Abstract

Stomata regulate exchange of water and carbon between plants and atmosphere. Guard cells which form stoma sense and respond to CO_2; however, the mechanisms and the protein that binds to CO_2 are still unknown. Such a response is particularly important due to constantly rising atmospheric CO_2. This study aimed to find the quantitative trait loci (QTL) related to stomatal CO_2 responsiveness in *Arabidopsis thaliana* after finding a right candidate pair for QTL mapping. Seeds of 10 different ecotypes were sown and then grown in a controlled growth chamber at 400 µmol mol^{-1} of CO_2 concentration. Then *in situ* leaf stomatal conductance (g_s) was measured at 200, 400 and 800 µmol mol^{-1} of CO_2 concentration with gas exchange instrument for one mature rosette leaf of each five-week-old plant. The g_s value which changed less than 2% over five minutes was recorded as the steady-state for each level of CO_2. Leaf mass per unit area (LMA), stomatal density, stomatal length and maximum stomatal conductance of CO_2 (g_{smax}) were also estimated for each plant. We finally found that stomatal apertures decline and increase at CO_2 levels above and below the growth concentration of 400 µmol mol^{-1}, respectively. ANOVA single factor revealed Col-0 and C24 were different in g_s at 400 µmol mol^{-1} and in relative response to increased CO_2 levels; they also differed in stomatal density and length and g_{smax}. This finding was confirmed by a second experiment. Thus, Col-0 and C24 were selected as the candidate pair for QTL analysis. Experimental population for QTL mapping was 42 samples of F2 generation from a cross between Col-0 and C24 grown in the same condition as before. g_s was measured for five-week-old plants at 400 and 800 µmol mol^{-1} of CO_2 concentration. However, genetic analysis was only performed for 12 samples, six samples that showed the highest and six that showed the lowest g_s, due to time constraints using 20 simple sequence length polymorphism markers. The locus of the trait stomatal CO_2 response could not be detected in this study although initial comparison of phenotypes and genotypes by QTLNetwork-2.0 indicated that the QTL is positioned on chromosome IV. However, plants with weak responses showed genotype of strong responses and vice versa, opposite to what expected.

Introduction

Stomata are crucial in regulating the exchange of water and carbon between the terrestrial vegetation and atmosphere. They play a major role in biogeographic distribution, ecosystem hydrology (Morison 1998, Gedney *et al*. 2006), plant air pollution sensitivity (Sitch *et al*. 2007) and also local and regional climate change due to land surface energy partitioning (Bonan 2008, de Boer *et al*. 2011). Stomatal aperture is formed by two guard cells in the plant epidermis which sense and directly respond to atmospheric CO_2 concentration ($[CO_2]$). Stomata permit simultaneous uptake of $[CO_2]$ meanwhile they control transpirational water loss and enable leaves to optimize water-use efficiency (WUE) during photosynthesis. WUE (uptake of $[CO_2]$ per unit of water loss) depends on CO_2 concentration in intracellular space (C_i). In fact, stomata respond to C_i which links stomata aperture to mesophilic demand of CO_2 for photosynthesis. (Mott 1988, Morison 1998, Cowan and Farquhar 1998)

Stomatal response to $[CO_2]$ was initially demonstrated in 1940 by Freudenberger (Morison 1998). During the last decades, $[CO_2]$ response of stomata has directed numerous studies towards understanding the stomatal physiology especially due to possible response of plants to increasing $[CO_2]$ and the importance of stomata in land plant evolution. It has been shown that elevation of ambient $[CO_2]$ in both controlled environment and free-air CO_2 enrichment (FACE) leads to partial stomatal closure which results in reduction of stomatal conductance (g_s) and increase of photosynthesis (Assmann 1999, Ainsworth and Rogers 2007, Hu *et al.* 2010). Moreover, elevation of $[CO_2]$ in FACE experiment has stimulated light-saturated photosynthesis in C_3 species C4 photosynthesis is not affected by CO_2) and varied with the environment and functional group (Ainsworth and Rogers 2007). Nonetheless, although RUBISCO (riboluse-1,5-biphosphate carboxylase/oxygenase) and PEPC (phosphoenolpyruvate carboxylase) are CO_2/HCO_3^--binding proteins; $[CO_2]$-triggered stomatal closure response has been shown to be independent of both RUBISCO activity and PEPC level (Hu *et al.* 2010). Also photosynthetic capacity and nitrogen source affect the capacity of carboxylation acclimation via modulating the response of photosynthesis to elevated $[CO_2]$ (Ainsworth and Rogers 2007).

For the stomata to close in response to environmental stimuli such as high $[CO_2]$, guard cell membrane requires to be depolarized (Assmann 1999) in which SLOW ANION CHANNEL 1 (SLAC1) plays a central role by controlling guard cell turgor pressure (Chen *et al*. 2010). Abscisic acid (ABA) is also crucial in signal transduction from the stimuli (Chen *et al*. 2010); however, the signal transduction pathways on the upstream of anion channel are not yet known (Assmann 1999, Schroeder *et al*. 2001, Ainsworth and Rogers 2007) although free Ca^{2+} in cytosol, membrane and ion channel, ATP derived via photosynthesis, levels of zeaxanthin in chloroplast, phosphorylation/dephosphorylation of protein and PH ingredient in cytoplasm and apoplast have been reported as parts of a signaling network in guard cell for stomatal response to ABA and light (Assman 1999, Ainsworth and Rogers 2007). Nonetheless, the protein that binds to CO_2 to control stomatal response is still unknown (Hu *et al.* 2010). However, it is known that the catalyst proteins carbonic anhydrase (CA) which act on the reversible reaction of $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$, functions in the upstream of CO_2 signaling pathway and CO_2/HCO_3^- transfers the signal to anion channel regulation (Hu *et al.* 2010). Also as the negative regulator when stomata close due to CO_2 induction, HIGH LEAF TEMPERATURE 1 (HT1) protein kinase is so far proposed as the only protein in *Arabidopsis* to function upstream of where ABA signaling pathway and CO_2 converge (Ainsworth and Rogers 2007, Hu *et al.* 2010). Therefore, further understanding of molecular mechanisms and discovering the CO_2–binding protein for regulation of stomatal CO_2 response is required.

Physiological short-term responses of stomatal conductance (g_s) to elevated $[CO_2]$ may differ from long-term effects of growth under elevated $[CO_2]$ on g_s. Regarding a direct short-term response to

elevated [CO_2] it is stomatal aperture that responds and g_s is reversibly reduced in most species (Morison 1998). On the other hand, growth under elevated [CO_2] causes long-term cumulative effects on plants development and structure, particularly in C_3 species (Ward and Kelly 2004). CO_2 concentration affects carbon fixation rate which consequently has an impact on plant growth, functioning and reproduction (Norby *et al*. 1999, Pritchard *et al*. 1999, Kinugasa *et al*. 2003, Ward and Kelly 2004). In the long-term, CO_2-induced change of g_s results from changes in stomatal density and size as well as the relative change in the opening degree of stomatal aperture (Morison 1998). Such changes subsequently influence water status and amounts of carbohydrates and nutrients (Morison 1998, Lodge *et al*. 2001). Furthermore, the C_i to C_a ratio has been reported to maintain constant during a long-term growth at elevated [CO_2] which indicates that stomatal and photosynthetic acclimations are in parallel when the concentration of [CO_2] is enhanced (Morison 1998).

Given the crucial role of stomata in environmental and agricultural sciences, this study was designed to elucidate the molecular basis of the evolutionary control of natural variation in stomatal CO_2 responsiveness in *Arabidopsis*. Molecular studies were conducted with the aim to identify yet un-identified genetic loci in controlling natural variation in stomatal responses to CO_2 among *Arabidopsis thaliana* ecotypes. *Arabidopsis* has well adapted to strongly contrasting climatic conditions and the genome is fully sequenced. This model plant is used due to its small size of genome, low amount of repetitive sequence (Leutwiler *et al*. 1984, Pruitt and Meyerowitz 1986), rapid life cycle, and abundant seed production. It is also a self-fertilizing species that makes the procedures straightforward.

The project was organized in two parts: I) selection of parents; II) mapping quantitative trait loci (QTLs) controlling the stomatal CO_2 response based on molecular markers. Part I aimed on finding two accessions showing the highest contrast in a short-term stomatal CO_2 response by in situ measurement of leaf stomatal conductance. Moreover, anatomical analysis (stomatal density and stomatal length) and also leaf mass per unit area (LMA) were included in part I. Part II was aimed to map QTL(s) in F2 generation derived by selfing from a cross between two accessions selected based on Part I.

Materials and Methods

A total of 10 accessions were selected and grown in identical conditions. Accessions were selected based on their climatic origins (Table 1) and were obtained from the stock center NASC (http://arabidopsis.info) except for Göt-0 which was collected in Göteborg city, Sweden. The plants were grown in a controlled growth chamber (PlantMaster, CLF Plant Climatics, Germany) under ambient CO_2 concentration, short days condition (8 hours photoperiod), 21.5°C and 18° C day and night temperature respectively, roughly 50% relative humidity and 180 µmol m^{-2} s^{-1} photosynthetical photon flux density (PPFD).

A number of four replicates were sown for each accession with a week interval between replicates to allow for sufficient time to evaluate all plants at the same age. The seeds were sown at high density in 6*6 cm pots and initially underwent a cold (+4°c to +8°c) and dark treatment for 48 hours before being transferred to the growth chamber. The plants were irrigated twice a week with tap water throughout the growing process and later treated with Conserve® (1 ml/liter) once a week to exterminate silverfish bugs (*Lepisma saccharina*). Two-week-old plants were then re-planted to one plant in each of two pots for each replicate, one for g_s measurement and another for LMA estimation, named as co-pot.

Table 1- *Arabidopsis thaliana* accessions and their original properties

Arabidopsis thaliana accessions	Country
Aitba-2	Morroco
Mt-0	Libya
Bla-2	Spain
Cvi*	Cape Verde Islands
Col-0*	Columbia, USA
Kin-0*	Kendalville, USA
Kn-0	Lithuania
Le-0	Netherlands
Göt-0	Sweden
C24*	Portugal

Source: www.arabidopsis.org
http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2011.04606.x/full
*: selected based on inclusion in the QTL study by Brosche *et al*. (2010)

Afterwards, one mature rosette leaf of each five-week-old plant with abaxial surface facing downward, was measured for g_s at three levels of CO_2 concentration (200, 400 and 800 µmol mol^{-1}) using a portable leaf gas exchange system, LI-6400XT, version 6 software (LiCor Inc., Lincoln, NE, USA; Figure 1). Both 200 and 800 levels were preceded and followed by the level 400, as the reference, to evaluate the relative responses to 200 and 800 µmol mol^{-1} of CO_2. Generally, the measuring levels were arranged as 400, 200 or 800, 400, 800 or 200 and 400. To choose either measuring 200 or 800 level first, the order was randomized for the first replicates, and the level alternated for the other replicates. Therefore, for each accession, there were finally two replicates with 200 and two with 800 µmol mol^{-1} of CO_2 measured first. The relative values of g_s for 200 and 800 levels were computed by dividing the g_s at these levels with the mean value of preceding and following g_s measurements at 400 µmol mol^{-1}. The conductance value that changed less than 2% over a 5-minute time period was recorded as the steady-state value for each CO_2 level. During g_s measurement, PPFD was kept constant at 150 µmol photons m^{-2} s^{-1} and the vapor pressure deficit (VPD) was controlled at a target value between 1.0 and 1.5 kPa, varying ≤0.03 kPa around the target. The adaxial to abaxial stomatal ratio was set to 1.

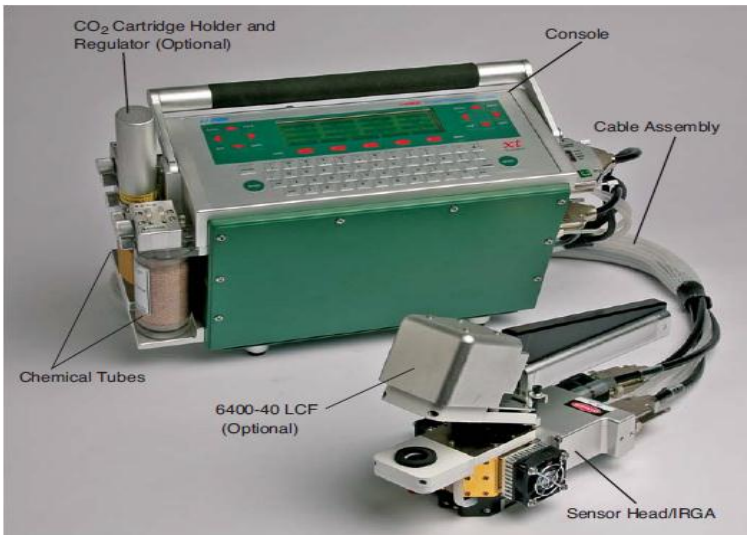

Figure 1- LI-6400XT, Portable Photosynthesis System

The order of plants to be measured was random and the measurements were conducted twice a day, one at 10:00 and another at 15:00 at the local time from late September to early November 2011. Two replicates of each accession were measured in the morning and the other two in the afternoon. Eventually, the leaf area which was enclosed in the chamber was calculated by a leaf area meter, WinFOLIA program version 5.1a (Regent Instruments INC., Quebec, Canada). Then the abaxial surface was nail polished and the peeling mounted on a microscopic slide for estimation of stomatal density and length.

Microscope camera AxioCam MRm (Germany, magnification of 10× / 0.3) using AxioVision Rel 4.8 program and also the software ImageJ 1.45 were utilized for estimation of stomatal density and stomatal length, respectively. Density was counted based on three images taken on the right side of the petiole, one image from the middle, one from the margin and another adjacent to the midrib. In addition, opening length of five stomata on each image was measured as well.

Moreover, maximum stomatal conductance (g_{smax}) was also estimated for the studied accessions following to estimation of g_{wmax} using the following formula (Franks and Beerling, 2009).

$$g_{wmax} = \frac{d}{v} \cdot D \cdot a_{max} \Big/ \left(l + \frac{\pi}{2} \sqrt{a_{max} / \pi} \right)$$

$g_{smax} = g_{wmax} / 1.6$

Where D = number of stomata per m^2; a_{max} = size of fully open stoma (m^2); l = depth of stomata tube (m); d_w = diffusivity of water vapor = 0.26 $m^2 s^{-1}$; v = molar volume of air = 24.47 $m^3 mol^{-1}$. The whole mass of each co-pot (excluding the leaves of less than 1 cm long) was used for calculation of LMA following to g_s measurement.

Finally, according to our findings in terms of difference in stomatal CO_2 responses and stomatal dimensions among accessions, most promising accessions were re-measured with the same method during December 2011 and January 2012 to confirm the results. The measured g_s responses to CO_2, stomatal density, stomatal length and g_{smax} were analyzed using ANOVA single factor test to compare pairs of accessions against each other. Then, to map the QTL(s) controlling the stomatal response to CO_2, the experimental population considered was the F2 generation from a cross between two most promising accessions derived by selfing. Seeds were grown and the plants were treated in the same method as before. A number of 42 individuals were available for the study. The g_s was similarly measured at the level of CO_2 that was found interesting based on Part I, i.e. relative responses to 800 µmol mol^{-1} CO_2 (see results below), during March and April 2012. The g_s was also measured for the F1 generation (n=3).

Afterwards, DNA from the experimental population was extracted from young leaf tissues. The DNA samples were then screened using 20 simple sequence length polymorphism (SSLP) markers available from The Arabidopsis Information Resource (http://www.TAIR.com) or designed from sequence data accessible through "1001 Genomes Project" (http://1001genomes.org). SSLP markers are PCR amplicons that cover small indels that exist in mapping populations, and they are visualized on agarose gels. The gel was made using SeaKem® LE Agarose and TBE buffer at a 3%, 3.5% and 4% concentration for length differences of more than 30 bp, between 20 and 30 bp, and less than 20 bp, respectively.

Initially, 12 individuals, six with the strongest and six with the lowest relative response to 800 µmol $mol^{-1}$of CO_2, were underwent genotyping. QTL was detected using QTLNetwork-2.0 (Brosché *et al*.

2010) developed by Yang *et al.* (2005) and then Yang, Zhu and Williams (2007). The software maps QTL with epistatic and QTL×environments (QE) interaction effects based on the mixed-model based composite interval mapping (MCMI). Composite interval analysis was accomplished with a probability into and out of the model of 0.05 and window size of 5 cM.

Results

We found all the accessions responsive to different CO_2 concentration. Stomatal conductance declined and increased at CO_2 above and below the ambient level, respectively. Pairs of accessions that demonstrated difference in absolute g_s at ambient [CO_2], and in responses to decreased and increased [CO_2] are shown in Table 2. Figures 2, 3 and 4 also illustrate g_s values and responses for each accession. Among the pairs that differed in g_s, Aitba-2 vs C24, Col-0 vs C24 and Cvi vs C24 were also different in both stomatal density and length, while Göt-0 vs C24 were only different in density (Table 3). Moreover, Col-0 and C24 were the only pairs with difference in g_{smax} among the pairs of accessions (Table 3). However, no difference was found in LMA between any of accessions. Further, when grouping the accessions in two groups of warm-origin (Aitba-2, Mt-0, Bla-2, Cvi, Col-0) and cool-origin (Kin-0, Kn-0, Le-0, Göt-0), there was no difference between the groups in g_s responses to decreased and increased levels of CO_2 concentration, neither in g_{smax}, stomatal density or stomatal length.

In general, we found three accessions, Col-0, C24 and Aitba-2, promising for QTL analysis (Table 2; Figures 2;3;4). In the first round, Col-0 showed the highest g_s (0.26 µmol m^{-2} s^{-1}) and C24 the lowest g_s (0.12 µmol m^{-2} s^{-1}) at the current atmospheric CO_2 concentration among the studied accessions ($p = 0.008$). They also showed different responses to increased [CO_2], i.e. 800 µmol mol^{-1} relative to reference, with the g_s of 0.49 for Col-0 and 0.62 for C24 ($p = 0.05$) (Figure 4). Also Aitba-2 vs C24 demonstrated a difference at decreased [CO_2] ($p = 0.01$). In addition, stomatal density was also significantly different between C24 vs Aitba-2 and vs Col-0. Aitba-2 and Col-0 together with Kn-0 had the highest stomatal length (13.14, 12.58, 13.38 µm, respectively) while C24 had the shortest length (10.02 µm). However, C24 vs only Col-0 showed difference in g_{smax} ($p = 0.005$). (Table 3)

The same results were also acquired from confirmation round. Again, Col-0 and C24 were significantly different in g_s (0.21 and 0.11 µmol m^{-2} s^{-1}, respectively) at the ambient [CO_2] while no difference was found between Aitba-2 and C24 in this regard (Table 2). In addition, C24 vs Col-0 and C24 vs Aitba-2 showed a statistical difference ($p = 0.0002$ and $p = 0.02$, respectively) in g_s at the increased [CO_2] (Table 2). Stomatal density and stomatal length were also estimated for abaxial surface in Col-0 and C24 resulting in significant difference in both factors ($p = 0.03$ for both). On average, C24 demonstrated a relative g_s of 0.70 and Col-0 a g_s of 0.46 at increased [CO_2] (30% and 54% of stomatal closure, respectively). Eventually, Col-0 and C24 were selected as the parents for QTL analysis at elevated [CO_2].

In F2 generation ($n = 42$) derived from a cross between Col-0 and C24, g_s at elevated [CO_2] relative to reference varied from low (0.33) to high (0.81). The majority of samples, however, showed a high level of conductance. The g_s in F1 exactly equaled the median conductance of F2 (0.57) and was also close to the mean (0.62). After phenotyping and genotyping of F2 plants, combined analysis of phenotypes and genotypes was accomplished by the software QTLNetwork-2.0. It revealed that the QTL is positioned on chromosome IV close to the GOT46 marker (Figures 5;6). However, we found that plants with weak responses had genotype of strong responses and plants with stronger responses had genotype of weak responses which was opposite to what we expected.

Table 2- Significant ($p \leq 0.05$) difference among studied accessions with respect to stomatal conductance (g_s) at [CO_2] = 400 µmol m^{-2} s^{-1} as well as relative stomatal conductance at increased (800 µmol m^{-2} s^{-1}) and decreased (200 µmol m^{-2} s^{-1}) [CO_2]

CO_2 levels	Relative g_s at decreased [CO_2]	g_s at [CO_2] = 400 µmol m^{-2} s^{-1}	Relative g_s at increased [CO_2]
Accession pairs	Mt-0 vs Göt-0 Mt-0 vs Aitba-2 Aitba-2 vs C24**	Mt-0 vs Bla-2 Mt-0 vs Col-0 Col-0 vs C24* Aitba-2 vs C24** Cvi vs C24 Göt vs C24	Mt-0 vs Col-0 Mt-0 vs Bla-2 Col-0 vs C24* Aitba-2 vs C24*** Bla-2 vs C24

*: found in both first and confirmation rounds
**: found in first round only
***: found in confirmation round only

Table 3- Stomatal dimensions of studied *A. thaliana* accessions (n=4)

Ecotype	Stomatal Length (µm)	Stomatal Density (mm2)	Stomatal Size	g_{smax} (mol m-2 s-1)	Leaf mass per area (g/m2)	g_s at ambient CO_2 (mol m-2 s-1)
Aitba-2	13,14	124	67,85	8,62	21,05	0,22
Mt-0	11,86	147	55,24	9,25	17,69	0,16
Bla-2	11,35	131	50,61	8,2	17,21	0,17
Cvi	12,23	131	58,74	8,51	25,39	0,18
Col-0	12,58	152	62,15	10,1	18,56	0,28
Kin-0	11,85	148	55,18	9,28	20,23	0,18
Kn-0	13,38	145	70,3	10,3	23,31	0,21
Le-0	11,43	161	51,27	9,76	24,86	0,20
Göt-0	10,26	143	41,36	7,79	20,48	0,19
C24	10,02	178	39,42	9,44	19,83	0,12

Table 4- List of markers used in this study, their physical positions on genome; forward and backward primers; expected base pairs in candidate accessions

Name	Chromosome	Position	PCR Left Primer	Right Primer	PCR Product Length (bp)	
					Col-0	C24
MN1.5	1	898430	TTATTATCAAGATCAAAGATTGTATGGTTT	CTTGTTTTTATATCTGTTTGGTTTAATTGT	308	320
CIW12	1	9621344	AGGTTTTATTGCTTTTCACA	CTTTCAAAAGCACATCACA	128	100
NGA280	1	20873698	GGCTCCATAAAAAGTGCACC	CTGATCTCACGGACAATAGTGC	105	85
NGA692	1	28836552	AGCGTTTAGCTCAACCCTAGG	TTTAGAGAGAGAGAGCGCGG	110	90
GOT10	2	693817	GCGGTGAGTATCTCATTGCAT	AAGCCCATTCCCTAAACCAC	214	165
GOT42	2	9249365	ATTCATTTGTCGCATTTGCT	CAACAACACGAGCCACACTC	207	188
GOT11	2	17493070	GGTTTCCATGTTGGCTCAGT	GAACCAACGATTGGGCTAGA	126	147
NGA172	3	786296	CATCCGAATGCCATTGTTC	AGCTGCTTCCTTATAGCGTCC	162	150
CIW11	3	9775545	CCCCGAGTTGAGGTATT	GAAGAAATTCCTAAAGCATTC	180	230
GOT47	3	16292750	AACATGTTTTTGATAATCATCCATC	CATCTAAAAGATCTCCAGATTAAGTGA	218	250
GOT39	3	22776727	TCGTCGTTGGAGGTCTTGAT	ACGGGACAAGAACCATTGAG	244	229
GOT46	4	372994	GCCTCTGTTATTTAGGCTGAAAAT	CAGCTGTTGGTCAAACTGATACA	224	267
GOT3	4	4644916	TCCTTAAAGCCCTTTGTTTATG	TTGTTGCTTTATTGGTTGGTTT	175	154
GOT37	4	8852180	ACGGTGTGTGCAATAACCAA	CCTCAGGCATCATCTCTCGT	432	469
GOT7	4	10977375	TCTTGAGCAAGGTGCAAAAA	TAACTTCCAGGCGGCCTTAT	144	135
GOT8	4	16272270	TCGAGAGAGGTGAGAAGGTCA	CACATCTCTCATTGGATGATGAA	138	150
T22D6-1ME	5	2584790	ACGCTTCCCAAAAATCCACAA	CTCGGGAGACTCGCACGAT	175	230
GOT43	5	8427955	ACTGGTGGGACCCGTTTAGT	CGAAGAAAGCCGAAGAAGAA	564	516
GOT38	5	15551620	CTCCGTGTCACCAATTTCAG	TGAAACCGAGAAATCGAAGG	245	230
CER456336	5	26095101	GGTCAATCCCAGGAAACTT	ATTAATGAAAATGGAGATT	210	160

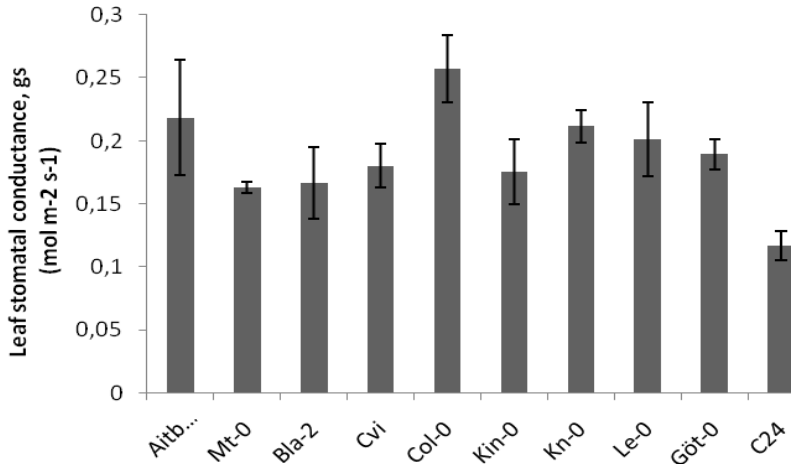

Figure 2- Leaf stomatal conductance (g_s) at reference concentration of CO_2 (400 µmol mol^{-1}) in studied *A. thaliana* accessions in first round (n=4; confidence interval=95%)

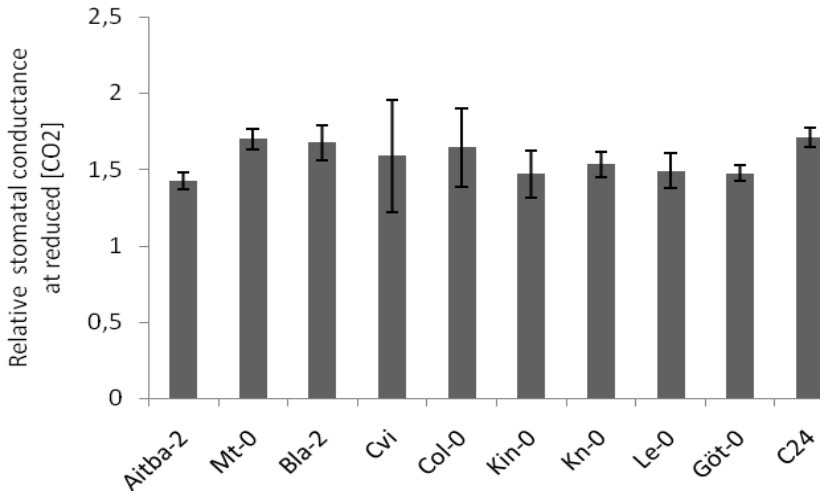

Figure 3- Relative stomatal conductance at decrease concentration of CO_2 (200 µmol mol^{-1}) in studied *A. thaliana* accessions in first round (n=4; confidence interval=95%)

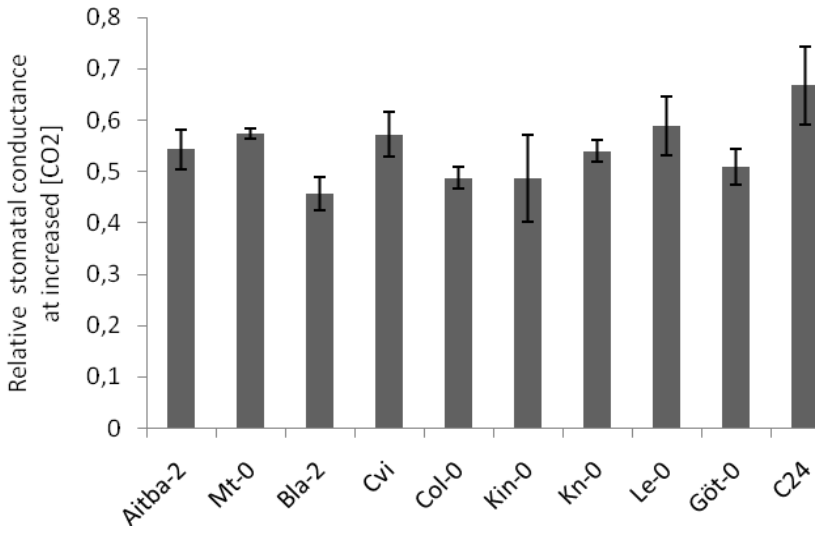

Figure 4- Relativ stomatal conductance at increase concentration of CO_2 (800 µmol mol^{-1}) in studied *A. thaliana* accessions in first round (n=4; confidence interval=95%)

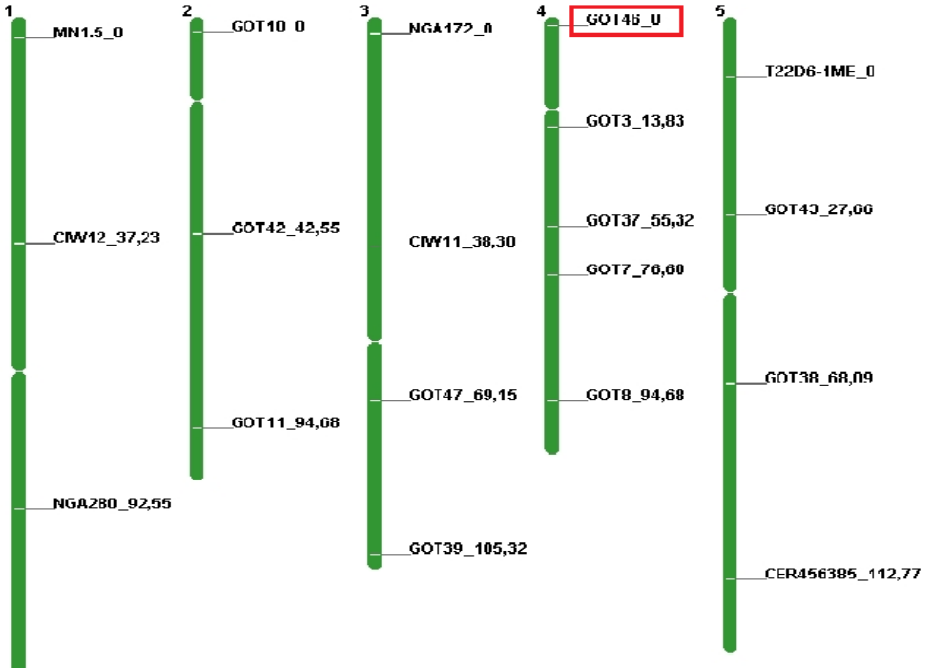

Figure 5- Markers used in this study and their positions on *A. thaliana* chromosomal set. The number after name of each marker indicates the genetic distance in cM between the markers calculated from 94 segregating Col-0xC24 F2 plants. GOT46 is the marker close to the QTL that was correlated with stomatal response to CO_2, although unreasonable because F2 plants with weak responses had genotype of strong responses and F2 plants with stronger responses had genotype of weak responses.

Discussion

tomata physiologically respond to elevated [CO_2] and control influx of atmospheric CO_2 into assimilation sites. Here, by looking at accessions of *A. thaliana*, we confirm the stomatal responsiveness to different concentrations of CO_2, and decrease and increase of g_s when CO_2 is above and below the ambient level, respectively, as reported before (Heath and Meidner 1957, Morison 1998). The reference (i.e. 400 µmol mol^{-1}) values of g_s for Kin-0, C24, and Col-0 were similar to those of reported by Brosche *et al.* (2010) but considerably lower for Cvi-0 (Figure 2). In addition to g_s difference at reduced and/or elevated [CO_2] observed between the accessions with different origins such as Mt-0 vs Göt-0, there were also accessions from similar climatic origins which showed to be statistically different in g_s; Mt-0 vs Bla-2 and Mt-0 vs Col-0 are such examples (Table 2). Therefore, climatic condition is unlikely to be a dominating cause of variation in g_s responses to [CO_2]. Moreover, based on opposite direction of differences in stomatal density and length in Table 3, we found that density and length counterbalanced each other with respect to calculated g_{smax}.

This study found that the *Arabidopsis* ecotypes C24 and Col-0 have contrasting stomatal CO_2 responses among the other ecotypes studied herein (Figures 2;4); thus, they are suitable parents for QTL mapping of stomatal CO_2 responsiveness. Interestingly, it has also been reported that these ecotypes differ in rates of photosynthesis hybridized progenies from crosses between C24 and Col-0 (lower photosynthesis in C24 compared to Col-0) exhibit heterosis in over-representation of chloroplast-located and photosynthesis-related proteins (Fujimoto *et al*. 2011). This was associated with enhancement of whole-plant photosynthesis and biomass. Also as plants have been found genetically different in photosynthesis (Flood *et al*. 2011) and since stomatal and photosynthetic

regulations are tightly linked (Ainsworth and Rogers 2007), there may be a link between these photosynthesis-related proteins and the proteins responsible for the differences in stomatal CO_2 responsiveness and/or stomatal dimensions between the C24 and Col-0 ecotypes found in the present study. This should be explored by future studies. Moreover, if the QTL related to stomatal CO_2 responsiveness and QTL for photosynthesis-related genes are explored then a new genetic network may be formed.

The QTL found in this study was unreasonable because in F2 generation plants with weak responses showed genotype encoding for strong responses and plants with strong responses had genotype of weak responses. Thus, as the pattern observed was opposite to what we expected, this QTL does not seem to explain variation in stomatal CO_2 responsiveness. It could be due to the low number (n = 12) of F2 plants experimented since we only screened the genotype for the six samples with highest and the six sample with lowest relative responses to 800 due to the time constraints. On the other hand, although there have been several investigations regarding molecular mechanism of stomatal response to [CO_2], the protein that binds to CO_2 is not yet known (Hu *et al.* 2010). Also there is no annotation of any genes on databases around where the present QTL was found, i.e. chromosome IV. For instance, the plant *SLAC1* anion channel (Chen *et al*. 2010) is positioned on chromosomes I, IV and V; *EPF1* and *EPF2* as negative regulators of stomatal development in Arabidopsis (Sugano *et al*. 2010) are on chromosomes II and I, respectively; *HT1* protein kinase, the regulator of stomatal movement, (Hashimoto *et al*. 2006) is positioned on all chromosomes, and *βCA1* is on chromosomes I, III and IV, and *βCA4* (β-carbonic anhydrases) on chromosomes I and IV both of which increase stomatal density and regulate stomatal movement in response to [CO_2] (www.ncbi.nlm.nih.gov/Entrez).

In this study the locus of the trait related to stomatal [CO_2] response could not be detected. Therefore, an experiment with higher number of individuals in the experimental population should be done and all the technical procedures should be well controlled to find this QTL. Because when the QTL and then the associated gene(s) are detected, a new window will be open to plant biology. It would be a remarkable finding which has both environmental and agricultural implications as the global temperature and concentration of atmospheric CO_2 is constantly increasing, and large areas are predicted to receive less precipitation.

Conclusion

Among a number of 10 natural accessions of *A. thaliana* studied herein, Col-0 and C24 were considered as the most suitable candidate pair for genetic mapping of putative loci for stomatal response to carbon dioxide. C24 was found to be almost as twice strong as Col-0 in response to short-term elevation of [CO_2]. However, the locus that controls natural variation in stomatal CO_2 responsiveness in *Arabidopsis* could not be verified by the present QTL mapping.

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