Quantitative trait loci analysis of nitrate storage in *Arabidopsis* leading to an investigation of the contribution of the anion channel gene, *AtCLC-c*, to variation in nitrate levels

Hisatomi Harada¹,*, Takashi Kuromori², Takashi Hirayama², Kazuo Shinozaki² and Roger A. Leigh³

¹ National Institute of Livestock and Grassland Science, 768, Senbonmatsu, Nasu, Tochigi, 329-2793, Japan
² RIKEN Genomic Sciences Center, 1-7-22 Suehiro-cho, Tsurumi-Ku, Yokohama 230-0045, Japan
³ Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK

Received 27 October 2003; Accepted 8 June 2004

Abstract

Storage of excess nitrate in the vacuole and its subsequent remobilization is an important aspect of a plant’s nitrogen economy, but the genes controlling the underlying processes have not all been identified and characterized. Cape Verdi Island (Cvi)/Landsberg erecta (Ler) and Columbia (Col)/Landsberg erecta recombinant inbred line (RIL) populations of *Arabidopsis thaliana* were used to identify quantitative trait loci (QTL) controlling natural variation in nitrate concentrations. One major and two minor QTLs were found for the Cvi/Ler population and one minor QTL for the Col/Ler RIL. These were designated NA1 to NA4. The major Cvi/Ler QTL (NA3) was located at the bottom of chromosome 5. No interaction among the QTLs was found by two-way ANOVA. By comparing *in silico* the locations of the QTLs with a physical map of the *Arabidopsis* genome, candidate genes for each QTL were identified. Several of these were anion channels of the *AtCLC* family. One of these, *AtCLC-c*, coincided with NA3 and its role was investigated using a mutant with a transposon insertion in *AtCLC-c*. Mutant plants homozygous for the insertion (designated clcc-1) had less than 5% of *AtCLC-c* mRNA compared with wild-type (WT) shoots. They also had significantly lower nitrate concentrations when grown at a range of external nitrate concentrations. The concentrations of chloride, malate, and citrate were also affected in the mutant. In wild-type plants, expression of *AtCLC-c* was down-regulated in the presence of nitrate, but ammonium had a much smaller effect while chloride and sulphate did not affect expression. These and published results suggest that multiple genes affect nitrate concentrations in plants and that *AtCLC-c* and other members of the *AtCLC* gene family play some role in this.

Key words: Anion channel, *Arabidopsis*, *AtCLC-c*, chloride, nitrate accumulation, QTL.

Introduction

Nitrate is a major nitrogen source for plants. When it is taken up in excess of immediate requirements, it is stored as free nitrate in the vacuole from where it can subsequently be remobilized when nitrogen supply is insufficient to meet demand (Martinoa *et al*., 1981; van der Leij *et al*., 1998). Although variations in nitrate storage between tissues, for example, leaf blades and petioles (Maynard *et al*., 1976; Steingröver *et al*., 1986) and between and within plant species (Baker *et al*., 1974; Ostrem and Collins, 1983; Reinink and Eenink, 1988; Reinink *et al*., 1994; Harada *et al*., 2003) have been reported, the underlying mechanisms contributing to this variation are not well understood. Greater understanding would not only give an insight into an important component of the nitrogen economy of plants, but could contribute to enhanced nitrogen-use efficiency in crops as well as decreasing human consumption of free nitrate in leafy crops, which can account for 70% of dietary...
nitrate intake (Ysart et al., 1999). It is thought that the intake of nitrate raises the risk of gastric cancer through the production of N-nitroso compounds in the stomach (Bruningfann and Kaneene, 1993a, b; Hill, 1999).

Studies using mutants and transgenic plants have revealed a number of genes that can affect the concentration of nitrate in a plant including those encoding nitrate reductase (Warner and Huffaker, 1989; Quilleré et al., 1994; Curtis et al., 1999; Scheible et al., 1997), a putative anion channel, AtCLC-a (Geelen et al., 2000), glutamine synthetase, and ferredoxin-dependent glutamate synthase (Hauser et al., 1994). However, other genes must also be involved because Loudet et al. (2003), using Arabidopsis Bay-0 and Shahidara recombinant inbred lines (RILs), identified eight quantitative trait loci (QTLs) for nitrate content on a dry matter basis. The genes contributing to these QTLs have not been identified nor tested to confirm their role in nitrate accumulation. In maize, Hirel et al. (2001) identified five QTLs for nitrate content in dry matter, one of which included a gene encoding glutamine synthetase.

In this study, a QTL analysis for shoot nitrate accumulation on a fresh weight basis was conducted using Arabidopsis Cape Verdi Islands and Landsberg erecta (Cvi Ler) and Columbia and Landsberg erecta (Col Ler) RILs. The role of a candidate gene, AtCLC-c, for one of the QTLs was tested using a transposon-tagged mutant. Phenotypic and gene expression analysis of the mutant and WT suggested that this gene does have a role in controlling nitrate concentrations in Arabidopsis.

Materials and methods

Plants and culture conditions

The seeds of RIL populations of the Arabidopsis thaliana parent ecotypes Cape Verdi Islands and Landsberg erecta (Cvi Ler, 98 lines) and Columbia and Landsberg erecta (Col Ler, 98 lines) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The other experiments were performed using ecotype Nossen.

For the QTL analysis, the Cvi Ler and Col Ler RILs were grown for 2 weeks on rock wool moistened with nutrient solution (1.5 mM KH2PO4, 0.25 mM K2HPO4, 1.5 mM MgSO4, 7.5 mM Ca(NO3)2, 67 mM Na2EDTA, 9 mM FeSO4, 10 mM MnSO4, 30 mM H3BO3, 1 mM ZnSO4, 1 mM CuSO4, 0.02 mM (NH4)6Mo7O24, and 0.1 mM CoSO4). The Cvi Ler RILs were grown in a greenhouse in Cambridge with 16 h light and 8 h dark supplemented with artificial light as needed. All other plants were grown in a growth chamber at 22 °C with continuous light (200 μmol m−2 s−1). When varied, the nitrate concentration was adjusted with Ca(NO3)2 and, if necessary, CaSO4 was supplied to maintain a minimum of 2.5 mM Ca. Chloride was supplied as Ca salts. For one experiment, plants were grown initially on an agar medium made up in the above nutrient solution and containing 5 mM nitrate, 5 mM chloride, 10 g l−1 sucrose and 9 g l−1 agar. After 12 d they were transferred to aerated nutrient solution with the same nitrate and chloride concentrations and grown for 4 d before harvesting. To prevent depletion of nutrients, the rock wool was washed with a 2X volume of the nutrient solution at 6, 4, and 2 d before sampling, or the culture medium was changed at similar times. At harvest, 6–10 plants were taken as one sample for chemical analysis. For molecular analysis, plants were grown on agar in a clear polystyrene box (100 mm long×140 mm wide×10 mm high). Six to ten plants were harvested as one sample. To study the effects of nitrate and other ions on gene expression, seeds were germinated on an agar plate with 2.5 mM Ca(NO3)2. After 6 d, the plants were transferred to a nitrate-free agar plate for a further 6 d and then 10 ml of water or treatment solution were added to the plate. RNA samples were prepared from plants collected at the specified times after treatment.

QTL analysis

A set of 241 and 106 molecular markers for Cvi Ler and Col Ler RILs with average spacing of approximately 2 cM and 5 cM, respectively, was used for the QTL analysis. The computer program MAPMAKER 3.0 (Lander et al., 1987) was used to calculate genetic distances between molecular markers for the Cvi Ler RILs. For the Col Ler population, the genetic map of molecular markers published by NASC was used (http://nasc.nott.ac.uk/). Another program, MQTL, (Tinker and Mather, 1995) was used to link QTLs to molecular markers. MQTL performs a simple interval mapping using the linear model described by Haley and Knott (1992), which gives results that are very similar to those obtained with the maximum-likelihood methods used in Mapmaker/QT (Lincoln et al., 1993). The test statistic threshold for QTL declaration was determined by a permutation test with 2000 replications to keep the probability of a type I error below 5% (Tinker and Mather, 1995). Three independent analyses of the variation in nitrate concentrations was conducted with each RIL population and there was broad agreement between them. The percentage of variance explained by each QTL was calculated using the nearest marker data. A cytoplasmic effect in the Cvi Ler population was not found by one-way ANOVA. Interaction between QTLs was calculated by two-way ANOVA.

DNA extraction, RNA extraction, reverse transcription of RNA, and real-time PCR

Preparation of DNA was performed as described by Lister et al. (2000). RNA was extracted with the TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription of RNA was conducted using a Truescript II Reverse Transcription Kit (Sawady). The 20 μl reaction mixture containing 1 μg RNA, 10 pmol oligodT primer, 20 nmol dNTP, 4 μl of 10X RT buffer, 20 units of RNase inhibitor, and 100 units of TrueScript II reverse transcriptase. Before the reaction, RNA was pretreated at 60 °C for 10 min and then cooled on ice. The reaction was started at 42 °C, increased to 60 °C over a 30 min period, held at 60 °C for 15 min and then cooled. The same amount of RNA was included in all reaction mixtures for reverse transcription. Thus, the relative AtCLC-c expression was standardized on a total RNA basis. In preliminary experiments, the patterns found in Figs 5 and 9 (see Results) were not changed when an actin gene was used as an internal standard.

The following primers were used in PCR and RT-PCR:

4RC: 5′-TCAAAATCCTGGCAGCAACTCTCAAATATAG-3′
5RC: 5′-AGCTAACATTGTCCTTCCAGAAAAGCTTG-3′
6RC: 5′-AACAGATCGTGAGGAGAGAGTGG-3′
7RC: 5′-CCTCTATATCATGATGGTCTCCTTC-3′
DS3R: 5′-CAAGGCTTTGCTATGGGCAAA-3′
DS3: 5′-CCGCCCGCGAGTAAATATAG-3′
DSS5: 5′-CTCTAAACACCCAGGGTCAGTCTTCTTCT-3′
DS3: 5′-TACCTCGGTTGAAATCGAT-3′.

PCR was performed in a 20 μl reaction mixture containing 1 μl of DNA template solution, 1 μl of Ex-Taq DNA polymerase (Takara), 1.6 μl of Ex-Taq buffer, 5 pmol dNTP, and 6 pmol of sense and anti-sense primers. The temperature program was 95 °C for 30 s followed by 35 or 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s or 60 s. Finally the solution was cooled to 4 °C.
Quantification of cDNA was performed by real-time PCR using a LightCycler 2.0 (Roche Diagnostics). The reaction mixture contained 1 μl of the medium from the completed reverse transcription reaction (see above), 60 nmol MgCl₂, 6 pmol of the 4RC and 5RC primers, and 2 μl of FastStart DNA Master SYBR Green I (Roche Diagnostics) in a final volume of 20 μl. The temperature programs was 95 °C for 10 min; 40 cycles of 95 °C for 10 s, 65 °C for 5 s, and 72 °C for 30 s. The reaction was then cooled to 40 °C. Relative amounts of AtCLC-c were quantified by using different dilutions of PCR product. The PCR product was checked by electrophoresis and melting point analysis. No fragments resulting from genomic DNA were detected.

Screening and genetic analysis of the clcc-1 mutant
A mutant, clcc-1, containing the maize Dissociation (Ds) transposon inserted into AtCLC-c in ecotype Nossen was obtained from the 16-0461-1 line using the in silico screening system developed at RIKEN (Kuromori et al., 2004). Hetero- or homozygosity for the clcc-1 locus in T₃ plants of line 16-0461-1 were distinguished by PCR. The plants were also tested for hygromycin resistance. The AtCLC-c of WT and heterozygote plants produced a 219 bp PCR fragment with primers 6RC and DS3R, while the insertional mutant produced no fragment. In addition, primers DS3-4 and DS3R gave a 244 bp fragment with the mutant and heterozygotes, but no product with WT DNA. The clcc-1 plants used in the experiments were homozygous for the transposon insertion into AtCLC-c.

Chemical analyses
Plants were harvested, weighed, and dried at 80 °C for 2 d. Nitrate was extracted from dried plants with deionized water for 2 h at room temperature. The nitrate concentration in the extract was determined by the method of Cataldo et al. (1975) for Cvi/Ler RILs or ion-chromatography for the other experiments. The latter used an Excelpak ICS-A23 column (Yokokawa) with a carrier of 3 mM Na₂CO₃ and a flow rate of 1 ml min⁻¹. The change in conductivity by nitrate and other anions was detected after Na₂CO₃ was removed by 15 mM H₂SO₄ in the suppressor. For total N analysis, dried plants were placed in a test tube and digested with H₂SO₄ containing 3% (w/v) salicylic acid. The tube was firstly heated at 120 °C for 4 h. To promote digestion, 0.1 ml of H₂O₂ was added twice. When the volume of ethanol was less than 0.1 ml, the tubes were cooled. The extract was diluted in distilled water and pretreated with 1.5 ml of hot 80% (v/v) ethanol in a boiling water bath. The organic acids in the extract were separated by high performance liquid chromatography using a 0.45 μm pore size membrane filter. The organic acids in the extract were separated by high performance liquid chromatography using a Shim-pak SCR-101H column (Shimazu) with a carrier of 4 mM p-toluene sulphonic acid at 40 °C. The change in the conductivity of the solution caused by an organic acid was detected after the addition of a solution of 4 mM p-toluene sulphonic acid, 16 mM bis(2-hydroxyethyl)aminomethane (hydroxymethyl)methane and 0.08 mM Na₂EDTA. Chloride, phosphate, and sulphate in the extracts were quantified by ion chromatography as described above.

Results
Genetic mapping and candidate genes for nitrate accumulation
QTL analysis was conducted using Cape Verdi Island (Cvi)/Landsberg erecta (Ler) (Alonso-Blanco et al., 1998) and Columbia (Col)/Ler (Lister and Dean, 1993) RIL populations. The nitrate concentration in the growth medium was set at 15 mM because, in preliminary experiments using Col, Ler, and Cvi ecotypes, this nitrate concentration gave higher broad sense heritability (the ratio of the genotypic variation to the total phenotypic variation) than lower concentrations, probably because 15 mM nitrate gave near-maximum nitrate concentrations in the shoots of the plants (not shown). Nitrate concentrations in both RIL populations were normally distributed (Fig. 1). The difference in nitrate concentrations between Cvi and Ler was 18 μmol g⁻¹ FW, while that between Col and Ler was 7 μmol g⁻¹ FW. Broad sense heritability was 0.760 and 0.574 for Cvi/Ler and Col/Ler RILs, respectively. The difference in mean nitrate concentrations for Ler in the two experiments is probably due to the different growth conditions used (greenhouse in Cambridge for Cvi/Ler, growth room in Japan for Col/Ler).

Using the MQTL computer program, (Tinker and Mather, 1995) three QTLs were found for the Cvi/Ler RILs. These were designated NA1, NA2, and NA3 and were located at the bottom of chromosome 4, and the top and bottom of chromosome 5, respectively (Fig. 2). Together, these QTLs accounted for 59% of the variance in nitrate concentrations in the Cvi/Ler population (Table 1). To test the interaction between these QTLs, the Cvi/Ler lines were grouped by a flanking marker for each of NA1,
NA2, and NA3 and the nitrate concentrations of the groups were compared (Fig. 3). The combinations of NA1–NA2 and NA2–NA3 were additive, while the effect of NA1 seemed to be influenced by the genotype of NA3. However, no significant interaction between NA1, NA2, and NA3 was detected by two-way ANOVA. With the Col/Ler RILs, one QTL for nitrate accumulation, located on chromosome 1, was identified (Fig. 2) and designated NA4. Although four QTLs were identified, the test statistics and QTL effect for NA3 were greater than those for the other QTLs suggesting that NA3 includes a major gene for nitrate accumulation.

Candidate genes for the four QTLs were screened in silico by comparing the locations of the QTLs with the loci of genes that might be involved in nitrate accumulation. The genes tested were those encoding enzymes of the nitrate assimilation pathway, nitrate transporters, anion channels, and tonoplast H+-pumps. The candidate genes were identified when their loci coincided with the QTL. Although the molecular markers for Cvi/Ler RILs populations could not be mapped to the physical and genetic map, common molecular markers between Cvi/Ler RILs and Col/Ler RILs (Alonso-Blanco et al., 1998) were used to locate NA1, NA2, and NA3 on the map for Col (Table 1).

NA1 overlapped with the loci of the artificial chromosome clones T4L20 (IV, 83 cM) and F20B18 (IV, 76 cM) encoding the genes of subunit c and G, respectively, of vacuolar H+-ATPase, a nitrate-sensitive enzyme, and also with clone F15J1 (IV, 84 cM) encoding a putative anion channel AtCLC-e. NA2 and NA3 covered the loci of F21E1 (V, 13 cM) and MYN8 (V, 109 cM) encoding ferredoxin-dependent glutamate synthase (Fd-GOGAT, GLU1) and NADH-dependent glutamate synthase (NADH-GOGAT), respectively. In addition, NA3 overlapped with clone K9P8 (V, 106 cM) encoding an anion channel AtCLC-c. NA4 coincided with the locus of F20N2 (I, 83 cM) coding a putative anion channel AtCLC-f.

Thus, three putative anion channels, AtCLC-c, AtCLC-e, and AtCLC-f, were potential candidate genes contributing to variation in nitrate accumulation. A disruption mutant of AtCLC-a has been shown to have a low nitrate content, suggesting that this gene has some involvement in determining tissue nitrate concentrations (Geelen et al., 2000). Therefore, further experiments investigated the contribution of AtCLC-c to NA3, the major QTL identified in the analysis.

![Fig. 2. Location of QTLs for nitrate concentrations in the Cvi/Ler (top and middle graphs) and Col/Ler (bottom graph) RILs. Test statistics were calculated using the MQTL computer program and the threshold test statistic for QTL declaration (horizontal lines) was determined by a permutation test with 2000 replications to keep the probability of a type I error below 5% (Tinker and Mather, 1995). The positions of some markers are shown.](image)

**Table 1. QTL for nitrate and potassium accumulation of Cvi/Ler and Col/Ler Arabidopsis RILs populations**

QTLs were analysed using MQTL, computer software (Tinker and Mather, 1994). The threshold for a type I error rate of 5% was calculated by 2000 times replicated permutation test. The QTL effect is expressed as Ler standard.

<table>
<thead>
<tr>
<th>QTL</th>
<th>RILs</th>
<th>Position (Chromosome:cM)</th>
<th>Flanking marker</th>
<th>Markers and position for Col/Ler RILs&lt;sup&gt;a&lt;/sup&gt; (Marker cM)</th>
<th>Test statistics</th>
<th>Threshold &lt;sup&gt;b&lt;/sup&gt;</th>
<th>QTL effect (μmol g&lt;sup&gt;-1&lt;/sup&gt; FW)</th>
<th>PVE&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA1</td>
<td>Cvi/Ler</td>
<td>IV:71</td>
<td>GB.490C</td>
<td>PG11(75)—mi369(104)</td>
<td>8.6</td>
<td>8.3</td>
<td>8.5</td>
<td>11</td>
</tr>
<tr>
<td>NA2</td>
<td>Cvi/Ler</td>
<td>V:6</td>
<td>AD.292L</td>
<td>G3715(7)—m217(19)</td>
<td>12.0</td>
<td>9.7</td>
<td>–9.4</td>
<td>13</td>
</tr>
<tr>
<td>NA3</td>
<td>Cvi/Ler</td>
<td>V:96</td>
<td>FD.345C</td>
<td>m331(104)—m435(109)</td>
<td>41.9</td>
<td>9.7</td>
<td>15.9</td>
<td>35</td>
</tr>
<tr>
<td>NA4</td>
<td>Col/Ler</td>
<td>I:86</td>
<td>mi230</td>
<td>m230</td>
<td>13.2</td>
<td>8.6</td>
<td>11.5</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup> Common markers for Cvi/Ler and Col/Ler RILs mapped by Alonso-Blanco et al. (1998) and numbers in parentheses indicate markers position of Col/Ler RILs expressed by centiMorgan.

<sup>b</sup> Percentage variance explained. Mapping data for DNA markers in RILs population is derived from NASC.
Isolation and molecular analysis of the AtCLC-c tagged mutant clcc-1

A collection tagged with the maize Dissociation (Ds) transposon was screened in silico for lines with insertions in AtCLC-c (Kuromori et al., 2004). This identified line 16-0461-1 and from 18 progeny of the T3 generation of this line, four plants homozygous for a transposon insertion into AtCLC-c were identified by PCR using the 6RC/DS3R and DS3-4/DS3R primer combinations. These plants gave no product with the 6RC/DS3R primers, but a 244 bp fragment with the DS3-4/DS3R combination. They were designated cclc-1 and were used in further experiments. Visually, these plants were indistinguishable from WT. Twelve of the plants from the T3 generation were heterozygous for the insertion (products with both the 6RC/DS3R and DS3-4/DS3R primer combinations) and showed a segregation ratio of 3:1 for resistance and sensitivity to hygromycin (by chi-square test). This strongly suggests that these T3 plants have only one transposon insertion. In further support of this conclusion, Martienssen (1998) has shown that 90% of Arabidopsis lines mutated with the Ds transposon carry a single Ds element and only 5% of the lines have multiple elements. Thus the genetic analysis strongly suggests that 16-0461-1 line has only a single transposon and this is in AtCLC-c.

The insertion site of the transposon was investigated by sequencing the PCR product amplified with the transposon border and AtCLC-c specific primer combinations DS3-4/DS3R and DS5-3/DS5L. The site was in the first exon between Gly 17 and Gly 18 (Fig. 4). Alignment of the AtCLC-c and AtCLC-c-1 genomic sequences derived from PCR amplification with primers DS5-3/DS5L and DS5L/DS3R showed that a stop codon was introduced 48 bp downstream of Gly 17 by the transposon insertion (Fig. 4b).

In RT-PCR, the primer combination of 6RC/DS3R produced a 219 bp cDNA fragment from WT plants while, as expected, no fragment was amplified for clcc-1 (Fig. 5a). By contrast, the primers 6RC/7RC and 5RC/4RC covering both sides of the insertion site produced RT-PCR fragments for both WT and clcc-1. This indicates that there is transcription to mRNA downstream of the insertion site in AtCLC-c-1. However, real-time PCR after reverse transcription showed that the expression levels of AtCLC-c in the mutant were only 5% and 0.3% of those in WT shoots and roots, respectively (Fig. 5b). Transcription of both sides of the insertion site suggests that clcc-1 might not be
a true knockout plant, but the real time PCR confirms that the transposon insertion greatly affects the quantity of \( \text{AtCLC-c} \) mRNA in \( \text{clcc-1} \).

**Nitrate accumulation in the \( \text{clcc-1} \) mutant**

Over a range of external nitrate concentrations, the \( \text{clcc-1} \) plants showed significantly and consistently lower nitrate concentrations in their shoots than WT plants (Fig. 6). This difference in nitrate concentration was maintained in the presence of 5 mM chloride and the \( \text{clcc-1} \) plants also accumulated less chloride than WT in this treatment (Figs 7, 8). However, when the plants were grown in the presence of 10 mM chloride, the difference between \( \text{clcc-1} \) and WT disappeared for both nitrate and chloride (Fig. 7). Chloride at both concentrations depressed nitrate accumulation. The shoot concentrations of some other anions, notably, citrate (decreased) and malate (increased) were also affected, compared with WT, when \( \text{clcc-1} \) was grown in a solution containing both 5 mM nitrate and 5 mM chloride (Fig. 8). For all anions, the concentrations in the shoots of the heterozygous \( \text{AtCLC-c/AtCLC-c-1} \) plants were not different from those in WT. The effects on malate and citrate were not investigated further but, together with the effects on chloride concentrations, the results indicate that the phenotype of \( \text{clcc-1} \) does not affect just nitrate concentrations. No significant differences were observed between WT and \( \text{clcc-1} \) for growth or the total concentrations of N, P, K, Ca, and Mg (data not shown). In the experiment shown in Fig. 8, the shoot chloride concentration in the three genotypes was not significantly different when analysed by Tukey’s multiple comparison test, but the difference between \( \text{clcc-1} \) and WT was significant when tested by ANOVA so the results do not contradict those in Fig. 7.

**Repression of \( \text{AtCLC-c} \) mRNA transcription by nitrate**

Decreased expression of \( \text{AtCLC-c} \) mRNA was observed within 0.6 h when \( \text{Ca(NO}_3)_2 \) was supplied to nitrogen-starved WT plants (Fig. 9). The decrease continued for 5 h (Fig. 9), but did not change thereafter (not shown).
Ammonium sulphate also decreased expression, but by a smaller amount than with Ca(NO₃)₂, indicating there is a nitrate-specific repression of the expression of AtCLC-c. Chloride and sulphate had no effect on the expression.

Discussion

QTL analysis of nitrate storage

Nitrate storage in vacuoles is affected by multiple processes including the relative rates of nitrate uptake, nitrate reduction and assimilation, and uptake into and export from the vacuole. Therefore, numerous gene products can potentially influence naturally occurring variation in free nitrate levels at the whole tissue level. In this study, QTLs for nitrate accumulation were demonstrated using Arabidopsis RILs and a major QTL and three minor QTLs were identified.

For the Cvi/Ler lines, NA1 to NA3 explained 59% of the total phenotypic variance (Table 1) and covered 78% (0.59/0.76) of the genetic variance since the broad sense heritability was 0.76. For Col/Ler lines, NA4 explained only 13% and 23% of the total phenotypic and genetic variance, respectively. The identification of only one minor QTL in the Col/Ler lines probably indicates there are only small phenotypic differences between relevant alleles in the parental lines. In addition, the broad sense heritability was relatively small and this will have limited the sensitivity with which minor QTLs could be detected. Loudet et al. (2003) working with a Bay-0 and Shahdara RIL population of 415 lines identified eight QTLs affecting nitrate concentration. One of these QTLs, NO10.8 (chromosome 5, 3.1 cM) overlapped with NA1, but the others did not match any of the QTLs identified here. This may partly be explained by the way nitrate concentrations were expressed. Here they were calculated relative to fresh weight while Loudet et al. (2003) used dry weight. The nitrate concentrations in dry matter were highly and positively correlated with tissue water content and half of the QTLs for nitrate content overlapped with QTLs for water content. Nonetheless, these earlier findings together with those described here, confirm that the variation in nitrate accumulation among Arabidopsis ecotypes is polygenic.

Candidate genes for the QTLs

Nitrate uptake is the first step in nitrate accumulation. There are at least three kinds of nitrate transporters in plants: a constitutive low-affinity transport system and constitutive and inducible high-affinity nitrate-transport systems (Forde...
and Clarkson, 1999). Recently, Harrison et al. (2004) found that expression of high-affinity nitrate transporters is positively correlated with shoot nitrate content in RILs of *Lotus japonicus*. However, none of the QTLs identified here overlapped with the loci of genes likely to encode these transporters. Further, none correspond with nitrate reductase genes, even though there is good evidence for a role for this enzyme in determining nitrate storage (Warner and Hüfner, 1989; Quilléré et al., 1994; Curtis et al., 1999; Scheible et al., 1997; TA Cuin and RA Leigh, unpublished results). However, the identification of QTLs depends on functional differences between the genes of the parent genotypes and the lack of correspondence between QTLs and the locus of a particular gene does not necessarily indicate a lack of involvement of that gene in the process investigated.

Hausler et al. (1994) reported that, in barley, the nitrate concentration is affected by GS and GOGAT activities and Hirel et al. (2001) found that one of the five QTLs they identified for nitrate content in maize was coincident with a locus encoding an isozyme of GS. NA2 and NA3 overlapped with the loci of Fd-GOGAT and NADH-GOGAT, respectively. Although a knockout mutant for NADH-GOGAT has been identified (Lancien et al., 2002), the most significant effects on growth and nitrogen assimilation were only seen when it was grown in 1% CO₂. In addition, the enzyme’s role is probably in nitrogen assimilation in roots and the leaf vasculature, with Fd-GOGAT being more important in leaf mesophyll cells where nitrate is stored (Lancien et al., 2002). Hence it is unlikely that the major QTL, NA3, is related to activity of NADH-GOGAT encoded by the gene that coincides with this QTL.

The rate of nitrate transport into and out of the vacuole will also determine the level of stored nitrate. Gene products likely to be important in this include primary V-ATPase and V-PPase H⁺-pumps which provide the driving force for ion transport across the tonoplast, and secondary transport systems such as anion channels for passive nitrate uptake into the vacuole at low vacuolar nitrate concentrations (Pope and Leigh, 1987), and H⁺-linked cotransporters mediating active nitrate transport into the vacuole (Miller and Smith, 1992). The activity of the V-ATPase and expression of its subunits are both influenced by nutrition and salinity, and the enzyme is nitrate sensitive (Perera et al., 1995; Lüttgte and Ratajczak, 1997; Fisher-Schliebs et al., 2000). NA1 overlapped the locus of the gene encoding subunits c and G of the V-ATPase. This may indicate some role for the V-ATPase in regulating nitrate concentrations in the vacuole, but because NA1 is a relatively minor QTL, this aspect was not pursued further.

Anion channels play a role in cell signalling, osmoregulation, plant nutrition, and metabolism (Barbier-Brygoo et al., 2000), and mediate nitrate transport across intracellular membranes including the tonoplast. The *AtCLC* genes encode a six-member family of anion channels and an Arabidopsis mutant disrupted in *AtCLC-a* showed a decrease in nitrate concentration without any effects on growth or the concentrations of chloride, sulphate, and phosphate (Geelen et al., 2000). Although the intracellular location and functional properties of *AtCLC-a* have not been elucidated, it has been shown to be induced by nitrate, making it a candidate for a tonoplast nitrate channel. NA1, NA3, and NA4 overlapped with the loci of *AtCLC-e, AtCLC-c,* and *AtCLC-f,* respectively, suggesting that these other family members may also play a role in nitrate storage. As NA3, the major QTL of the four QTLs identified, overlapped with the locus of *AtCLC-c,* the possible role of this gene was investigated using an insertional mutant, clcc-1.

### Role of AtCLC-c in nitrate storage

The clcc-1 mutant has a transposon insertion within the N-terminal part of *AtCLC-c* open reading frame. The insertion of the transposon dramatically decreased *AtCLC-c* mRNA transcription and/or stability although there remains a small possibility that the function of *AtCLC-c* was not completely lost. The homozygous clcc-1 plants showed normal growth and development, but had decreased nitrate concentrations compared with WT, confirming a potential role in nitrate storage. Thus both *AtCLC-c* (Figs 6, 7) and *AtCLC-a* (Geelen et al., 2000) have some role in nitrate accumulation. However, unlike the *AtCLC-a* mutant, the cclc-1 plants also had altered concentrations of other anions, notably chloride, malate, and citrate, which may indicate that *AtCLC-c* has a broader anion specificity than *AtCLC-a.* This is supported by the observation that *AtCLC-c* complemented a chloride channel mutant in yeast (Schwappach et al., 1998). In addition, the transcription of *AtCLC-a* and *AtCLC-c* is regulated differently by nitrate. The transcription of *AtCLC-a* was stimulated by nitrate (Geelen et al., 2000) while that of *AtCLC-c* was repressed. However, the repression by nitrate did not seem to have a dramatic effect on the nitrate concentration since the heterozygous plants showed a similar nitrate concentration to the WT plants.

Thus the QTL analysis has revealed a number of loci involved in nitrate accumulation that are additional to those identified by Loudet et al. (2003) and the loci of a number of plausible candidate genes coincide with each of the QTLs identified here. The phenotype of clcc-1 is consistent with a role for *AtCLC-c* in nitrate storage. However, further evidence, such as functional characterization, confirmation of its cellular location, and complementation of the mutant with WT *AtCLC-c* will be required to confirm this role fully.

### Acknowledgements

The work in Cambridge was performed while HH was holding a fellowship provided by the Japan Science and Technology Agency.
References


Lister C, Dean C. 1993. Recombinant inbred lines for mapping RFLP and phenotypic markers in Arabidopsis thaliana. The Plant Journal 4, 745–750.


