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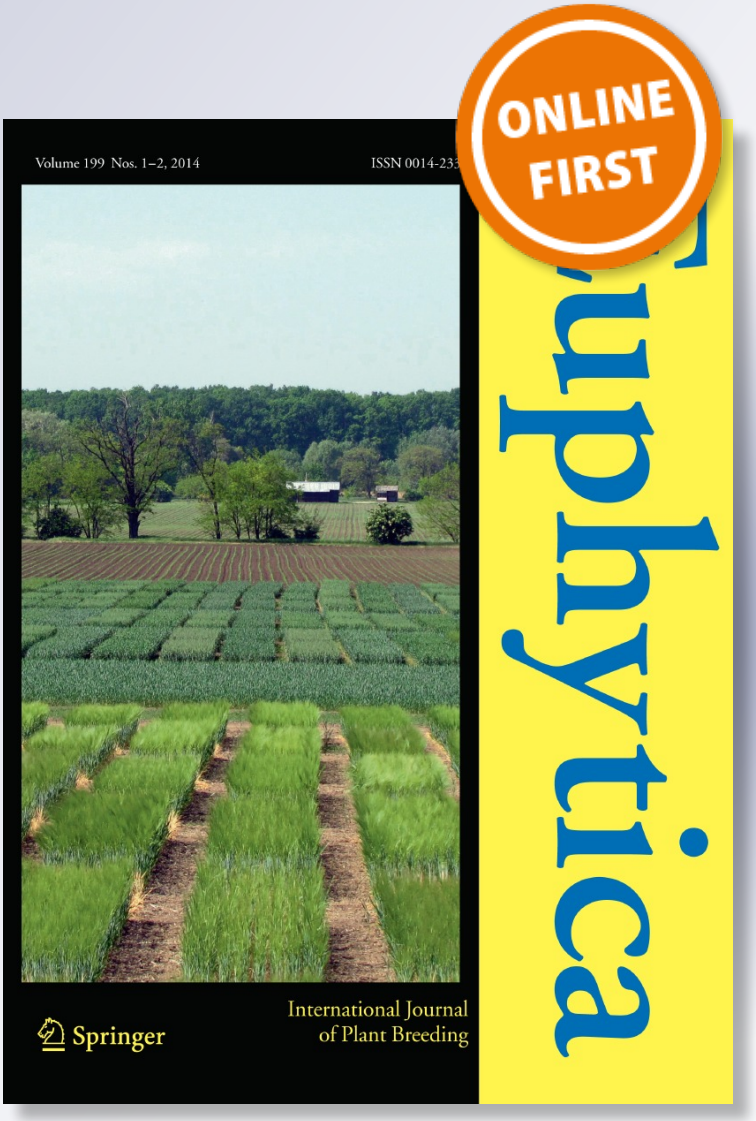

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QTL for chlorophyll fluorescence of barley plants grown at low oxygen concentration in hydroponics to simulate waterlogging

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Abstract Waterlogging is a major factor limiting barley grain yield worldwide. Climate change will likely increase this water stress in Northern Europe. Breeding for waterlogging tolerance (WLT), as for other abiotic stresses, is difficult, but identification of genetic markers linked to genes affecting WLT could facilitate the breeding process. To identify a suitable marker population, parents of 14 double-haploid (DH) barley populations were tested for segregation of biomass growth reduction in waterlogged soil. The most interesting was found in the offspring from crossing cv. Psaknon and breeding line (SLUdt1398 × Mona[4]). Hence, 120 DH-lines derived from this cross were phenotyped for the chlorophyll fluorescence parameter quantum yield (QY) of electron transport of PSII from leaves of hypoxia-stressed plants and further genotyped with 384-SNP Illumina GoldenGate Bead Array. Five quantitative trait loci (QTL) for QY, with a narrow sense heritability of 0.87, were identified on chromosomes 4, 6 and 7H. They had additive effects ranging from 0.74 to 1.35 % with LOD scores from 3 to 12 and explained variance from 6 to 29 %. The major alleles for high QY were from cv. Psaknon; i.e., QY was low if the alleles from cv. Psaknon were not present. Based on leaf necrosis and residual biomass data, the four most interesting QTL may be also in two other populations with completely different progeny, which shows a certain stability of these QTL. The possibility of using marker assistant selection for WLT is discussed, as is possible concurrent improvement of drought tolerance and grain yield.

Keywords Chlorophyll fluorescence · *Hordeum vulgare* · Hypoxia · Quantitative trait loci (QTL) · Waterlogging

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Introduction

Barley (*Hordeum vulgare* L.) is very susceptible to waterlogging (Setter and Waters 2003). This limiting factor affects barley production worldwide and, with expected climate change, grain yield loss will likely increase. The problem in Northern Europe is not yet as acute as in Asia and many other parts of the world, but is expected to increase. Differences in waterlogging tolerance (WLT) are among the factors determining grain yield. Breeding for grain yield in barley has therefore led indirectly, to improved WLT in the last

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50 years (Bertholdsson 2013). However, direct screening should probably be more efficient.

Similarly to other abiotic stresses, WLT is a complex trait and lack of useful efficient selection methods is an obstacle in breeding programmes. The methods currently used are indices based on leaf chlorosis (Li et al. 2008), germination ability (Takeda and Fukuyama 1987), biomass growth reduction (Zhou et al. 2012) and yield components (Xue et al. 2010; Walker et al. 2013). Most of these traits are difficult to use in breeding, especially in field selection with high genotype-by-environment interaction. Selection at genetic level is now possible with the identification of quantitative trait loci (QTL) associated with the stress, but genotype-by-environment interactions are still a problem, since QTL analysis depends on phenotypic characterisation, which in most cases is influenced by the environment (Wójcik-Jagla et al. 2013). The efficiency of QTL is therefore very much dependent on accurate phenotyping, but also on the trait used for screening and its heritability.

To date, many QTL have been identified for different abiotic stresses and crops, such as drought in wheat and rice (Ahmed et al. 2013) and maize (Qiu et al. 2007). With only seven chromosomes, barley has been widely used in different QTL analyses (Ahmed et al. 2013; Close et al. 2009) and also for WLT (Li et al. 2013). Li et al. (2008) identified several QTL linked to leaf chlorosis, plant survival and plant residual biomass, while Zhou et al. (2012) found four QTLs linked to an index calculated from scores of leaf chlorosis and plant survival of waterlogged plants in outdoor tanks filled with soil. The latter method was used by Zhou (2011) to obtain more accurate phenotyping. Bertholdsson (2013) developed a hydroponic method with cultivation of plantlets in O_2-depleted nutrient solution, which allows many plants to be tested in a limited space under fully controlled growth conditions. In his study, the growth reduction under hypoxia stress was correlated with results obtained in soil studies, and also with the chlorophyll fluorescence parameter quantum yield (QY) of electron transport for photosystem (PS) II (Genty et al. 1989). The hydroponic system may be totally artificial, but reflects what actually happens in the first days when the soil is waterlogged and a situation of hypoxia occurs (Agarwal and Grover 2006).

Under hypoxia, the growth processes are slowed down because of low energy supply. Tolerant plants seem to be able to maintain or conserve energy, but also escape mechanisms are common (Hattori et al. 2009; van Veen et al. 2013). Chlorophyll fluorescence is widely accepted as an indicator of the activity of PSII, which is involved in electron transport in plant photosynthesis and is particularly sensitive to stresses (Fracheboud et al. 2004; Guo et al. 2008; Gu et al. 2012). Various fluorescence parameters have been found to be closely correlated with carbon assimilation under different stresses in rice (Guo et al. 2008), lucerne (Smethurst and Shabala 2003), soybean (Yin et al. 2010), wheat (Zhang et al. 2010) and maize (Fracheboud et al. 2004), as well as grain yield in barley (Bertholdsson 2013) and, under optimal conditions intriticale (Hura et al. 2009). Fluorescence parameters has also been used for mapping QTL in relation to drought stress in wheat (Czyczyło-Mysza et al. 2011), rice (Gu et al. 2012) and barley (Guo et al. 2008; Wójcik-Jagla et al. 2013), salt tolerance in barley (Aminfar et al. 2011) and biomass and yield in wheat (Czyczylo-Mysza et al. 2013). However, to our knowledge there have been no previous efforts to associate chlorophyll fluorescence with WLT, besides research by Pang et al. (2004) and Bertholdsson (2013), who used it to characterise WLT of barley cultivars.

Residual biomass was strongly correlated with QY in the study by Bertholdsson (2013), suggesting a direct relationship QY could thus be used instead of the more labour-demanding residual biomass. The hydroponic cultivation method in combination with leaf fluorescence measurements was therefore used in the present study for phenotyping a double-haploid (DH) population where the parents segregated both for waterlogging in soil and QY. The DH population was further genotyped by SNP markers and the fluorescence data were analysed for QTLs and compared with QTL results from other populations phenotyped for leaf necroses, plant death and residual biomass.

Materials and methods

Plant materials and phenotyping

A barley population selected from 14 available DH populations consisting of 120 DH lines derived from a cross between cv. Psaknon (mother) and a line derived from the cross between the *Hordeum spontaneum*

accession SLUdt1398 and cv. Mona with four backcrosses to Mona (SLUdt1398 × Mona^4) (father) was used in this study. The parents of this population showed the most contrasting results concerning water logging tolerance (WLT).WLT in pot grown material was 98.1 % of Psaknon and 71.1 of (SLUdt1398 × Mona^4). WLT was measured as the decrease in seedling biomass after 18 days of waterlogging compared with plants grown without the stress. The plants were 12 days old at start of waterlogging. For further details of waterlogging trials in pots see Bertholdsson (2013). The hydroponic system and fluorescence measurement method described by (Bertholdsson and Kolodinska-Brantestam 2009) and Bertholdsson (2013) were used for phenotyping DH lines for QY. An important detail not found in Bertholdsson (2013) is that during waterlogging treatments in hydroponics the water level was raised above the seeds. In each hydroponic container (55 × 35 × 18 cm), 11 DH lines and a standard barley cultivar (cv. Henni) were grown in four replicates, with eight plants per replicate. The inner six plants were used for the QY measurements; i.e., a total of 24 plants per DH line. The plants in the first strip at each end of a frame and the first plant in each end of the strip were border plants. Mean values were calculated using the GLM procedure of the Statgraphics Plus software (Manugistics, Inc, Rockville, MD, USA). Unusual residuals with studentised residuals greater than three were discarded. All lines were measured a second time, but in a different combination than before. If the internal standard deviated from its normal value of 0.44, a third replicate was used. The mean value was used as phenotypic data for the DH lines for the QTL analysis. Narrow sense heritability (h^2) was calculated from variance components of the two separate QY determinations according to (Cochran 1957).

Genotyping and QTL analysis

Genomic DNA was isolated from 10-day-old seedlings using the DNeasy Plant Mini kit (QIAGEN, USA) according to the manufacturer's instructions. Leaf segments of 100 mg were collected from the first leaf of seedlings grown in agar. The samples, two per DH line, were frozen in liquid nitrogen and kept at −80 °C until the time of extraction. The quality and quantity of the DNA isolated were analysed using spectrophotometric measurements and gel electrophoresis. One of the replicate samples was then sent to the James Hutton Institute, Scotland, for gene mapping analysis and the other was kept as back-up.

Since we planned to use the Illumina GoldenGate Veracode Bead Assay technology (Illumina Inc, http://www.illumina.com), the Barley 384-plex Oligo Pool Assay (OPA) (Close et al. 2009; Moragues et al. 2010) was used for testing for segregation among the parents. This OPA is a subset of 384 SNPs selected from 3,072 SNPs representing sites with polymorphism at positions located in coding gene sequences more or less equally distributed along the seven chromosomes of barley (Close et al. 2009). It was found that 196 of the 384 SNPs (50.9 %) were polymorphic between the parental genotypes, and hence the OPA was used for SNP genotyping. A linkage map was constructed and QTL were identified for QY using QTL IciMapping Ver 33 (Wang et al. 2012). During the linkage mapping, the 196 SNP were anchored and grouped according to the integrated map from several populations. This resulted in 18, 28, 28, 28, 43, 31 and 20 markers being grouped to the seven barley chromosomes, respectively The markers were ordered by the nnTwoOpt algorithm. The map was further rippled with the SARF criterion and a window size of 5. For QTL identification, ICM-ADD mapping (inclusive composite interval mapping for additive QTL) was used and a significant threshold of likelihood of odds (LOD) of 2.83 was found by running 1,000 permutations with a Type 1 error of 0.05.

Results

Phenotypic and genotypic variation in the DH lines

The frequency distribution of QY of hypoxia-stressed lines in hydroponic culture did not fit a normal distribution (Chi Square = 34.934, $P = 0.000254$), but slightly skewed to sensitivity (skewness = −0.75) and with two peaks making the distribution more flat (kurtosis = −1.132) (Fig. 1). The population mean was 0.416, with a standard deviation of 0.025. This result suggests that several loci are involved, but the two-peak distribution indicates the presence of loci with major effects. The estimated narrow-sense heritability was 0.87, and the R^2 of the QTL model was 0.48. The QY of the parent cv. Psaknon was 0.440 ± 0.0082 and of SLUdt1398 × Mona^4 0.378 ± 0.0082 ($P < 0.0001$, $N = 24$). Several DH

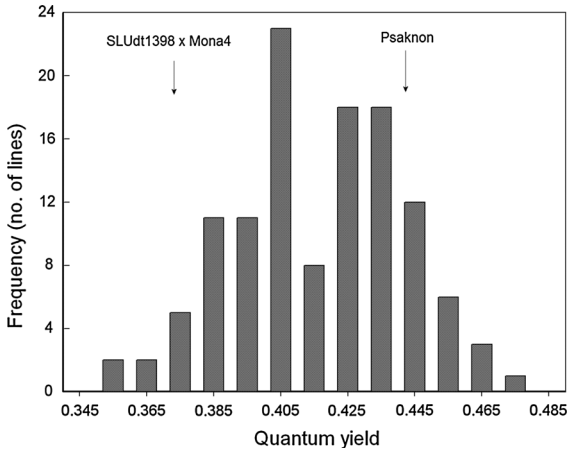

Fig. 1 Frequency distribution of quantum yield (QY) under hypoxia conditions of 120 double-haploid barley lines from the mapping population, cv. Psaknon × (SLUdt1398 × Mona^4). Arrows show the QY of the tolerant mother (cv. Psaknon) (*right*) and the sensitive father (SLUdt1398 × Mona^4) (*left*) SEM of QY = 0.013. Chi Square = 34.934, $P = 0.000254$

lines showed transgressive segregation for QY than the tolerant parent, but fewer were more sensitive than the sensitive parent SLUdt1398 × Mona^4.

QTL controlling quantum yield

Five significant QTLs were detected (Fig. 2), all of them originated from cv. Psaknon. QTL-QY1 on 6HL, with a peak close to the SNP marker 11_10175 had a LOD score of 12 and accounted for 29 % of the variation (Table 1). It was followed by QTL-QY2 close to the SNP marker 11_11014 on 7HS. It accounted for 20 % of total QY variation. The two QTL had an additive effect of 0.0137 and 0.0113, respectively. QTL-QY3 and QTL-QY4, on 6HL and 4HL, respectively, had minor negative additive effects. The QTL-QY5, located on 6HS, had also a minor positive additive effect.

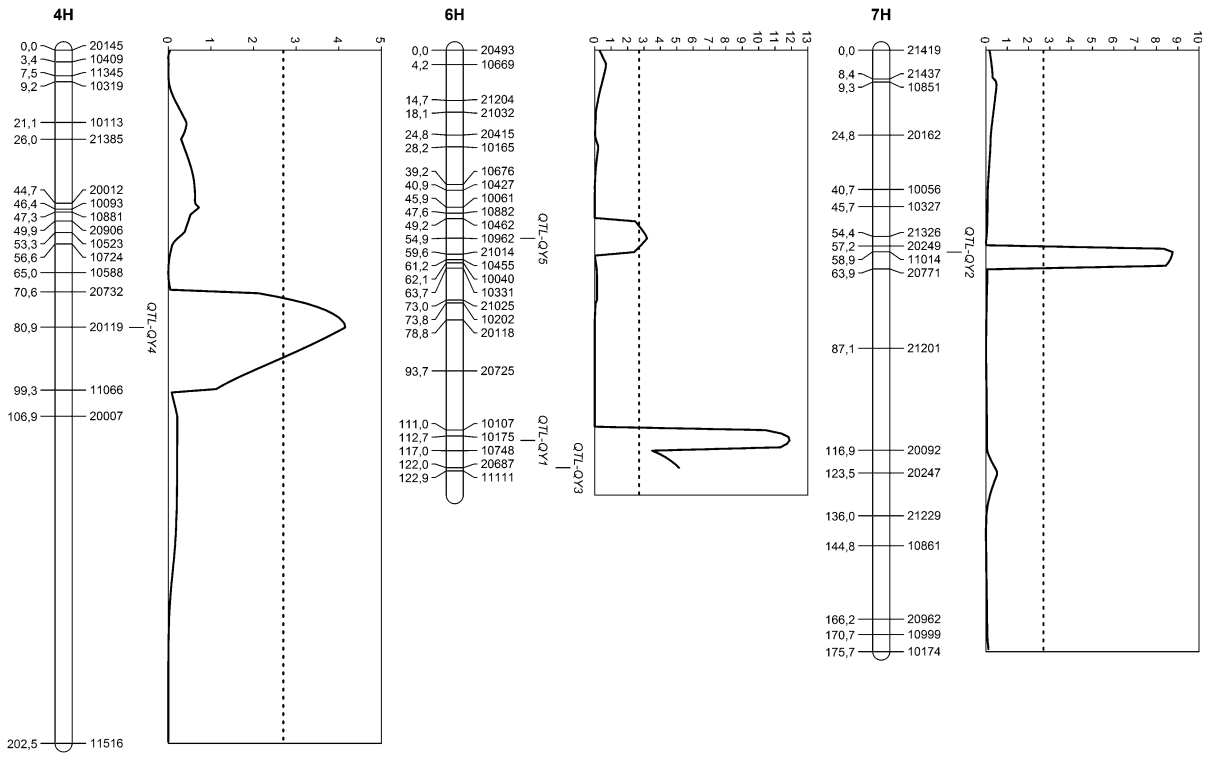

Fig. 2 LOD score profile across the linkage maps of the double-haploid population cv. Psaknon (tolerant) × (SLUdt1398 × Mona^4) (sensitive) by 196 SNP markers. LOD scores > 2.83 are significant QTL of quantum yield of hypoxia-stressed plants

Table 1 Characteristics of the quantitative trait loci (QTL) detected explaining quantum yield (QY) related to waterlogging in the tolerant cv. Psaknon × sensitive (SLUdt1398 × Mona[4]) population

QTL	Chr	Peak position (cM)	Left marker[a]	Position (cM)	Right marker[a]	Position (cM)	LOD[b] score	PVE[c] (%)	Add effect
QTL-QY1	6HL	114	10175	112.7 (119.0)	10748	117.0 (123.8)	11.89	29.09	0.0137
QTL-QY2	7HS	59	11014	58.9 (60.7)	20771	63.9 (87.2)	8.76	19.69	0.0113
QTL-QY3	6HL	122	20687	122.0 (124.9)	11111	122.9 (128.5)	5.14	10.74	−0.0084
QTL-QY4	4HL	81	20119	80.9 (99.3)	11066	99.3 (113.9)	4.15	8.53	−0.0073
QTL-QY5	6HS	55	10962	54.9 (54.6)	21014	59.6 (54.6)	3.19	6.44	0.0064

Positions within brackets are from an integrated map of several populations (Close et al. 2009)

Chr chromosome, *LOD* likelihood of odds, *PVE* phenotypic variation

[a] BOPA C name of line 11

[b] Significant threshold of LOD = 2.83, $P < 0.01$

[c] *PVE %* Proportion of the total variance explained by each QTL in percentage

Table 2 Allele frequency of the QTL associated with water logging tolerance in the 20 lines with highest and lowest quantum yield (QY) of a total of 120 double-haploid offspring barley lines

	QTL-QY1	QTL-QY2	QTL-QY3	QTL-QY4	QTL-QY5	QY[a]
Lines with highest QY						
Alleles from cv. Psaknon	0.65	0.85	0.5	0.55	0.65	0.453
Alleles from (SLUdt1398 × Mona[4])	0.35	0.15	0.5	0.45	0.35	
Lines with lowest QY						
Alleles from cv. Psaknon	0.3	0.1	0.45	0.2	0.15	0.372
Alleles from (SLUdt1398 × Mona[4])	0.7	0.9	0.55	0.8	0.85	
Additive effect	0.0137	0.0113	−0.0083	−0.0073	0.00644	

[a] Mean quantum yield of 20 double-haploid lines with highest and lowest fluorescence, respectively

Examination of the 20 lines with the highest and lowest QY clearly showed that the QTL responsible for tolerance to low oxygen concentration in the nutrient solution were from cv. Psaknon (Table 2). Should these QTL were not present QY was particularly low; if present the frequency of lines with high QY was high but not to the same extent as the frequency of lines with low QY without the alleles. For QTL-QY3, one of the QTL with a negative additive effect showed no differences among those with high or low QY (Table 2). A classification of all lines with the presence/absence of QTL and their combinations revealed that the genomic regions with positive effect on QY play a major role in controlling this trait in spite of the presence of QTL with negative effects (Fig. 3). In lines where a single QTL is present/absent QY differed in percent from −0.6 for QTL-QY3 to 6.2 of QTL-QY2. With alleles either from the father or mother at QTL-QY1 and QTL-QY2 QY differed with 8.2 % and if also the third positive QTL was included the mean QY values differed with 11.2 % (see QTL125, Fig. 3). By instead including the negative QTL the differences were 21.2 and 13.1 % for QTL-QY3 and QTL-QY4, respectively. These differences increased up to 22.4 % by counting lines where all loci had the same alleles either from the father or the mother (Fig. 3). Even though this difference may be bias due to linkage and sample size, it is expected to be in the same direction given the estimated additive effects.

Discussion

The DH barley population used is a cross between cv. Psaknon and a breeding line with genes mainly from Mona, but also from a *Hordeum spontaneum* accession from Israel. Cv. Psaknon is an old cultivar originating

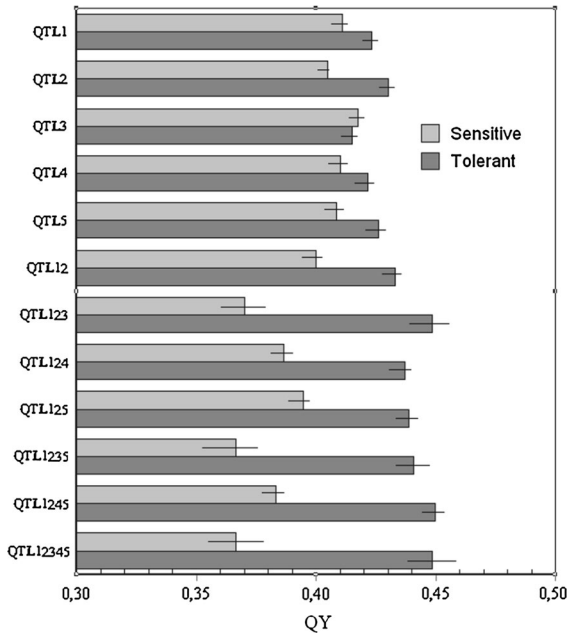

Fig. 3 Mean quantum yield (QY) of lines with alleles from the sensitive parent (SLUdt1398 × Mona[4]) (*light grey*) and tolerant parent (cv. Psaknon) (*darker grey*) at loci of the different QTL. QTL12 means lines with alleles coming from the same parents in both QTL-QY1 and QTL-QY2 and so on. Bars = 2 SE. (Color figure online)

from Australia, with the *Mlp* resistance genes for powdery mildew and *rhynchosporium*. There is some evidence that cv. Psaknon is also waterlogging-sensitive, but not consistent in its response (Wignarajah et al. 1976). For example, in the present study cv. Psaknon delivered the alleles for tolerance to hypoxia. However, plants tolerance/sensitivity to hypoxia is dependent on water deep and length of waterlogging regimes and may hence differ with methods used. The sensitive parent in this study, SLUdt1398 × Mona[4], has resistant genes for netblotch and scald. There is no information available about its WLT, but with genes from *H. spontaneum* one could expect tolerance to other abiotic stresses such as drought and salt (Forster et al. 2000).

With the implementation of high-throughput SNP genotyping in barley, the bottleneck in genomic studies of abiotic stresses is now phenotyping (Xu and Crouch 2008; Close et al. 2009; Ahmed et al. 2013). WLT is related to many processes in the plant and for each of these processes; traits can be identified and used in phenotyping (Colmer and Voesenek 2009). The objective of the present study was to identify QTL for selection of WLT at the seedling stage. Tolerance at this stage is very important in northern Europe, which can experience alternating periods of dry and wet soil during the spring. In lowland areas, on compacted soils or in fields lacking a drainage system, young barley plants are often yellowed, with low biomass growth. These two traits are early signs of sensitivity to waterlogging and are used in many phenotyping studies. They are secondary effects, primarily of low oxygen concentration causing an energy crisis, followed by a carbohydrate crisis, toxicity, accumulation of reactive oxygen species (ROS) and water deficit (Colmer and Voesenek 2009), and thus a perfect trait at first sight for phenotyping. However, a disadvantage is extensive environmental interactions and costly field trials. In the hydroponic method developed by Bertholdsson (2013), many seedlings can be tested in a limited space and time with minimal environmental influences. By measuring QY already after four to five days of stress, the basic energy crisis is addressed. Since QY is highly correlated with residual biomass of seedlings after 18 days of waterlogging, it could thus be used to predict the probable residual biomass several weeks later in water-logged soil (Bertholdsson 2013).

The value of QTL identified as important depends on their additive effects and whether they respond well in different backgrounds. In this study a few main QTL showing high additive effects were found and fortunately these QTL seem also to have been identified in other populations phenotyped for biomass growth reduction and yellowing. In order to make comparisons with other populations a consensus map is needed and for barley there are several such available (Wenzl et al. 2006; Close et al. 2009). The SNP markers in the Illumina chip used in the present study have been carefully selected for use in modern barley breeding populations and the markers in the present population were mapped similarly to the consensus map from Close et al. (2009). Of the five significant QTL identified, QTL-QY2 on chromosome 7H at 60.7 cM was noted before in the DH population cv. Franklin × TX9425 and probably also in cv. Franklin × cv. Yerong (Li et al. 2008). The QTL affected leaf chlorosis negatively in cv. Franklin and positively in the waterlogging tolerant cultivars TX9425 and Yerong. In the population used in the present study, QY was low when the alleles came from (SLUdt1398 × Mona[4]) and high when they came from cv. Psaknon (Table 2). QTL-QY2, with a rather

high additive effect, may therefore be relevant for more than the present population and, with the flanking markers very close to each other, it should be easy to develop a PCR marker for use in marker selection (MAS). Furthermore, QTL-QY2 and also QTL-QY4 on 4H are located in similar positions to a QTL identified in TX9425 × cv. Yerong by Li et al. (2008) based on dry matter reduction after 3 weeks of waterlogging stress. QY is used as an indirect way of predicting the biomass growth reduction of waterlogged seedlings and the genes responsible may be the same.

The correlation between residual biomass and QY was as high as $R^2 = 0.67$ ($P < 0.001$) for a reference material of 12 cultivars, hence 67 % of the variation was due to the genotypes studied (Bertholdsson 2013). Most of the QTL identified by Li et al. (2008) in cv. Franklin × cv. Yerong were also identified in semi-field trial with steel tanks, where the more accurate phenotyping resulted in higher LOD scores (Zhou 2011). Using an improved method with a less artificial field test and better scoring with an index including leaf necrosis, plant residual biomass and plant survival, QTL were also identified on 6H in similar positions as in QTL-QY1 and QTL-QY3 in the present study. Putatively these two detected QTL were also identified using the above field test in another DH population with cv. Franklin and the tolerant cv. YuYaoXiangTainErleng (Zhou et al. 2012). Hence, most of the interesting QTL were found in two Asian populations and now also in a European population, which could indicate a certain stability of these QTL.

QTL-QY1 and QTL-OY3 appeared to be close to each other, providing positive and negative effects, respectively. Despite that the QTL with positive effect played a major role in the expression of QY, it would be of great importance to not indirectly select for QTL-QY3 when doing MAS for QTL-QY1. One possible strategy to apply is to identify the recombinant lines at that genomic region and to use for breeding purposes the lines without QTL-QY3. We have identified tree candidate lines that carry both QTL-QY1 and QTL-QY2, but not QTL-QY3.

With the linkage maps we constructed in our study, it was not possible to determine the full size of QTL-QY3. Despite of being a QTL with negative effect, it would be of interest to saturate that genomic region of chromosome 6HL to better determine the position of such QTL.

In a meta-analysis of QTL associated with tolerance to abiotic stresses in barley, one meta-QTL (MQTL) for waterlogging were located to 1H, eight on 2H, two on 3H and one on 5H, where no QTL were found in this study for QY (Li et al. 2013). A possible explanation could be that these QTL affect other genes responsible for changes in the metabolic processes causing toxicity (Zeng et al. 2013), ethylene production initiating aerenchyma formation or some of the other processes not directly related to energy production (Agarwal and Grover 2006; Colmer and Voesenek 2009; Ahmed et al. 2013). However, the meta-analysis did confirm the presence of QTLs on 4H and 7H. The QTL on 6H for waterlogging found by Zhou (2011) and in this study were not confirmed by the meta-analysis, however, but there were MQTLs for drought tolerance located at 6H, close to the positions of QTL-QY1, QTL-QY3 and QTL-QY5. In an earlier study of 12 barley cultivars subjected to drought and waterlogging stress, ten cultivars showed the same response to both stresses (Bertholdsson 2013), indicating that similar genes are involved in several stresses. This is true particular for drought and hypoxia, for example, rice experiences dehydration stress following de- submergence due to reduced hydraulic conductivity in leaf sheaths (Setter et al. 2010). QTL studies concerning salt stress also show similarities with the hypoxia stress. Zhou et al. (2012) found co-located QTL for waterlogging and salt tolerance. QTL-QY4 at 4H was also co- located with a MQTL for salt tolerance, strengthening this further. QTL-QY5, with a LOD peak value of 55 cM, is located close to a QTL controlling salt tolerance found by Long et al. (2013) that co-localised with biomass growth, chlorophyll content and leaf senescence. They used association mapping of 192 barley cultivars from all over the world, including 92 cultivars from Europe. Changes in the latter two parameters would also have a direct effect on chlorophyll fluorescence.

Zhou et al. (2007) showed that WLT was mainly controlled by additive genes with high heritability, and was thus suitable for screening in early generations. The QY of our short-term waterlogged seedlings also showed high narrow sense heritability ($h^2 = 0.87$). With PCR-based markers developed for the QTL, it should thus be possible to select genotypes with higher WLT. Before these markers are implemented in breeding programmes, however, some further evaluations are needed by practical selection (Xu and Crouch 2008). Both WLT and QY have been found to be positively correlated with grain yield (Bertholdsson

2013). Thus, there is no conflict between WLT, high QY and grain yield, as also indicated by an involuntary improvement in WLT by breeding for yield in the Nordic countries (Bertholdsson 2013).

Even though plant breeders have already been successful in adapting barley cultivars to waterlogging and probably also drought by selecting for yield, direct marker selection would be more efficient; i.e., if the QTLs are not already present. A wider range of cultivars could be evaluated and used as parents in crossings and the risk of discarding interesting genotypes would be reduced by selection in a young generation (F_2 or F_3), whereas selection for yield is done from F_5 when there are seed enough to do yield trials. Grain yield is currently the main breeding target and will continue to be so in the future, so marker-aided selection for stress tolerance, including waterlogging, could save time to reach this goal.

In the study five significant QTL were identified. Even though the two major QTL explained 29 and 20 %, respectively, and because of their high additive effect, these two QTL play a major role in controlling QY. Thus it is important that a combination of PCR markers are developed to reach optimal selection efficiency. To be able to use the markers in breeding, the QTL need to be validated first in the 'real field situation' to see if the markers are highly correlated with yield under stress conditions. Further these markers can be run across different genetic backgrounds and across different environments. Also to have more robust markers, tightly linked markers or ultimately gene-based diagnostic markers could be needed.

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