Identification of the Submergence Tolerance QTL
Come Quick Drowning1 (CQD1) in Arabidopsis thaliana

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Abstract

Global climate change is predicted to increase water precipitation fluctuations and lead to localized prolonged floods in agricultural fields and natural plant communities. Thus, understanding the genetic basis of submergence tolerance is crucial in order to improve plant survival under these conditions. In this study, we performed a quantitative trait locus (QTL) analysis in Arabidopsis to identify novel candidate genes for increased submergence tolerance by using Kas-1 and Col (gl1) parental accessions and their derived recombinant inbred lines (RILs). We measured survival after submergence in dark for a 13-day period and used median lethal time, LT50 values for the QTL analysis. A major QTL, the Come Quick, Drowning (CQD1) locus, was detected in 2 independent experiments on the lower arm of chromosome 5 involved in higher submergence tolerance in the parental accession Kas-1. For fine-mapping, we then constructed near isogenic lines (NILs) by backcrossing the CQD1 QTL region. We also analyzed QTL regions related to size, leaf number, flowering, or survival in darkness and none of the QTL related to these traits overlapped with CQD1.

The submergence tolerance QTL, CQD1, region detected in this study includes genes that have potential to be novel candidates effecting submergence tolerance such as trehalose-6-phosphate phosphatase and respiratory burst oxidase protein D. Gene expression and functional analysis for these genes under submergence would reveal the significance of these novel candidates and provide new perspectives for understanding genetic basis of submergence tolerance.

Subject area: Genomics and gene mapping

Keywords: Arabidopsis thaliana, Col (gl1), darkness, flooding, Kas-1, QTL, submergence tolerance

As a result of seasonal floods, waterlogging and submergence lead to losses of around 20% of annual crop yield (Normile 2008). With unpredicted fluctuations in precipitation levels as a result of global climate change, the area of agricultural fields and natural plant communities affected by floods will gradually increase and become a problem worldwide (Arnell and Liu 2001; Hirabayashi et al. 2013). When flooded, plants are unable to acquire the oxygen necessary for respiration as a result of limited gas diffusion under water, and...
anoxic/hypoxic conditions lead to rapid mortality as a result of starvation (Bailey-Serres and Voesenek 2008). In order to overcome catastrophic effects of floods on crops and plant communities, it is important to broaden the knowledge on physiological and genetic basis of submergence tolerance.

There has been extensive research focused on identifying the genetic basis of submergence, hypoxia and anoxia tolerance; particularly in frequently flooded rice varieties, Arabidopsis, in other crop species, as well as naturally flooded wetland species. The discovery of a set of ethylene response factor (ERF) genes, SUB1A and SNORKELI controlling different survival strategies (namely low oxygen quiescence and escape strategies), in rice led to a better understanding of how plants survive under various flooding regimes (Xu and Mackill 1996; Xu et al. 2006; Hattori et al. 2007; Hattori et al. 2009). Both SUB1 and SNORKEL genes are members of the group VII (B-2) subfamily of ERF genes that are distinguishable by their DNA binding domains and N-end motifs (Nakano et al. 2006). Orthologs of this subfamily were shown to be up-regulated in poplar under hypoxia (Kreuzwieser et al. 2009), additionally in Arabidopsis thaliana they are involved in hypoxia/anoxia sensing and tolerance (Hinz et al. 2010; Licausi et al. 2010, 2011; Gibbs et al. 2011). Studies on close relatives of Arabidopsis, Rorippa species, from naturally flooded habitats, also suggest significance of these genes under low oxygen sensing (van Veen et al. 2014).

Another adaptation to low oxygen environments is aerenchyma formation, which increase porosity and gas diffusion in many plant species adapted to low oxygen stress (Voesenek and Bailey-Serres 2015). In rice, aerenchyma formation is associated by reactive oxygen species through down-regulation of Metallothionein 2b (Steffens et al. 2012) and, in wheat, up-regulation of respiratory burst oxidase homologs (RBOHs) that amplify ROS-mediated signaling (Yamauchi et al. 2013). Moreover, a recent study also identified a gene, *trehalose-6-phosphate phosphatase* in rice seedlings; by altering developmental patterns enhancing starch mobilization, this gene leads to higher anaerobic germination tolerance (Kretzschmar et al. 2015). A transcriptome-wide expression study in *Rumex* species revealed genes important in ethylene-mediated petiole elongation (van Veen et al. 2013). Among these are shade avoidance related genes, expansins and xylologuecandotransglucosylase-hydrolases that are involved in cell wall modifications possibly important in elongation. These studies show that low oxygen stress tolerance relies on various adaptations that regulate gene expression and the subsequent physiology. Thus, in order to understand the genetic basis of this diverse palette of adaptations, it is crucial to reveal novel adaptive genes by using natural variation in low oxygen tolerance for model organisms as well as naturally flooded species.

*Arabidopsis thaliana* has been extensively used in quantitative trait loci (QTL) analysis for discovery of novel genes controlling quantitative traits (Alonso-Blanco et al. 2009; Koornneef et al. 2011). Broad genetic tools and short generation times make A. thaliana an excellent model for QTL analysis to link phenotypic variation to genotype. Natural accesses of Arabidopsis display an impressive geographical distribution spanning many diverse ecological conditions. These accesses with broad morphological variation set a valuable system to unravel adaptations to diverse environmental conditions. In addition, with the launch of the Arabidopsis 1001 genomes project available genetic information on these Arabidopsis accessions will accelerate the discovery of genes controlling phenotypic variation (Weigel and Mott 2009; Cao et al. 2011; Long et al. 2013). Furthermore, knowledge on Arabidopsis can contribute to crop improvements in crucifer (mustard) family members (Mitchell-Olids 2001; Schrani et al. 2007).

Considering its advantages, Arabidopsis was used in many studies that focused on anoxia/hypoxia responses. However, very few have studied the effects of submergence directly (Lee et al. 2011; Peña-Castro et al. 2011; Vashisht et al. 2011; Hsu et al. 2013; Chen et al. 2013; van Veen et al. 2016). Although *A. thaliana* is typically not flooded in its natural habitats, Vashisht et al. (2011) showed that 86 accesses show considerable natural variation for submergence tolerance. This natural variation could be a fundamental resource to discover novel genes effecting submergence tolerance in Arabidopsis by QTL analysis or association studies (Flint and Mort 2001; Bergelson and Roux 2010).

In this article, we performed a QTL analysis to identify loci controlling variation in submergence tolerance in 2 accessions of *A. thaliana*, Kas-1 and Col (gl1) using recombinant inbred lines (RILs). In correspondence with results of Vashisht et al. (2011), we selected Columbia (Col) and Kashmir-1 (Kas-1), as these accessions showed considerable variation in their submergence survival and also have an available mapping population composed of 96 RILs and 119 genetic markers and was used previously in mapping aluminum tolerance, powdery mildew disease tolerance and flowering time genes successfully (Wilson et al. 2001; Wolyn et al. 2004; Li et al. 2006; Sánchez-Bermejo et al. 2014). In order to find QTL involved in submergence tolerance, we performed survival assays with a selected set from 96 RILs and the parental lines and used lethal median time (LT50) values in our submergence QTL analysis. We found a single QTL on chromosome 5 that we have named the *Come Quick Drowning I* (CQD1) locus, which explains the variation between the parental accessions. We validated the effect of the QTL with confirmation assays, and used back cross populations for further fine-mapping.

**Materials and Methods**

**Plant Material**

Seeds of the parental accessions, Kas-1 (from Kashmir) and Col (gl1) (the standard Col-0 accession with the glabrous1 mutation that inhibits trichome formation) and 128 F6 Kas-1/Col (gl1) RILs were obtained from Nottingham Arabidopsis Stock Centre (NASC, UK). Seeds of all RILs were sown on soil/perlite mixture (1:2, Peters Professional, Scotts Europe BV, Heerlen, The Netherlands) and kept at 4 °C for 4–5 days in dark for stratification and later put in a greenhouse at 20 °C under natural light supplemented with 600-W SON-T lamps (Philips, Eindhoven, The Netherlands) when necessary. After seeds germinated, they were transferred to 55 mm mesh pots and F7 seeds were used in the following experiments.

**Submergence Assay in Soil/Perlite Medium**

Two survival assays for submergence in dark were performed with parental accessions and 1 survival assay with 6 randomly selected RILs to test the experimental setup. Seeds of the parental accessions and RILs were sown on soil/perlite mixture (1:2, Peters Professional, Scotts Europe BV) and kept at 4 °C in dark for 5 days for stratification. They were then transferred to a greenhouse until germination. The greenhouse was at 20 °C (±2 °C) with a 14 h light photoperiod under natural light supplemented with 600-W SON-T lamps (Philips, Eindhoven, The Netherlands). Twice the amount of seedlings necessary for the experiments were transplanted to single pots of 70 mm with the same soil/perlite mixture supplied with nutrient solution.
When plants were at 7–8 leaf stage, sand was added on top of the topsoil to prevent floating of soil/perlite mixture during submergence. Four hours after the light period started, plants were submerged in 16 L buckets that were filled with rainwater 1 day prior to the start of the submergence treatments (for acclimation of water temperature). After all the plants were submerged, the buckets were covered with opaque black plastic bags to eliminate effects of light during the experiment and prevent algae growth. All experiments were conducted in a greenhouse with the same conditions as used for the growth period. At predetermined time-points, 10–15 plants (as mentioned above) were removed from the buckets and placed in another greenhouse with the same conditions for a recovery period of 14 days. Survival was scored for the parental accessions and 6 RILs according to the presence of newly growing green parts as an indication of surviving and active meristem tissues.

Submergence Assay in MS/Agar Medium

Seeds of parental accessions were soaked in 15% commercial bleach (5.25–6.15% sodium hypochlorite) and 0.05% Tween 20 (Sigma–Aldrich Chemie B.V., Zwijndrecht, The Netherlands) solution for 8 min and washed 5–7 times with sterile MilliQ water. They were placed on 0.8% agar (Duchefa Biochemie B.V., Haarlem, The Netherlands) with half-strength Murashige-Skoog medium (Duchefa Biochemie B.V.) supplied with 20 μM nystatin (Duchefa Biochemie B.V.) in magenta boxes in a laminar flow in order to avoid contamination. Sixteen seeds were placed in each box and then kept in 4 °C in dark for 4–5 days. Boxes were then transferred to a greenhouse at 20 °C (±2 °C) with 14 h photoperiod under natural light supplemented with 600 W SON-T lamps (Philips, Eindhoven, The Netherlands) when necessary. Two days after germination, lids of magenta boxes were slightly opened to increase air circulation. When plants were at the 7–8 leaves stage, each plate was individually inspected and some plants were removed in order to have homogeneous-sized plants. They were submerged in 400 mL demi-water 4 h after the photoperiod started. For assessing submergence tolerance of the parental accessions, 3 different lighting conditions were used during submergence: dark, light, and shade. For submergence in darkness, black opaque plastic bags were used to cover boxes. For shade conditions, a shade cloth was used to reduce the light intensity to 10% of ambient light conditions. At each predefined time-point, water was removed from the boxes by piercing a small hole at the bottom. Survival was scored after a 10- to 14-day recovery period with the same criteria as in soil/perlite experiments.

Submergence Assay for QTL Analysis

QTL experiments were done with MS/agar medium as described above, except submergence treatments were done only in the dark based on results from parental line experiments. In addition, air controls in the dark were included in the experiment, using black opaque plastic bags. For air controls in dark, the plastic bags were removed at several time-points and survival was scored similarly after a 14-day recovery period. Two independent experiments were performed for the QTL study. For the first QTL survival assay, RILs used were selected according to Li et al. (2006) and germination success, leading to 83 lines for the first experiment. A random smaller subset of 35 RILs was used in a second QTL survival assay in order to confirm the results of the first assay. In order to have similar plant sizes for the QTL experiments, we performed a growth experiment in which we grew and categorized RILs into 5 groups according to their timing of germination and seedling growth. Accordingly, sowing was done over a 5-day period according to size categories in which slow growing RILs were sown on the first day and fast growing RILs were sown on the last day. For each line, there were 5 time-points and 2 magenta boxes per time-point with up to 16 plants in each. Air controls in dark were taken out of plastic bags at only one time-point (13 and 9 days, respectively for the 2 experiments) and there were 2 replicate plates for each line. Submergence survival data was used to calculate median lethal time, LT₅₀ values, for each RIL (as explained below) and these data were used in the QTL analysis as an indication of submergence tolerance. For air controls in dark, survival (%) at a single time-point (13 and 9 days, respectively for the 2 experiments) was used to detect QTL related to survival in darkness.

For size measurements, pictures were taken just before the start of the submergence experiments. Number of leaves was counted and surface area of plants was measured for QTL analysis in order to test if these parameters had an effect on the submergence tolerance. Size measurements were done with ImageJ software (Abramoff et al. 2004). We grew RILs for categorizing them according to their growth rates for the submergence QTL experiments, and in this categorization test we also scored flowering after 30 days as “flowering” or “non-flowering.” These binary data were used in a QTL analysis in the mapping population to test if we could detect previously published QTL by Li et al. (2006).

QTL Confirmation

Ten RILs were selected for confirmation of the major QTL CQD1 (see Results section) and further characterization of their growth. Two of these had a Col (gl1) background and Col (gl1) QTL (CS84887, CS84922), 3 had Col (gl1) background and Kas-1 QTL (CS84943, CS84964, CS84984), 3 had Kas-1 background and Col (gl1) QTL (CS84986, CS84994, CS84997), and the last 2 had Kas-1 background and the Kas-1 QTL (CS84931, CS84934). Selection of these lines was done according to the percentage coverage of Kas-1 or Col (gl1) genotype: lines with the most Kas-1 or Col (gl1) markers were selected. These lines were analyzed in detail for submergence and dark survival, soluble carbohydrate content, and dry weight. We used 4–5 boxes for each time-point (6 time-points) for submergence and 3 boxes for dark controls (6 time-points). Survival analysis and calculation of LT₅₀ values were done for both survival in darkness and submergence with MS/agar medium. Values in Figure 4a–d represent the means and standard errors calculated from the averages of each of the 3–4 lines used within the groups.

Before the plants were submergence, shoots of 5 plants from each of these 10 lines were pooled for dry weight and soluble carbohydrate measurements. This was repeated 2 times for each line. After freeze drying, plants were weighed and then 20–40 mg of ground tissue was suspended in 0.5 mL 70% MeOH in water (v:v), vortexed and boiled for 5 min. After placing the tubes in an ultrasonic bath for 15 min, samples were centrifuged (10 min at 10000 rpm) and the
supernatants were transferred to new tubes. Pellets were extracted once more, excluding the boiling step. Supernatants of each sample were combined and 70% MeOH was used to bring the final volume to 1 mL. For HPLC quantification, 20 µL of extract was diluted in 980 µL of MilliQ water and carbohydrate measurements and data analysis were performed as described previously (Van Leur et al. 2008; Akman et al. 2012).

NIL Construction and Testing
NILs were generated by backcrossing the CS84997 RIL used in the confirmation experiment that had the highest Kas-1 background and the Col (glll) QTL. The line was crossed to the parental accession Kas-1 in order to increase the overall Kas-1 genomic background and break the Col CQD1 QTL region into smaller blocks by recombination (see Supplementary Figure 1 for details of NILs). BC1 plants (heterozygotes for QTL region) were used for another round of backcrossing with Kas-1. Two hundred BC2 plants were grown, from which 93 plants were analyzed for recombinants by genetic marker analysis. DNA was extracted from the leaf tissue using the CTAB extraction method (Doyle and Doyle 1990). Genotyping of BC2 parent population lines was done with the 3 markers within the QTL region (SNP44607808, NGA129, and MSAT5.12). Twelve out of the sampled 93 plants showed recombination between these 3 markers. Some of these lines had similar recombination patterns and only one plant per recombinant type was used for self-pollination to construct BC3S1 lines. This led to the construction of 5 different recombinant genotypes. For each recombinant BC2 parental line, 20 seedlings (BC3S1) were transplanted and 7–20 of these were used for DNA isolations for selecting homozygotes for the recombined QTL region. These lines were then genotyped with 6 newly designed markers (see below) and one of the original markers (NGA129). From each BC3S1 parent, we selected 2 homozygous pairs: one of the individuals within a pair included a seedling with a partial homozygous Col (glll) QTL allele and the other that was homozygous for the Kas-1 QTL allele for comparisons within lines. The same procedure of NIL construction was repeated with an individual plant selected from RILs (CS849493) with mostly Col (glll) genomic background and the Kas-1 QTL (used in QTL confirmation). This individual was crossed with Col (glll) parental accession in order to increase Col (glll) background and break the QTL into smaller regions by recombination. NILs were constructed as explained for the reciprocal NIL construction above and a total of 5 NIL pairs were created. Seeds of these recombinant NIL pairs (BC3S1), adding up to 20 genotypes per background (40 genotypes in total), were used for further phenotyping experiments.

Marker design for fine-mapping: Whole genome sequences of Col-0 and Kas-2 are available from the 1001 genomes project (http://signal.salk.edu/atq1001/index.php). We constructed 6 new cleaved amplified polymorphic sequence markers for these accesses in the QTL region. Single nucleotide polymorphisms in Kas-2 were also present in our parental accession Kas-1. The genes for markers, primers, and restriction enzymes are listed in Supplementary Table 1. These markers and one of the original markers, NGA129 were used in marker assisted selection of recombinant NILs. A PCR reaction was set for all the markers and restriction enzyme assays were performed as described by the supplier (Fermentas GmbH, Leon-Rot, Germany).

Survival assays of each NIL was done with 5 time-points for submergence tests (4, 6, 8, 10, and 12 days), with each time-point consisting of 4 replicated boxes. Two replicates per time-point per line were used for dark controls. Assays were done similarly with seeds kept in sterile water after sterilization at 4 °C in dark for 6–7 days in order to break the dormancy. After seeds were placed in magenta boxes, they were put in a greenhouse immediately. Additionally, 8 seeds from one recombinant with Col (glll) insertion and 8 seeds with Kas-1 genotype (both from the same parent BC1) were sown in one box in order to enable simultaneous analysis of the 2 lines within a box to assess effects of the Col (glll) QTL region insertion in the Kas-1 background. For the same recombinant, the second line with Kas-1 insertion at the same loci was also done with the Kas-1 parental genotype and was treated as a separate line in the survival analysis. The LT50 values were calculated as described above separately for the 3 lines for each recombinant. The average LT50 value was calculated for all lines with the same genotype for a particular marker.

NILs constructed had a high dormancy and did not germinate after the standard vernalization period. In order to break dormancy of the NILs used in these survival assays they were put back at 4 °C for 5 additional days, 7–10 days after moving into a greenhouse. After this period, almost all seeds germinated successfully. The delay of germination 1 (DOG1, AT5G45830) QTL on chromosome 5 could be causing this delayed germination since it is close (by 800 kb) to the QTL region and is thus in linkage with the QTL.

Statistical Analyses
Survival data were used to calculate LT50 values (median lethal time), that is, the time-point at which 50% of the plants died, with the Weibull regression model by implementation in Excel (Hosmer and Lemeshow 1999). A Weibull regression model was fitted to survival data for each parental accession or RIL and calculated LT50 values were used in further analyses. Each of the 2 boxes with a single RIL was used as a biological replicate in the regression analyses. In order to test if survival between the 2 accesses were significantly different in the initial tests, we used generalized linear models with R. In our model, we used survival data from both accesses for each condition (dark, shade, light in MS/agar media and the 2 tests in the soil/perlite media as in Table 1) and included accession as a fixed variable to test significance of its effect on survival (survival – day + accession).

Statistical analyses including ANOVA post hoc (Tukey’s B) tests that were done to test for significant differences among groups in LT50 values for submergence and dark survival, dry weight, and soluble carbohydrates for QTL confirmation tests were performed with SPSS 16.0 for Mac (SPSS Incorporated, Chicago, IL).

QTL analyses were performed with Windows QTL Cartographer Version 2.5 (Wang et al. 2011). We used composite interval mapping (CIM) with 2 cM intervals using a 10 cM window and 5 background cofactors that were selected via a forward and backward stepwise regression method. A thousand permutations were performed to estimate α = 0.05 threshold values (Doerge and Churchill 1996) for detecting significant QTL. The linkage map and QTL were constructed by MapChart 2.2 (Voorrips 2002).

We used linear mixed effect models to test marker and NIL LT50 associations by using R package “lme4” and “lmer” function. In each model, we related the LT50 values as the response variable to the presence of absence of 1 of the 5 QTL markers as a fixed effect. In order to account for differences in LT50 values for each of the NIL pairs included in the experiment, we incorporated random effects for each NIL pair (BC3S1) nested within maternal source plants (BC1).
Table 1. Median lethal time (days), $LT_{50}$ values for all submergence experiments done with the parental accessions

<table>
<thead>
<tr>
<th>Medium</th>
<th>Lighting</th>
<th>During submergence</th>
<th>After submergence</th>
<th>$LT_{50}$ (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil/perlite</td>
<td>Dark</td>
<td>Artificial lighting on</td>
<td>11.95 ± 1.69</td>
<td>2.89**</td>
</tr>
<tr>
<td>Soil/perlite</td>
<td>Dark</td>
<td>Less artificial lighting</td>
<td>16.18 ± 2.18</td>
<td>5.76***</td>
</tr>
<tr>
<td>MS/agar</td>
<td>Dark</td>
<td>Artificial lighting on</td>
<td>9.73 ± 0.21</td>
<td>3.27***</td>
</tr>
<tr>
<td>MS/agar</td>
<td>Shade</td>
<td>Artificial lighting on</td>
<td>11.88 ± 0.2</td>
<td>2.06**</td>
</tr>
<tr>
<td>MS/agar</td>
<td>Light</td>
<td>Artificial lighting on</td>
<td>10.79 ± 0.12</td>
<td>2.09***</td>
</tr>
</tbody>
</table>

** $P < 0.001$, *** $P < 0.001$.

Figure 1. Survival assay for Col (g/l) and Kas-1 with (a) the pot system, (b) magenta box system, and (c) correlation of median lethal time ($LT_{50}$) of 6 random RILs in soil/perlite and MS/agar medium.

**Results**

**Kas-1 Was More Tolerant to Submergence than Col (g/l)**

We performed several submergence survival assays to evaluate submergence tolerance of parental accessions Kas-1 and Col (g/l). We used 2 different rooting media: soil/perlite in a pot and MS/agar in magenta boxes to test if using these different media have an effect on survival. In all our tests, there was a significant difference in survival between the 2 accessions (Table 1). In 2 independent experiments performed with soil/perlite medium, Kas-1 was more tolerant to submergence with $LT_{50}$ values of 11.95 and 16.18 days for pots and agar media, respectively, compared to 9.06 and 10.42 days for Col (g/l) under the same conditions (Figure 1a, Table 1). Since survival assays with soil/perlite medium had time and space limitations, we tested MS/agar assays with both parental lines in order to select fast and robust conditions for QTL experiments in which thousands of plants could be phenotyped for submergence tolerance simultaneously. Our tests with MS/agar medium showed that a similar trend of Kas-1 being more tolerant was also present even though survival was generally lower than on soil/perlite for both accessions (Figure 1b).

Independent of the lighting conditions during submergence (submergence in dark, shade, or light), Kas-1 was always more tolerant to submergence although there was variation among different treatment types. Both accessions survived the longest when plants were submerged in shade (Table 1). The difference between $LT_{50}$ values for the 2 accessions was higher when submergence was performed in dark. We compared survival of 6 randomly selected RILs submerged in dark with both soil/perlite and MS/agar medium, and showed that there was a strong positive correlation in $LT_{50}$ values calculated for the 2 growth media (Figure 1c).

**Submergence Tolerance QTL on Chromosome 5**

Two independent survival assays were performed for the QTL analysis. In the first assay, 83 RILs and the 2 parental accessions were used. $LT_{50}$ values for the parental lines were 6.5 days for Col and 9.7 days for Kas-1. The $LT_{50}$ values for the RILs varied between 1.3 and 14.1 days and showed a normal distribution with some lines having transgressive phenotypes beyond the values of the parental lines (Figure 2). The second confirmation assay was performed with 55 RILs and the $LT_{50}$ values varied between 1.7 and 6.8 days. Plants showed higher mortality in the second assay and $LT_{50}$ values from the 2 assays showed an average of 3 days difference. Despite this variation between the 2 assays, results of both QTL analyses revealed a submergence tolerance QTL on the lower arm of chromosome 5 linked to markers “SNP44607808”, “NGA129,” and “MSAT5.12” (Figure 3). We named this QTL the Come Quick, Drowning 1 (CQD1) locus. Survival in dark, size of the plants, leaf number before submergence and flowering showed correlation with several markers indicating QTL, but none of these overlapped with the submergence tolerance QTL on chromosome 5 (Figure 3, Table 2). One QTL for survival in dark was linked to the SNP markers “21607463” and “21607700” on chromosome 1. Both experiments had an overlapping dark survival QTL linked to markers “MSAT4.39” and “CIW5” and SNP marker “44608028” and “44606623”, overlapping with a flowering QTL. On chromosome 4, linked to “NGA1139” and SNP “44606688” another dark survival QTL was detected. One plant size QTL was found linked to 2 SNP markers “44607824”, “21607157” and “NGA361”, “MSAT2.7” on chromosome 2. A single QTL was detected for number of leaves on chromosome 1 linked to “MSAT1.13”, “NGA692” and SNP marker “21607030” overlapping with a flowering QTL. Positions, 1-LOD score intervals and effects of QTL are given in Table 2.

**QTL Confirmation: The Effect of the QTL Was Larger with Kas-1 Background**

For confirmation of the submergence tolerance QTL, 10 RILs were selected for confirmation of the QTL by performing survival assays with more replicates and time-points. The difference
between LT_{50} values for RILs with Col (gl1) background with and without the Kas-1 QTL were compared. Similarly, RILs with Kas-1 background with and without Kas-1 QTL were compared. Consistent with the QTL analyses, there was a significant improvement in submergence survival by the presence of the Kas-1 QTL on chromosome 5 in these RILs (Figure 4a). This effect was higher in a Kas-1 background than in a Col (gl1) background. There was no effect of the QTL on dark survival when the background was Col (gl1) (Figure 4b). We observed a higher dark mean survival due to the presence of the Kas-1 QTL in Kas-1 background but this difference was not statistically significant (at P < 0.05). Dry weight (Figure 4c) and soluble sugar status (Figure 4d) just before submergence did not vary for different backgrounds and QTL, and thus cannot explain the higher survival achieved by the Kas-1 QTL.

Fine-Mapping
As already indicated above, some seeds of NILs exhibited strong dormancy. The delay of germination 1 (DOG1, AT5G45830) allele from Kas-1 on chromosome 5 was the likely cause of this delayed germination, since it is close (by 800 kb) to the QTL region and presumably dragged along with the QTL during backcrossing. Backcrosses led to the establishment of 5 different recombinant NIL pairs in each parental genetic background. In the interval of

Figure 2. LT_{50} values for RILs used in the QTL study for (a) first QTL experiment and (b) second QTL experiment. The parental lines are indicated with arrows in (a). The parental lines were not used in the second experiment (b).

Figure 3. QTL detected by submergence and dark treatments in the QTL experiments and QTL associated with growth-related parameters (flowering, leaf number, and size).
the first 4 markers (estimated size approximately 1.04 megabases), we did not observe any recombinants. Marker-LT\textsubscript{50} association tests by using linear mixed effect models revealed an effect on survival only in the lower end of the QTL region, linked to markers M51760 and M52910 and constituting a 1.02 megabase region that includes potential candidates for the increased survival from the Kas-1 allele (\(P = 0.0029, 0.0122\); FDR-corrected \(P\) values= 0.0145, 0.0306; for the 2 markers, respectively).

### Discussion

Survival in a prolonged flooding is determined by a complex orchestration of distinct physiological and gene expression alterations at various stages of the stress. Thus, it may be difficult to relate changes in physiology and gene expression at very early stages of submergence tolerance to later stage survival. With this study, we identified genomic regions that increase submergence survival by performing survival assays for mature Kas-1/Col (\(gl1\)) RILs followed by a recovery period for a QTL analysis.

#### Submergence and Post-Submergence Light Conditions Effect Survival

Although we observed variation between submergence assays we performed with the parental accessions, Kas-1 was more tolerant to submergence independent of the media used or lighting conditions during submergence. These results are consistent with Vashisht et al. (2011) who also showed higher submergence tolerance in Kas-1 compared to Col-0 with similar assays.

Both parental lines survived longer when they were submerged in light or shaded conditions than in dark. In line with other studies, this indicates that Arabidopsis is capable of underwater photosynthesis (Lee et al. 2011; Vashisht et al. 2011), possibly leading to a higher survival by postponing starvation as seen with other plant species (Vervuren 2003; Mommer et al. 2006; Herrera 2013). In addition, the larger difference between the parental lines in submergence survival in the dark compared to light or shade might have been due to more effective scavenging of light in the less tolerant Col (\(gl1\)) leading to higher photosynthesis under water. Higher internal oxygen levels produced by photosynthesis in turn might have led to

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### Table 2. Results of composite interval mapping analysis for submergence tolerance, dark tolerance, size, and number of leaves for Kas-1/Col (\(gl1\)) RILs

<table>
<thead>
<tr>
<th>Trait</th>
<th>Chr.</th>
<th>Position</th>
<th>1-LOD interval</th>
<th>Additive effect</th>
<th>LOD score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submergence tolerance exp1 (days)</td>
<td>5</td>
<td>84.8</td>
<td>75.2–90.3</td>
<td>0.92</td>
<td>4.04</td>
</tr>
<tr>
<td>Submergence tolerance exp2 (days)</td>
<td>5</td>
<td>80.8</td>
<td>76.3–86.8</td>
<td>0.53</td>
<td>2.66</td>
</tr>
<tr>
<td>Dark tolerance exp1 (% survival)</td>
<td>4</td>
<td>2</td>
<td>0.0–4.1</td>
<td>25.55</td>
<td>7.75</td>
</tr>
<tr>
<td>Dark tolerance exp2 (% survival)</td>
<td>4</td>
<td>8</td>
<td>79.3–89.2</td>
<td>−7.15</td>
<td>2.84</td>
</tr>
<tr>
<td>Rosette size (cm(^2))</td>
<td>2</td>
<td>68.3</td>
<td>65.5–70.3</td>
<td>−8.63</td>
<td>3.21</td>
</tr>
<tr>
<td>Flowering</td>
<td>1</td>
<td>99.3</td>
<td>97.9–101.3</td>
<td>28.15</td>
<td>3.21</td>
</tr>
<tr>
<td>Leaf number</td>
<td>1</td>
<td>99.3</td>
<td>96.8–101.3</td>
<td>−0.41</td>
<td>4.17</td>
</tr>
</tbody>
</table>

Minus signs indicate a QTL that increases the trait value when the QTL is from Col (\(gl1\)) accession.

#### Figure 4. LT\textsubscript{50} values for RIL groups (a) under submergence, (b) under darkness, (c) dry weight, and (d) soluble carbohydrates of RIL groups at the start of the experiments. Significant differences are indicated for post hoc ANOVA results as “a” and “b” at \(P < 0.05\). (e) Schematic overview of chromosome 5 of the RIL groups used in the experiments. Arrow represents the location of the QTL. Light grey represents Col(\(gl1\)) background and dark grey represents Kas-1 background.
higher survival in this accession, which might have decreased the difference between the 2 accessions under light or shaded conditions.

Survival differences between the 2 soil/perlite medium assays, in which post-submergence lighting conditions were different, indicated that light also has an influence on survival after submergence during recovery period. The lower survival achieved when artificial lighting was present could be due to the inability to adapt to a sudden increase in light intensity, resulting in photo-oxidative damage to the photosynthetic machinery. When coupled with rapid accumulation of reactive oxygen species upon re-eration, this can hamper growth, which is normally achieved by photosynthetic acclimation and the subsequent carbohydrate production necessary for recovery after severe floods (Luo et al. 2009, 2011).

Candidate Genes in the QTL Region
A common submergence tolerance QTL, Come Quick Drowning 1 (CQD1), on chromosome 5 was detected in both QTL assays. Consistent with results of Li et al. (2006), 2 other QTL were mapped for flowering to chromosome 1 and 4, respectively. The Col (gl1) allele for the QTL on chromosome 1 delayed flowering and the effect on delay caused by Kas-1 on chromosome 4 constituted a major QTL. Col (gl1) has a loss of function deletion polymorphism on the FRIGIDA (FRI) gene that is responsible for the molecular basis of this QTL effect (Li et al. 2006). The QTL for leaf number overlapped with the flowering QTL on chromosome 1. These 2 traits related to growth parameters might be controlled by a common locus. A biomass QTL was detected in this region for Col-0/C24 RILs (Lisec et al. 2008) and might be the same locus controlling leaf number and flowering. The flowering QTL on chromosome 4 overlapped with the dark tolerance QTL from the 2 experiments. The locus causing a delay in flowering might increase the tolerance to stresses since it will also delay resource allocation to flowering. When plants undergo a stress, they might be able to use those resources to acclimatize and become more tolerant to darkness.

With our initial QTL analyses, we have defined the CQD1 locus to a 2.04 Mb region and a 1.02 Mb region by fine-mapping. Within such a relatively large region there are numerous possible candidate genes. Several of these were found to be differentially regulated under submergence in Col-0 (Lee et al. 2011) in the indicated region including ethylene response factor 2 (AREF2, AT5G47220), respiratory burst oxidase protein D (RBOHD, AT3G47910) and trehalose-6-phosphate phosphatase (AT3G51460). Of these genes, the latter 2 show variation in the inferred amino acid sequences between Kas-1 and Col alleles (Table 3), which might affect submergence tolerance. Increased trehalose content, which is a low-abundance carbohydrate, is correlated with a higher tolerance to several abiotic stresses (Chen and Murata 2002; Garg et al. 2002; Nuccio et al. 2015). An up-regulation is observed in Col-0 for trehalose-6-phosphate phosphatase under hypoxia and submergence (Liu et al. 2005; Lee et al. 2011). Moreover, a homolog of this gene was found to be important in anaerobic germination tolerance in rice (Kretzschmar et al. 2015). Thus, this gene is a strong candidate for controlling the difference in submergence tolerance between Kas-1 and Col (gl1). One of the other candidates, RBOHD, was shown to mediate signaling for diverse stresses such as wounding, heat, cold, high-intensity light, and salinity accompanied by the accumulation of reactive oxygen species (Miller et al. 2009). It is possible that RBOHD also controls responses to submergence stress, since RBOH homologs were shown to be involved in ROS-mediated aerenchyma formation in wheat (Yamauchi et al. 2013). Additionally, a mitochondrial substrate carrier family protein (ACP2, AT5G51050) and an auxin-responsive GH3 family protein (AtGH3.8, AT5G51470) are localized in this region. These genes, although not differentially regulated, might have an effect on submergence tolerance since they include single to several amino acid polymorphisms between the parental accessions. Carbohydrate and ATP levels can be determinants of submergence tolerance and ACP2 was shown to be acting as a Ca2+-regulated ATP-Mg/Pi transporter in Arabidopsis (Stael et al. 2011). During hypoxia, when mitochondrial oxidative phosphorylation is limited, ACP2 could balance ATP levels as a transporter and increase submergence tolerance. Ethylene is a major hormone for low oxygen signaling as it accumulates during low oxygen stress (Bailey-Serres and Voesenek 2008). Auxin-responsive GH3 genes constitute a large superfamily divided into 3 groups depending on their substrate for adenylation, either jasmonic acid, indole-3-acetic acid, or salicylic acid (Wang et al. 2008). The substrate of AtGH3.8 is still unknown, however other members of group II GH3s were shown to be involved in salicylic acid mediated

<table>
<thead>
<tr>
<th>Locus</th>
<th>Amino acid polymorphisms</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT5G51050</td>
<td>Yes</td>
<td>Mitochondrial substrate carrier family protein</td>
</tr>
<tr>
<td>AT5G51190</td>
<td>Yes</td>
<td>AP2 domain-containing transcription factor, putative</td>
</tr>
<tr>
<td>AT5G51350</td>
<td>Yes</td>
<td>Leucine-rich repeat transmembrane protein kinase, putative</td>
</tr>
<tr>
<td>AT5G51390</td>
<td>Yes</td>
<td>Unknown protein</td>
</tr>
<tr>
<td>AT5G51460</td>
<td>Yes</td>
<td>ATTPPA (Arabidopsis thaliana trehalose-6-phosphate phosphatase); trehalose-phosphatase</td>
</tr>
<tr>
<td>AT5G51470</td>
<td>Yes</td>
<td>Auxin-responsive GH3 family protein</td>
</tr>
<tr>
<td>AT5G51550</td>
<td>Yes</td>
<td>Phosphate-responsive GH3 family protein</td>
</tr>
<tr>
<td>AT5G51720</td>
<td>No</td>
<td>Similar to Os07g046720</td>
</tr>
<tr>
<td>AT5G51760</td>
<td>No</td>
<td>AHG1 (ABA-hypersensitive germination 1); protein serine/threonine phosphatase</td>
</tr>
<tr>
<td>AT5G51830</td>
<td>No</td>
<td>PkB-type carbohydrate kinase family protein</td>
</tr>
<tr>
<td>AT5G51890</td>
<td>No</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>AT5G51910</td>
<td>Yes</td>
<td>TCP family transcription factor, putative</td>
</tr>
<tr>
<td>AT5G52250</td>
<td>Yes</td>
<td>Transducin family protein/WD-40 repeat family protein</td>
</tr>
<tr>
<td>AT5G52300</td>
<td>Yes</td>
<td>LTI65/RD29B (responsive to dessication 29B)</td>
</tr>
<tr>
<td>AT5G52310</td>
<td>Yes</td>
<td>COR78 (cold regulated 78)</td>
</tr>
<tr>
<td>AT5G52450</td>
<td>No</td>
<td>MATE efflux protein-related</td>
</tr>
<tr>
<td>AT5G52710</td>
<td>Yes</td>
<td>Heavy-metal-associated domain-containing protein</td>
</tr>
<tr>
<td>AT5G52900</td>
<td>Yes</td>
<td>Similar to unnamed protein product (Vitis vinifera)</td>
</tr>
<tr>
<td>AT5G52910</td>
<td>Yes</td>
<td>ATIM (timeless)</td>
</tr>
</tbody>
</table>

All amino acid polymorphisms detected are amino acid replacements.
pathogen resistance (Jagadeeswaran et al. 2007; Nobuta et al. 2007). Since plants become more prone to pathogen infections after a heavy stress, AtGH3.8 might play a role in the recovery period by increasing resistance to pathogens after plants are de-submerged. Ethylene response factors (ERFs), especially group VII ERFs were shown to be key factors in low oxygen stress tolerance (Hinzel et al. 2010; Iacusa et al. 2010, 2011; Gibbs et al. 2011). An AP2 domain-containing transcription factor (ATSG51190) on the lower end of chromosome 5 might also increase submergence tolerance by inducing gene expression similarly to group VII ERFs, since these 2 groups have close homology within the ERF gene superfamily (Nakano et al. 2006). This gene and other candidates such as ATSG51460 (ATTPPA), ATSG51720 (similar to rice gene Os07g0467200), ATSG51760 (AHG1), and ATSG51830 (p5kB-type carboxyhydride kinase family protein) were also induced under 12 h of hypoxia in 7 day-old seedlings (Branco-Pierce et al. 2005) indicating roles in low oxygen response.

In conclusion, we detected a submergence tolerance QTL, CQD1, on the lower end of chromosome 5 by screening Kas-1/Col (gl1) RILs for submergence tolerance. This region includes several genes with potential as candidates effecting submergence tolerance. Since the functional validation is not available, the significance of these candidate genes is only suggestive. However, further confirmation on the effects of these genes to assess their significance and role for increased submergence tolerance is underway. Expanding these results by analyzing the natural variation present in other Arabidopsis accessions through association mapping and experimental gene expression studies can be powerful tools to understand the genetic control of submergence tolerance and improving survival of natural plant communities and our crops in the changing climate.

Supplementary Material
Supplementary data are available at Journal of Heredity online.

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Data Availability
Data deposited at Dryad: http://dx.doi.org/10.5061/dryad.bt713

References


