2002; Kreuzwieser, Papadopoulou & Rennenberg 2004). In addition, no consistent model is yet available for hypoxia tolerance (Drew 1997; Vartapetian & Jackson 1997). For instance, in the case of *Q. robur*, previous studies showed that the response of seedlings to waterlogging

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includes short-term responses as well as long-term acclimation processes. Short-term responses include changes in root-cell sugar metabolism, with increased activity of fermentation pathway enzymes (Parelle *et al*. 2006). Long-term acclimation relies on the formation of hypertrophied lenticels and of adventitious roots (Colin-Belgrand, Dreyer & Biron 1991; Schmull & Thomas 2000; Parelle *et al*. 2006). The large variability observed in seedling survival during waterlogging could result from an intraspecific diversity in the ability to develop these acclimation traits.

We developed an experiment aiming at detecting QTL for a large number of traits induced by waterlogging. QTL detection is classically used with quantitative traits as recorded at a given time under one or several environmental conditions. However, some traits of interest follow a time course that could provide supplementary information for QTL detection. Detecting QTL for modelled parameters describing the time course of such traits is one possible approach (Zhang *et al*. 2005). However, such a modelling approach is not always available for complex traits. Direct QTL detection using a multi-environment analysis (Jansen *et al*. 1995) does not require preliminary modelling. Such analyses are currently used to assess the stability of QTL under different environmental conditions (Jermstad *et al*. 2003; Reymond *et al*. 2003) or across years (Lerceteau, Szmidt & Andersson 2001). In the present experiment, successive dates of sampling were used as the different environments in a multi-environment QTL mapping model. This technique increases the power of detection of the QTL and the precision of mapping (Jansen *et al*. 1995). Additionally, the method provides useful information on the time course of the allelic substitution effect of a given QTL.

The analysis was focused on the morphological response of *Q. robur* to waterlogging. We monitored the time course of leaf epinasty (i.e. increase of the angle that petioles form with stems), which is known to be highly specific of hypoxia responses (Vartapetian & Jackson 1997). We also measured the time course of the formation of hypertrophied lenticels, which are thought to play a major role in hypoxia tolerance in *Q. robur* (Parelle *et al*. 2006). At the end of the treatment, the formation of adventitious roots was recorded as a candidate trait to assess the level of tolerance, as is reported for numerous herbaceous species (Vartapetian & Jackson 1997). As a matter of fact, adventitious roots are known to be formed massively on *Q. robur* in response to waterlogging (Colin-Belgrand *et al*. 1991; Parelle *et al*. 2006). The time courses of leaf chlorophyll content and of diameter at collar, and several traits characterizing plant biomass and leaf structure, were quantified as indicators of general plant performance. These measurements were also aimed at following the time course of the overall plant response to waterlogging and to obtain further information for interpreting the relevance of the detected QTL.

Our main objectives were therefore to document (1) the genetic determinism of responses to waterlogging in *Q. robur*, (2) the organization of the genomic regions involved in this determinism and (3) the time courses of the allelic substitution effect of some of the detected QTL.

MATERIAL AND METHODS

Plant material and waterlogging conditions

Rooted cuttings of *Q. robur* were grown in a greenhouse from December 2003 to June 2004 in 4 L pots containing peat and sand mixture. The rooted cuttings comprised three clonal replicates of 120 full sibs. These full sibs were a chosen subsample of the mapping population of *Q. robur* obtained from the controlled cross between two *Q. robur* individuals located at INRA-Pierroton (South-western France, see details in Scotti-Saintagne *et al*. 2004). The subsample of individuals was selected among those with the largest number of identified genetic markers. Individuals were randomized in 15 incomplete blocks of 24 plants each. Individual pots were placed in groups of four in larger containers. These containers were filled with water up to the soil level from 21 June to 9 August 2004 (51 d or 7 weeks). The water level was adjusted daily with dis-oxygenized water obtained by bubbling N_2 in water tanks (Parelle *et al*. 2006). Oxygen content was monitored at days 2, 9, 17 and 42 of the waterlogging treatment, in piezometric tubes inserted in each pot. Dissolved oxygen content was already low in tubes close to the roots after 2 d of waterlogging (2.7 ± 0.1 mg L^{-1}) and decreased further to 1.3 ± 0.1 mg L^{-1} during the treatment. This corresponds to severe hypoxic conditions as compared to water equilibrated with free air (8.5 mg L^{-1} at similar temperature). The oxygen content in the free water around the pots was higher, but similarly decreased from 5.9 ± 0.1 to 2.3 ± 0.1 mg L^{-1} during the treatment. The trees were fertilized with Nutricote (N/P/K, 13/13/13; Fertilizer Co Ltd, Chisso-Asan, NY, USA) at 4 g $L^{-1}_{substrate}$ during active growth (before waterlogging, August 2003). During waterlogging, dissolved nitrate was estimated in the water table of 24 randomly selected trees using Quantofix test sticks (Machery-Nagel, Düren, Germany). After 31 d of treatment, a severe N deficiency due to nitrate leaching and denitrification induced by waterlogging was detected. A liquid fertilizer (Plantprod, Brampton, Canada) at 50 mg of nitrogen per litre was applied evenly to all individuals after 31 d of waterlogging.

Recorded traits

The chlorophyll content in leaves was measured twice a week from 6 d before waterlogging to 47 d of treatment. Three to five measurements were made on three fully developed leaves of the last growth flush developed before waterlogging on each tree, using a chlorophyll content metre (CCM; Opti Science, Tyngsboro, MA, USA). The same leaves were monitored during the whole experiment. After 50 d of waterlogging, the three leaves were sampled for water and nitrogen content and leaf mass-to-area ratio (LMA) determination. Three pooled and dried leaves were ground with a ball mill (MM 200; Retch, Haan, Germany), and the nitrogen content was estimated with a Carlo Erba elementary analyser (Carlo Erba, Milan, Italy). The diameter at collar was measured twice a week on all individuals,

Table 1. Development stages for hypertrophied lenticels assessed from their colour

Description of the colour of the hypertrophied lenticels	Colour level	Levels observed on whole plant	Colour classes
Black disc visible on the bark, corresponding to the start of hypertrophy and splitting of the bark	1	1	1
		1 > 2	2
		1 > 3	3
A white spot growing in the middle of the black disc due to appearance of suber cells by the hole formed by the cracking of the bark	2	2 > 1	4
		2	5
		2 > 3	6
Totally white lenticel	3	3 > 1	7
		3 > 2	8
		3 > 1	9
		3 > 4	10*
		3 > 5	11*
Large and purple*	4*	4 > 3	12*
		4	13*
		4 > 5	14*
Large and black*	5*	5 > 3	15*
		5 > 4	16*
		5	17*

Colour classes were defined as a function of the relative fraction (indicated by the symbol >, e.g. 1 > 2 a majority of lenticels at stage 1 and some at stage 2) of the different colour levels observed on the whole plant. *Colour level and classes that were only observed at the end on the treatment, and in consequence not used for the estimation of the development stages for 'visible' hypertrophied lenticels.

2 cm above the water table from 10 d before waterlogging to 46 d of treatment. The occurrence of leaf epinasty was recorded using three ordinal classes: above perpendicular, perpendicular to stem axis and less than perpendicular, and three numerical values were assigned to these classes: 120°, 90° and 60°, respectively, in order to obtain a quantitative variable necessary for QTL analysis.

During the full time course of waterlogging, visible hypertrophied lenticels (i.e. those occurring above and those occurring below the water table and accessible to observation) were monitored on each plant. We used two complementary criteria to describe lenticel development: total count and colour. The latter criterion was used as an estimator of the degree of hypertrophy of the lenticels, and encompassed five levels (see Table 1 for details). Using the colour scale we defined nine different classes to describe the population of lenticels on each individual (Table 1). We used the date at which colour class 9 was reached as an indicator of the rate of development of hypertrophied lenticels. At the end of the experiment (7 weeks of waterlogging), we observed that a large number of hypertrophied lenticels were hidden below the soil level. In consequence, the total amount of hypertrophied lenticels was recorded. In addition, the diameter and the thickness of hypertrophied lenticels above and below the water level were visually assessed.

After 7 weeks of waterlogging, we recorded the number of severely wilting individuals. Finally, all trees were randomly harvested during 1 week (days 51 to 55 of waterlogging) and the water level was marked on each stem. Small stem sections (corresponding to the zone of formation of hypertrophied lenticels) were sampled and immediately frozen in liquid nitrogen for bark anatomy observations. The roots were cleaned with tap water and adventitious roots were counted, sampled and their length measured. Dry mass was measured on fine, main and adventitious roots separately, and on leaves and stems. Leaf dry mass and LMA of a sample of leaves were used for the calculation of total leaf area of each individual.

Anatomical observations

Twenty-four trees were selected in order to cover independently the range of hypertrophied lenticels and of adventitious roots, together with two replicates of three clones of plants with either no, few or many adventitious roots. We also selected two replicates of three clones grown under normoxic conditions as controls. A levelled cross-section of bark tissue just beneath a hypertrophied lenticel (when there was at least one on the collar of the plant) was observed by variable pressure scanning electron microscopy (VP-SEM, model Leo 1450 VP; Leo Electron Microscopy Inc., NY, USA).

Statistical and QTL analyses

Statistical analyses were performed using Statistica version 7.1 (Statsoft, Tulsa, OK, USA), and QTL detection was done using Multi-QTL version 2.5, available at http://www.multiqtl.com (Haifa, Israel). Charts of linkage maps and QTL were made using the MapChart version 2.1 (Voorrips 2001). Hierarchical linkage clustering (HLC) was performed using TIGR-Mev version 3.1 available at http://www.tm4.org (Saeed *et al*. 2003). For all analyses

(including QTL analyses), the tests were considered significant when *P*-value was below 0.05.

Statistical analyses for correlations among traits

Correlations among traits were tested with a linear model and comparisons among multiple groups were done with an analysis of variance (ANOVA) followed by Tukey–Kramer mean-comparison test. Adventitious root dry mass was log-transformed in order to respect the postulates of ANOVA. For comparisons between two categories of individuals, we used parametric analyses for quantitative traits, and non-parametric ones for ordinal traits. As the data for diameter and thickness of hypertrophied lenticels did not follow the assumptions of the Student's tests, and as transformation did not improve the distribution, only non-parametric tests were performed for these two traits. Comparisons between above- and below-water hypertrophied lenticels were made with a Student's *t*-test for dependent samples (number of lenticels) and with sign tests (ordinal traits).

Genetic map

The *Q. robur* clones used in this study originated from an intraspecific F_1 family composed of 278 full sibs. To overcome the unknown allelic phase in offsprings, we used the so-called double pseudo-testcross mapping strategy (Grattapaglia & Sederoff 1994), which only considers alleles segregating in the female background (monomorphic in the father, except for microsatellites) to build the female map and only alleles segregating in the male (monomorphic in the mother, except for microsatellites) to build the male map. Details of the map construction dedicated to QTL location are given in Scotti-Saintagne *et al*. (2004). A majority of gaps present in an earlier version of the map were filled for the present study. The local order of markers was re-analysed by keeping an LOD threshold of 3. Nineteen co-dominant markers common to both maps were used as bridges to align male and female linkage groups (LGs). For both sexes, 12 LGs were identified, corresponding to the 12 chromosomes of *Quercus*.

QTL detection

The presence of a block effect was tested using the following statistical model:

$$Y_{ij} = \mu + C_i + b_j + \varepsilon_{ij}$$

where Y_{ij} is the observed value of the trait, μ the overall mean, C_i the random effect of clone i, b_j the fixed effect of block j and ε_{ij} the error term. To estimate the block-corrected clonal means for QTL analysis, the least square means for C_i were used. If no block effect was detected, b_j was omitted from the model.

Assumptions of ANOVA were fulfilled for each variable, except for the number and dry mass of adventitious roots, and for oxygen content, which were log-transformed to fulfil the assumptions.

The multi-interval mapping (MIM) method (Jansen & Stam 1994; Zeng 1994; Kao, Zeng & Teasdale 1999) was used to identify QTL, allowing individual QTL to be detected independently of background noise and already detected QTL. Multi-QTL software uses statistical models to estimate the significance of QTL, where models with one and two QTL per LG are proposed. The single QTL model tests the null hypothesis that there is a single QTL on the LG against the absence of QTL, by permutation test (1000 permutations, Churchill & Doerge 1994). The two linked QTL model tests the null hypothesis of the presence of two QTL on the LG: (1) against the hypothesis of the absence of QTL and (2) against the hypothesis of the presence of a single QTL. The two tests were performed by permutations (1000 permutations). The MIM procedure of Multi-QTL allows attributing the appropriate model to each LG. As permutation tests in the Multi-QTL software calculate the significance of the QTL at the chromosome level, the significance for all detected QTL was recalculated at the genome level, as a function of the overall number of markers in the map and the number of markers in each LG (Brendel *et al*. 2002; Scotti-Saintagne *et al*. 2004). QTL with a *P*-value < 0.05 at the genome level were considered to be significant. The confidence intervals of the position were estimated by a bootstrap method (1000 re-samplings, Visscher, Thompson & Haley 1996). The allelic substitution effect of the QTL and the percentage of explained variance (PEV) were calculated with the MIM procedure. When the allelic substitution effect of a QTL was not significantly different from 0, we considered the QTL not significant, even though its *P*-value at the genome level was < 0.05.

QTL detection was performed in a two-step procedure: (1) a MIM was performed among the LGs, using a two linked QTL model on each LG and (2) a MIM with a two linked QTL model was performed on LGs for which significant two linked QTL were detected, and a single QTL model for the other ones.

For leaf chlorophyll, epinasty, diameter at collar and dissolved oxygen, we used a multi-environment model (Jansen *et al*. 1995) with each sampling date considered to be a different environment. The model of Jansen *et al*. (1995) does not require independence among individuals in the different environments, which allows to use this model to assess a time course for the allelic substitution effect of the QTL. The data were normalized in order to take into account the heterogeneity of variances among the different dates. We used the same procedure for the classical single-environment models, except that we considered that the QTL were significant if the effect was significantly different from 0 for at least one date.

As leaf-epinasty was monitored using an ordinal scale, we tested whether the transformation into a quantitative variable did not affect the QTL detection. A marker-by-marker Mann–Whitney *U*-test was performed, and all QTL detected by MIM were confirmed ($P < 0.05$ at the chromosome level for at least one date) except for those found on LG 8 on the male map (LG8M).

Correlation clustering of the time patterns of QTL effects

For traits analysed with the multi-environment model, the time courses of allelic substitution effects were compared to detect significant patterns. Absolute values of allelic effects were used and the patterns were normalized to a variance of 1 and a mean of 0. Temporal patterns were sorted by similarity using an HLC algorithm with the absolute Pearson's correlation coefficient as an entry (Plomion *et al.* 2000). This algorithm builds a hierarchical tree (HLC tree) of the different patterns as a function of their correlation coefficients. Clusters were built by imposing a distance threshold to the HLC tree, corresponding to the minimum absolute Pearson's correlation coefficient necessary to obtain a significant correlation. Finally, the coefficients of determination were reported in a correlation matrix, which was ordered according to the HLC tree and divided in the different significant clusters detected.

This method was used for each trait independently. As the different traits were not measured on the same days, inter-trait comparisons were made after switching to a weekly resolution of the time course of the effects.

RESULTS

Time course of the responses of *Q. robur* to waterlogging

The diameter at collar increased during the course of the treatment (Fig. 1a), with two transient slow downs (at the beginning of waterlogging and just before the addition of fertilizer on day 31). The leaf chlorophyll content increased during waterlogging, particularly after fertilization (Fig. 1b). The leaf angles between petioles and stems decreased rapidly during the treatment, revealing an increase in epinasty (Fig. 1c). The number of visible hypertrophied lenticels increased linearly during the treatment (Fig. 2a). Lenticel development, estimated from the changes in colour, took place mainly during the first 20 d of waterlogging (Fig. 2b), after which all lenticels had reached the maximum development stage.

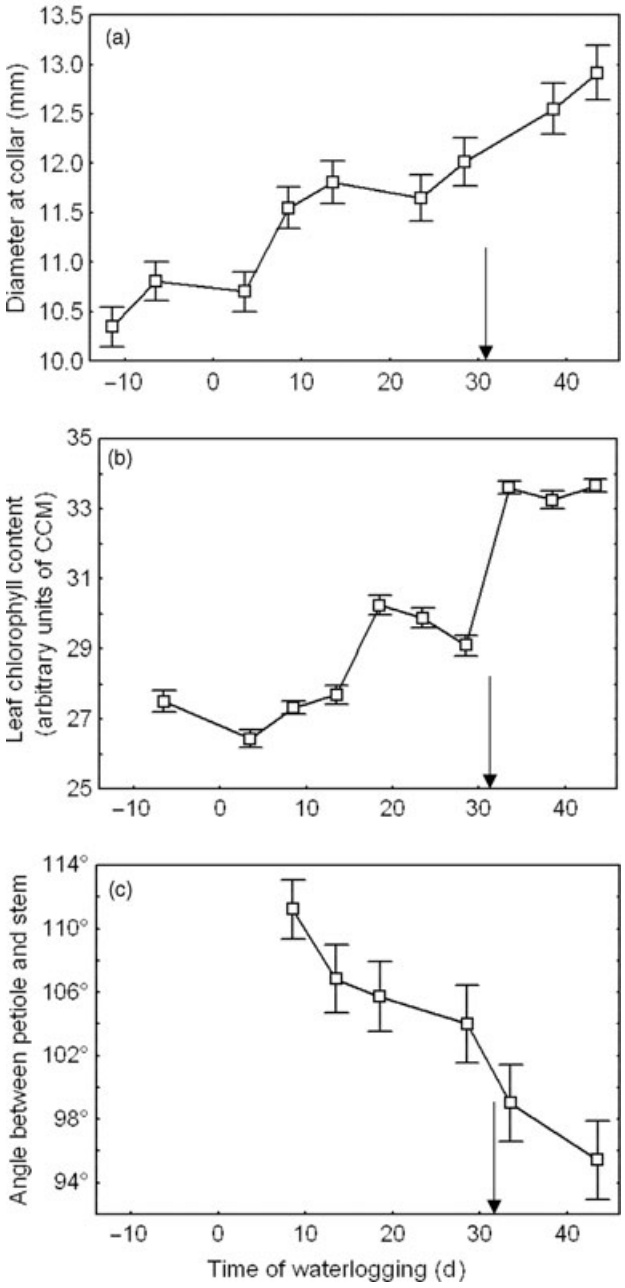

Figure 1. Time course during waterlogging of (a) diameter at collar, (b) leaf chlorophyll content [arbitrary chlorophyll content meter metre (CCM) units] and (c) leaf epinasty (defined as the angle that leaves formed with the stems). The arrow indicates the date when liquid fertilizer was added to the waterlogged plants. Means with confidence intervals at 0.95. See text for details.

Responses after 7 weeks of waterlogging

Adventitious root formation and hypertrophied lenticels

At the end of the experiment, that is, after 7 weeks of waterlogging, we could not detect any correlation between parameters describing lenticel development and adventitious root formation: the two groups of traits were completely independent. The largest fraction of hypertrophied lenticels (69%) was located below the water table. The diameter and thickness of hypertrophied lenticels were significantly larger below than above water (Fig. 3). At the end of the treatment, there was a significant correlation between the number of visible and the total number of hypertrophied lenticels ($R^2 = 0.34$, $P < 0.001$). Nevertheless, only 15% of those under water had been visible during the course of waterlogging.

Anatomical observations

No lacuna could be detected in the parenchyma beneath lenticels or the equivalent zone on controls of the 30

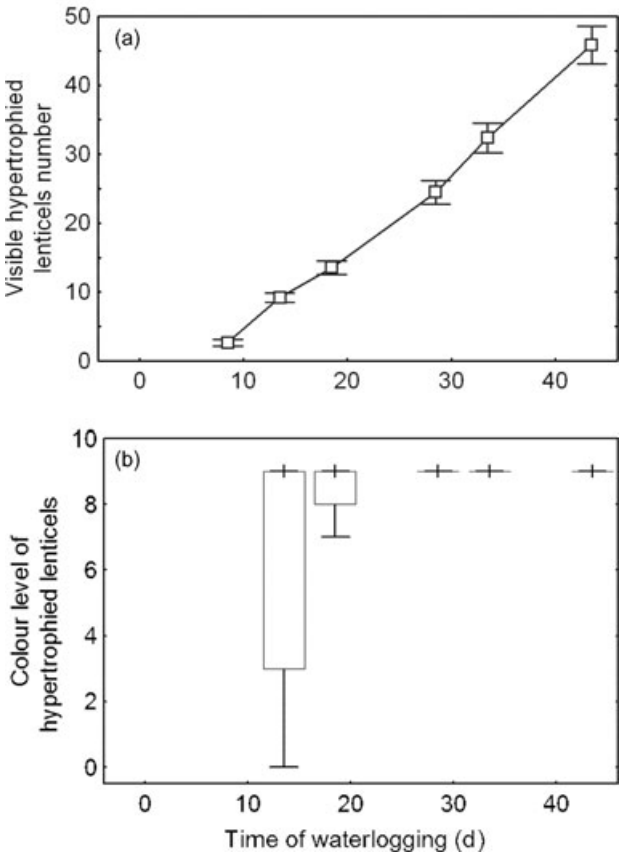

Figure 2. Time course of the number of visible hypertrophied lenticels during waterlogging. (a) Total number (means and confidence intervals). (b) Development stage estimated with the ordinal scale of colours (see Table 1 for details), medians+, quartiles (75% of the individuals) boxes, non-outlier range whiskers (95% of the individuals).

observed individuals (Fig. 4), regardless of development stage of hypertrophied lenticels or adventitious roots. On the contrary, support tissue was detected in the cortical parenchyma and formed discontinuous concentric circles

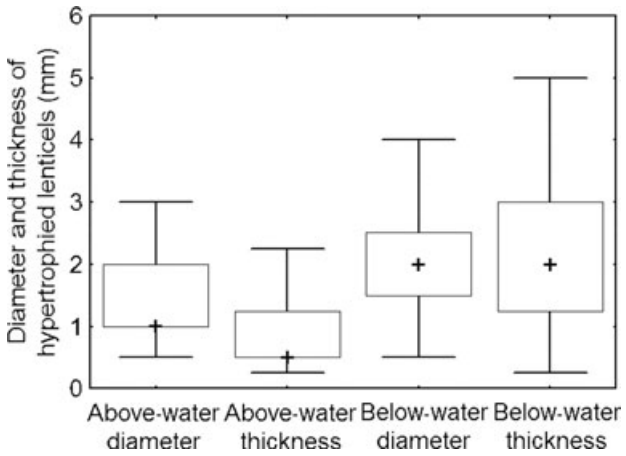

Figure 3. Diameter and thickness of hypertrophied lenticels located on stems and roots above or below the water level in individuals of *Quercus robur* submitted to 7 weeks waterlogging. Visual estimation, medians+, quartiles (75% of the individuals) boxes, non-outlier range whiskers (95% of the individuals).

around the stele in stressed as well as in control individuals (Fig. 4).

Detected QTL: distribution, number and PEV

QTL for responses to waterlogging were detected on all LGs of the female and male maps, except on LG 6 male (LG6 M) and LG 8 female (LG8F) (Supplementary Information S1 and S2). More QTL were detected with the multi-environment than with the classical single-environment method. Indeed the former allowed to detect QTL with lower PEV (< 9%) than the latter (Supplementary Information 1).

QTL were detected for traits unambiguously induced by waterlogging, many of them being located on the male map (Supplementary Information S2). There were five QTL for

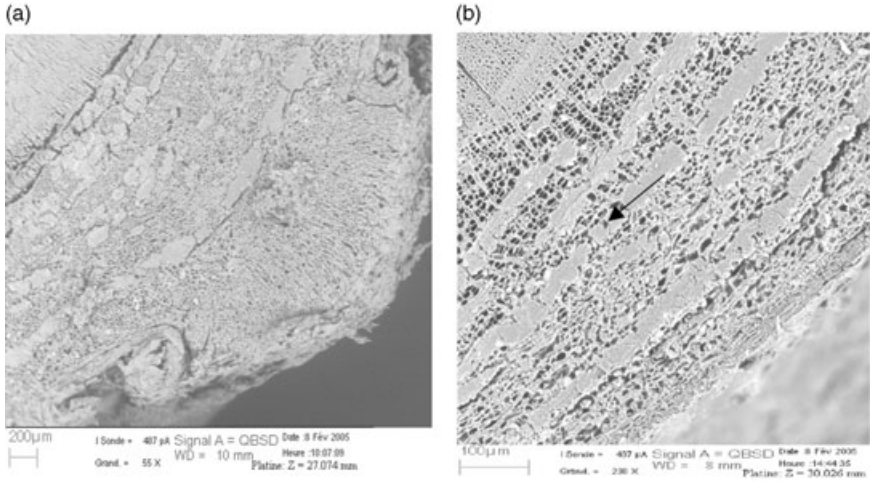

Figure 4. Anatomy of hypertrophied lenticels and beneath bark tissue observed in *Quercus robur* roots after 7 weeks of waterlogging (variable pressure-scanning electron microscopy). Support tissue was present in the bark as indicated by an arrow in the figures. (a) Bark tissue beneath a hypertrophied lenticel. (b) Bark of a control plant.

epinasty distributed on four different LGs of the male map (3, 8, 9, 11) with maximum PEV (mPEV) ranging from 7.8 to 12.2% depending on the date (Supplementary Information S1). A QTL was found on LG9M for dissolved oxygen content in the water table with an mPEV of 12.2%, and two QTL for hypertrophied lenticels were detected on LG2M (total number) and LG3M (rate of development) with PEV of 14.8 and 10.4%, respectively. Surprisingly, no QTL was detected on either map for adventitious roots, whether mass, number, total length or mean length were taken into account. This occurred despite a significant clone effect for adventitious root mass and number explaining 6.6% of the total variance (data not shown).

Significant QTL were also visible for biomass accumulation on the male map, and for the shoot/root ratio on both male and female maps, with PEV of 15.4 and 12.1, respectively. A QTL for leaf nitrogen content was detected on both maps (PEV of 12 and 16.7%). The traits analysed with the multi-environment procedure produced a large number of QTL. For chlorophyll content, 23 QTL were distributed over all LGs on the male and female maps, except LG 8 (Supplementary Information S2); mPEV (mPEV: maximum of all dates) was between 3.5 and 16.3%. For diameter at collar, 18 QTL were distributed over all LGs except LGs 4 and 8 (Supplementary Information S1 and S2) and displayed mPEV between 3.7 and 20% (see Supplementary Information S1 and S2 for further details on detected QTL).

Important genomic regions involved in control of waterlogging responses

We focused on genomic regions for which at least one QTL was detected for a trait specific to hypoxia response (such as oxygen content, epinasty and lenticel development) with a PEV above 9% (Fig. 5). When several additional QTL presented overlapping confidence intervals and when their localizations were within one adjacent interval of those QTL, they were considered genetically close. Five such genomic regions were identified on the male map, and none on the female map:

Genomic region 1. On LG2M (at 78.1 cM, Fig. 5), a QTL for the total number of hypertrophied lenticels had a PEV of 14.8%, and QTL for main root dry mass and diameter at collar were close.

Genomic region 2. On LG3M (at 0 cM, Fig. 5), a QTL for the rate of development of hypertrophied lenticels (PEV = 10.4%) was close to a QTL for epinasty (mPEV = 7.8%), one for leaf chlorophyll content, and four QTL for growth parameters.

Genomic region 3. On LG9M (at 8.8 cM, Fig. 5), a QTL for dissolved oxygen with an mPEV of 9.3% was close to a QTL for diameter at collar and one for leaf chlorophyll content.

Genomic region 4. On LG9M (at 54.8 cM, Fig. 5), a QTL for epinasty (mPEV = 9.9%) was close to a QTL for leaf chlorophyll content.

Genomic region 5. On LG11M (at 57.9 cM, Fig. 5), a QTL for epinasty (mPEV = 11.6%) was close to a QTL for leaf chlorophyll and nitrogen contents, LMA and diameter at collar.

Temporal patterns of QTL effects

The multi-environment QTL analysis provided a variety of temporal patterns for the allelic substitution effect of QTL for epinasty, chlorophyll content and diameter at collar. The number of lenticels monitored during the course of the experiment yielded no QTL. Substitution effects followed very different temporal time courses. For example, the effect patterns of cluster 2 for leaf chlorophyll content were a linear increase during the first 20 d of treatment followed by a constant value while those of cluster 4 linearly increased, or decreased over the whole period (Fig. 6b). Some of these effect patterns were tightly intercorrelated, both on an intra-trait as well as on an inter-trait level (Figs 6 & 7). We found one cluster for epinasty, four for diameter at collar and four for chlorophyll content, each containing two to eight QTL (Fig. 6). Only 10 out of the 45 effect patterns analysed could not be classified (i.e. were not significantly correlated to other patterns). Some of the clustered effect patterns were negatively correlated. For example, in cluster 4 of leaf chlorophyll content (Fig. 6b), the first five QTL followed positively, and the last three negatively correlated temporal patterns. During the inter-trait analysis, six mixed-trait clusters were detected, three (5, 9, and 11; Fig. 7) contained effect patterns of QTL for epinasty, which is highly specific for hypoxia response.

Some QTL displayed similar locations on the male and female maps: a QTL for diameter at collar on LG7, and a QTL for leaf chlorophyll content on LG10 (Supplementary Information S2). Intra-trait clustering revealed that in the two cases, the QTL clustered with similar effect patterns (positive correlation for the first, and negative for the second). Inter-trait clustering showed that a QTL for leaf chlorophyll content and a QTL for epinasty on LG11M clustered with similar effect patterns and were very close on the genetic map (Figs 5 & 7b).

DISCUSSION

Physiological relevance of waterlogging response QTL in *Q. robur*

Five genomic regions containing each at least one QTL for a trait specific to hypoxia responses were detected. We focused on QTL with a high PEV, keeping in mind that the small number (120) of genotypes from the F_1 progeny used here probably induced a threefold overestimation of PEV (Beavis *et al*. 1994; Brown *et al*. 2003).

The use of artificial dis-oxygenation of water resulted in severe hypoxia. Nevertheless, we detected an apparent

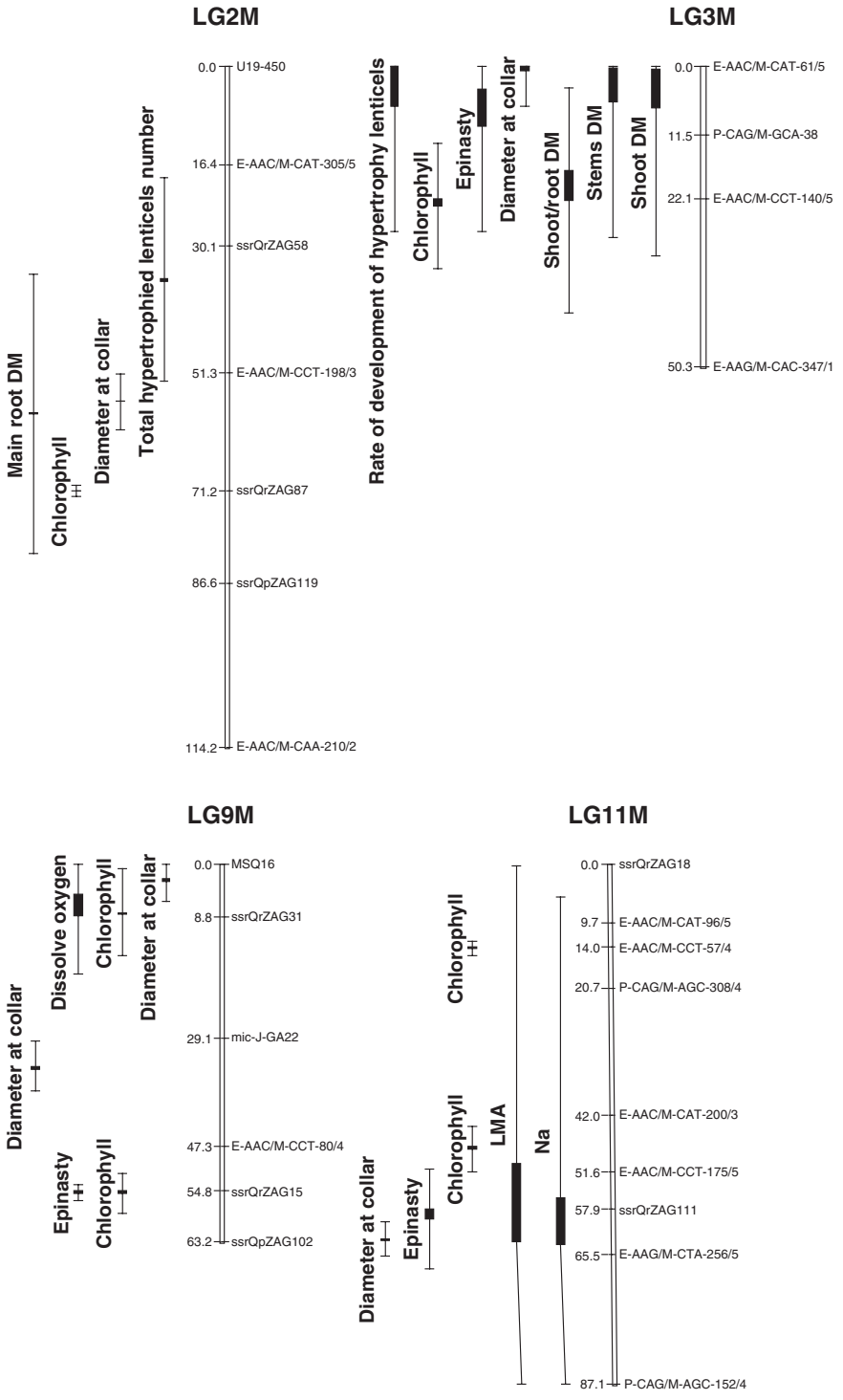

Figure 5. Genetic map of markers in centimorgan and quantitative trait loci (QTL) position among linkage groups (LGs) on which several QTL were identified at a short distance. Estimation of the position by permutation and bootstrap, and confidence intervals at 0.95. LMA, leaf mass-to-area ratio; DM, dry mass.

genotypic variability of oxygen content among pots, which led to a QTL (genomic region 3). This QTL can only be explained by the occurrence of a diversity of rates of O_2 consumption by roots of the different individuals, which is an indirect index for root activity under hypoxia. As a matter of fact, no such apparent genotypic effect was detected for dissolved oxygen measured in the free water around the pots. Whereas the variability of O_2 content in pots may have induced a slight variation of stress among genotypes, no correlation was detected between the presence of adaptive structures (i.e. hypertrophied lenticels and adventitious roots) and oxygen content. It may therefore safely be assumed that the differences in O_2 content were too small to induce a significant artefacts for QTL detection.

During hypoxia, adaptive structures such as hypertrophied lenticels and adventitious roots developed at the base of the main stem, the collar and the upper roots, as

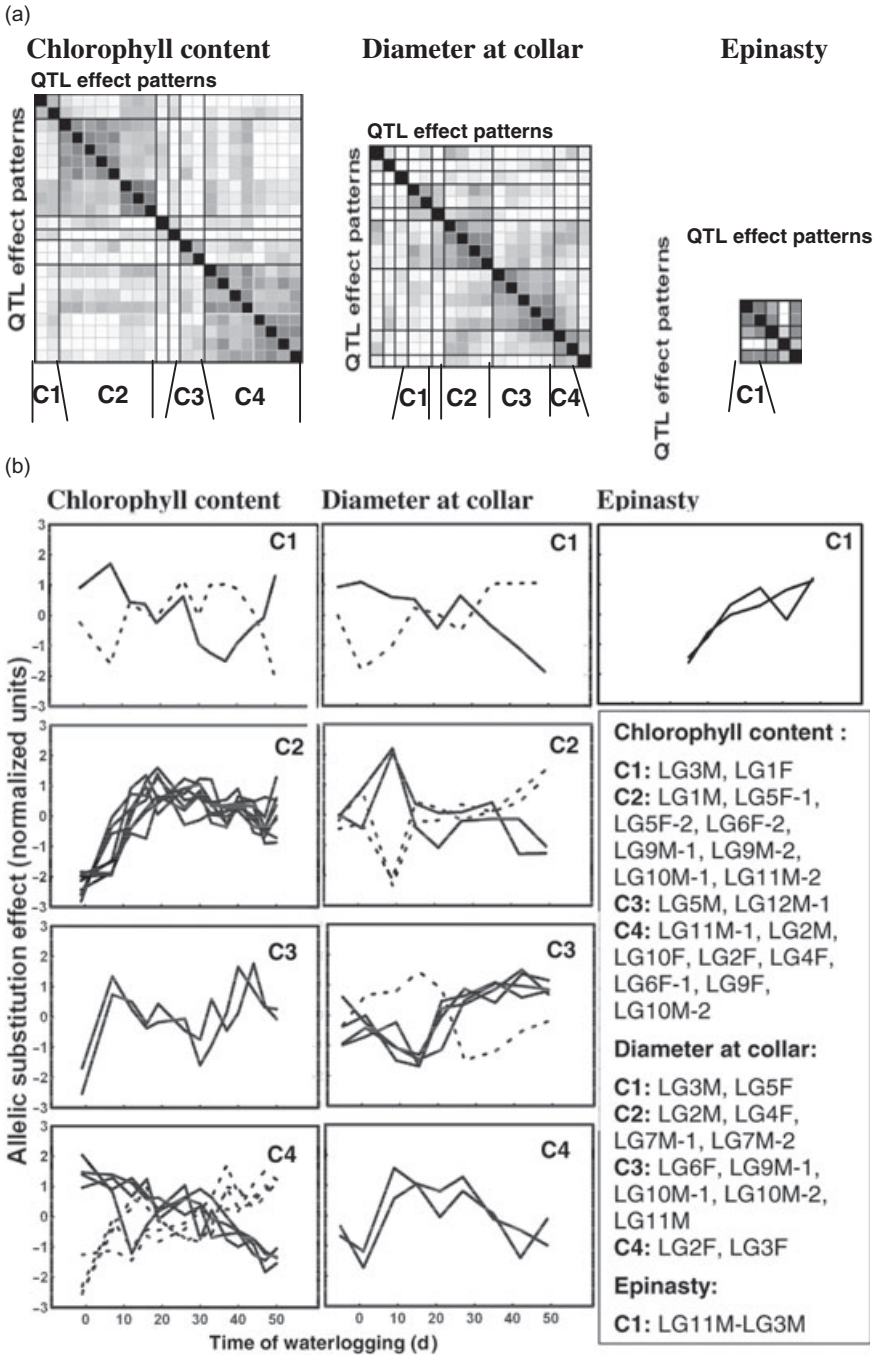

Figure 6. Intra-trait clusters of effect patterns. (a) Matrices of correlation among time patterns of quantitative trait loci (QTL) effects, and clusters C1 to C4 evidenced for leaf chlorophyll content, diameter at collar and epinasty (effects normalized to a mean of 0 and an SD of 1 as a function of duration of waterlogging treatment). Colour intensity indicates square Pearson's correlation coefficient between the effect patterns of two QTL. Effect patterns were sorted by similarity using a hierarchical linkage clustering algorithm with the absolute Pearson's correlation coefficient as an entry. Black lines indicate clusters of QTL with correlated effect pattern. (b) Clusters of effect patterns evidenced for leaf chlorophyll content, diameter at collar diameter and epinasty. QTL are abbreviate from their linkage group (LG) while M or F refer to the male and female map, respectively. When two linked QTL were detected on the same LG they were noted –1 for the QTL close to LG start, and –2 for the second. Positively correlated QTL were symbolized with a similar line, and negatively correlated ones with different line (full or dotted lines).

previously observed on *Q. robur* and *Q. petraea* (Colin-Belgrand *et al*. 1991; Parelle *et al*. 2006), on *Quercus macrocarpa* (Tang & Kozlowski 1982b), and on many other trees (e.g. Newsome, Kozlowski & Tang 1982; Tang & Kozlowski 1982a, 1984; Nunez-Elisea *et al*. 1999). The total lack of correlation between traits characterizing hypertrophied lenticels and adventitious roots was unexpected. In fact, hypertrophied lenticels and adventitious roots are thought to build a highly porous zone conducting oxygen through aerenchyma to the root system of herbaceous (Jackson & Drew 1984) or tropical woody plants (Joly 1991). During anatomical observations, no root primordia were detected beneath lenticels, supporting the hypothesis that adventitious roots do not develop beneath lenticels. These two traits are probably not induced directly by the same signal. In addition, we did not detect any lacuna in the bark beneath the hypertrophied lenticels, confirming observations on different *Annona* species (Nunez-Elisea *et al*. 1999), and on *Alnus glutinosa*, a highly waterlogging tolerant tree of temperate Europe (Armstrong & Armstrong 2005). Lacuna had been observed on other tree species (*Ulmus americana*, *Pinus halepensis*) adapted to waterlogging (Angeles, Evert & Kozlowski 1986; Yamamoto, Kozlowski & Wolter 1987). On the contrary, we observed

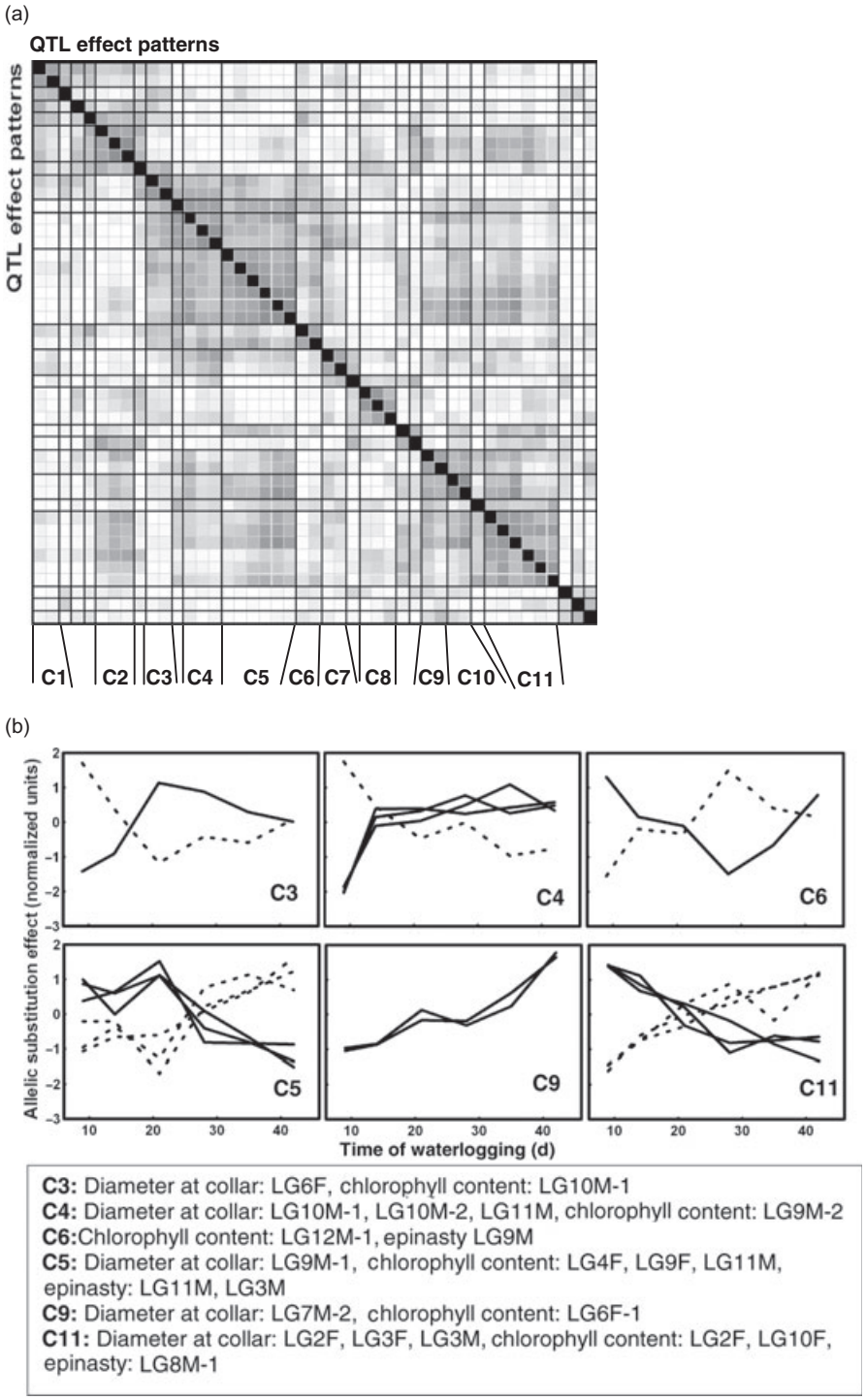

Figure 7. Inter-trait clusters of effect patterns. (a) Matrix of correlation among time patterns of effects of quantitative trait loci (QTL) of leaf chlorophyll content, collar diameter and epinasty (effects normalized to a mean of 0 and an SD of 1 as a function of duration of waterlogging treatment). Colour intensity indicates square Pearson's correlation between the effect patterns of two QTL. Effect patterns were sorted by similarity using a hierarchical linkage clustering algorithm with the absolute Pearson's correlation effect pattern. (b) Clusters of effect patterns grouping different traits. QTL are abbreviate from their linkage group (LG) while M or F refer to the male and female map, respectively. When two linked QTL were detected on the same LG they were noted –1 for the QTL close to LG start, and –2 for the second. Positively correlated QTL were symbolized with a similar line, and negatively correlated ones with different line (full or dotted lines).

support tissues, which could act as barrier to gas diffusion. All these observations emphasize that oxygen diffusion from lenticels to roots is not very likely to occur in *Q. robur* under hypoxia. The largest fraction of hypertrophied lenticels was located below the water level, as previously observed on oaks (Tang & Kozlowski 1982b; Parelle *et al.* 2006) and other tree species (Topa & McLeod 1986b, 1988), and the lenticels formed above the water level were smaller and less developed. In consequence, it may be safely concluded that hypertrophied lenticels are formed mainly in, or close to, a hypoxia zone (below the water level). No clear hypothesis could be formulated about the function of these hypertrophied lenticels. These observations underline that the QTL detected for hypertrophied lenticels (genomic regions 1 and 2) are highly specific to hypoxia stress. However, the detection of QTL for growth parameters in

close vicinity to the former on the genome, suggests either that growth was differentially affected by hypoxia among clones, and that the corresponding QTL reflect different levels of tolerance, or conversely, that these QTL reflect clonal and hypoxia-independent differences of vigour among clones. Indeed, the number of lenticels in particular may depend on stem and root dimensions. There is currently no possibility to demonstrate which of these conflicting hypotheses is true. There is no information available in the literature on the variability of hypertrophied lenticels density.

Whereas the function of hypertrophied lenticels is still a matter of debate, the role of adventitious roots in hypoxia tolerance is well understood: they grow close to the top of the substrate where oxygen availability is largest and allow plants to sustain water and nutrient absorption, while deep roots die (Topa & McLeod 1986a; Vartapetian & Jackson 1997; Drew, He & Morgan 2000). Despite the occurrence of clonal effects for the number and biomass of adventitious roots formed on the individuals, no QTL could be detected for adventitious root formation, whatever parameter (number, length or biomass) was taken into account. The clonal variance was low and an environmental background noise may have hindered the detection of a QTL.

Epinasty, the increase of the angle that the petiole forms with the stem, is a highly specific trait for oxygen deprivation and waterlogging (Vartapetian & Jackson 1997), and has never been observed in other stress situations. The QTL detected for this trait (regions 2, 4 and 5) should be highly specific for root hypoxia. Interestingly, the genomic region 2 contained a QTL with a high PEV for the development rate of hypertrophied lenticels as well as for epinasty, suggesting that this region should be of particular interest for future investigations.

The continuous increase of diameter at collar throughout the 7 weeks of waterlogging demonstrated the capacity of *Q. robur* to sustain growth even under severe root hypoxia. Hypertrophy of collar, resulting also in an increase in diameter, has been reported to be a trait related to hypoxia response (Yamamoto & Kozlowski 1987; Yamamoto *et al*. 1987). However, most of the detected QTL for diameter growth are probably related to growth control under unstressed conditions such as those detected for oaks (Scotti-Saintagne *et al*. 2004) and chestnut (Casasoli *et al*. 2004). The observed increase of chlorophyll content during the first 2 weeks of the stress could be due to the phenological development of leaves. The slight decrease observed after 18 and 28 d, followed by an increase after fertilization, could be linked to nitrogen availability, which is severely lowered in hypoxic soils due to denitrification (Zhang & Wienhold 2002; Venterink, Hummelink & Van den Hoorn 2003). Thus, fertilization probably counteracted this phenomenon, allowing the chlorophyll content to increase. Leaf chlorophyll content is highly linked to leaf nitrogen content (e.g. Niinemets & Kull 1997), and thus depending on nitrogen availability under root hypoxia (Kreuzwieser, Furniss & Rennenberg 2002). In consequence, some of the QTL detected might represent the capacity of plants to maintain their nitrogen uptake despite hypoxic root conditions.

The physiological relevance of most of the QTL for waterlogging response, in particular for collar diameter and leaf chlorophyll content, was difficult to interpret. In order to classify the QTL and obtain further insight in their relevance, we used a clustering method based on multi-environment analysis.

Multi-environment analysis and clustering of effect patterns

Traits analysed using the multi-environment model produced a large number of QTL, especially QTL with small effects, as compared to the single environment analysis. This conformed to the theory described by Jansen *et al*. (1995) and Korol, Ronin & Nevo (1998), which predicts an increase of detected QTL with low effects, compared to the classical single-environment MIM. Additionally, a large percentage of the QTL detected using the multi-environment approach were precisely located on the map (small confidence intervals).

The estimation of an allelic effect for each environment (in this case the sampling dates) allowed us to estimate the temporal variation for each individual QTL (called 'effect pattern'). The clustering of the effect patterns produced a few groups with a striking similarity, at the intra- as well as on the inter-trait level.

Some of these QTL with similar effect patterns were QTL for the same trait located in corresponding regions on the male and female maps. This supports the hypothesis that the same QTL were detected on the male and the female map. However, the possibility that different genes display similar effect patterns in the same genomic region cannot be excluded.

In one cluster, two QTL were located at close vicinity on the map. Nevertheless, in the majority of the cases QTL from a given cluster were distributed over the whole map. If it is assumed that each QTL is related to a single gene, then the clustering of effect patterns would suggest a parallel change in the effect of an allele of these genes within the family. Whether this parallelism can be explained by a coordinated response of a gene network to the hypoxic stress signalling, needs to be confirmed by further experiments. In this respect, the correlation coefficients among effect patterns would be an interesting tool for identifying such gene networks.

Furthermore, the inter-trait clustering highlighted two co-localized QTL with similar effect patterns, which supports the hypothesis that the same locus was detected by the QTL of the two traits. Three clusters (C5, C9 and C11) contained QTL for leaf chlorophyll content, diameter at collar (two traits not specific of waterlogging response) and epinasty, which is specific of waterlogging response. This could suggest that the QTL for leaf chlorophyll content and diameter at collar within this cluster are also involved in response of *Q. robur* to the hypoxia treatment.

CONCLUSION

Five main genomic regions containing QTL specific to hypoxia response (epinasty, hypertrophied lenticels development and dissolved oxygen content) were identified, suggesting a genetic determinism for the diversity of waterlogging responses within this pedunculate oak family. Additionally, the multi-environment model and the clustering analyses of time courses of allelic QTL effects suggested further QTL potentially involved in the waterlogging response of *Q. robur*. In consequence, the strategy developed here is relevant for the identification of loci involved in the waterlogging response of *Q. robur*, despite the fact that the mechanisms of tolerance to waterlogging are still poorly known in forest tree species. The loci obtained on the genetic map are a powerful resource for the future detection of candidate genes.

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SUPPLEMENTARY MATERIAL

The following supplementary materials are available for this article:

Supplementary Information S1. List of the QTL detected for the different traits of response to waterlogging in a *Quercus robur* full-sib family.

Supplementary Information S2. Position of QTL on linkage groups (LGs) of the genetic map of the male and female *Quercus robur* parents. QTL position was estimated by permutation and bootstrap, and confidence intervals at 0.95. Dotted lines between LG of male and female map indicate common markers that allow to compare the relative position of the QTL between the two maps.

These materials are available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-3040.2006.01629x
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