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CHARACTERIZING NEW *ARABIDOPSIS THALIANA* MUTANTS AFFECTED IN TRANSPIRATION



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Abbreviations

ABA	abscisic acid
abi	*ABA-insensitive* (PP2C constitutive expression mutant)
aba	*ABA-deficient* (ABA biosynthesis mutant)
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
EMS	ethyl methane sulfonate
FAD	flavine adénine dinucleotide
ht1	*high leaf temperature 1 (*mutant affected in a protein kinase)
NB-LRR	nucleotide binding site- leucine rich repeat
PCR	polymerase chain reaction
rcd	*radical-induced cell death* (ozone-sensitive mutant)
ROS	reactive oxygen species
RTSC	rapid transient stomatal closure
slac1	*slow anion channel-associated 1* (mutant affected in ion transport)
slah1	*slow anion channel associated homologue 1*
T-DNA	transferred DNA
TAIL-PCR	thermal asymmetric interlaced-PCR
O_3	ozone
ost	*open stomata* (mutant affected in guard cell signaling transduction)

Term followed by * in the text is explained in the glossary.

Glossary

EMS mutagenesis : chemically induced point mutations. Mutants are not considered as genetically modified organisms.

T-DNA insertion mutagenesis: *Agrobacterium tumefaciens* transformation consisting of introducing randomly distributed T-DNA inserts in the plant genome.

1. INTRODUCTION

Several international research groups tend to elucidate plant response to environmental constraints such as abiotic factors. Particularly, the Ecologie et Ecophysiologie Forestières Group (UMR 1137, UHP, Nancy1, France) and the Plant Signal Research Group (Technology Institute, Tartu University, Estonia) collaborate on the stomatal regulation theme.
Stomata are foliar organs that consist of two guard cells forming a pore. Plants can be epistomatous or hypostomatous, having stomata located either on the upper (adaxial) or the lower (abaxial) side of the leaf, respectively. Majority of plants, however, are amphistomatous, that is having stomata on both surfaces (Lawson, 2009), like the flowering plant *Arabidopsis thaliana* belonging to the *Brassicaceae* family.
A stomatal pore acts as a gate connecting the atmosphere and the interior of the leaf, thus allowing gas fluxes between the two compartments. Indeed, carbon dioxide (CO_2), as one substrate of the photosynthesis enters the leaves mainly through these pores (Boyer *et al.*, 1997). Stomata also mediate oxygen (O_2) and water efflux; this necessary transpiration, promotes the hydraulic flux through the whole plant, and consequently the allocation of nutrients within the plant. They also play an important role in the global water cycle at the biosphere level. Stomata are also a gate for the release of hormones, such as ozone-induced ethylene (Rodecap & Tingey, 1986) and other volatile compounds (Niinemets & Reichstein, 2003). Furthermore, atmospheric pollutants such as ozone (O_3) can penetrate the leaves through open stomata (Heath, 1980)

Stomatal functioning is under circadian control. Stomata are able to sense and respond to environmental changes (reviewed by Hetherington & Woodward, 2003). Opening is induced by light (Shimazaki *et al.*, 2007) and low CO_2 (Vavasseur & Raghavendra, 2005) whereas closure is induced by darkness and in response to drought (Medrano *et al.*, 2002 ; Luan, 2002), through ABA signaling. Also above ambient concentrations of CO_2 (Heath & Kerstiens, 1997) and ozone (Kollist *et al.*, 2007) are shown to induce stomatal closure.
Changes in air humidity induce changes in leaf-air vapor pressure difference (VPD). It has been shown that increasing VPD (lower relative humidity) closes stomata but several theories are still in competition to explain underlying sensing and transduction mechanisms (Assmann *et al.*, 2000).
Early on, the photosynthetic capacity was mentioned to be closely related to stomatal conductance as it varies in nearly the same proportion as the rate of assimilation of CO_2, resulting in a constant intracellular CO_2 concentration (Wong *et al.*, 1979). It is consistent with the hypothesis that it is the intercellular concentration of CO_2 which is sensed rather than the CO_2 concentration at the leaf surface or in the

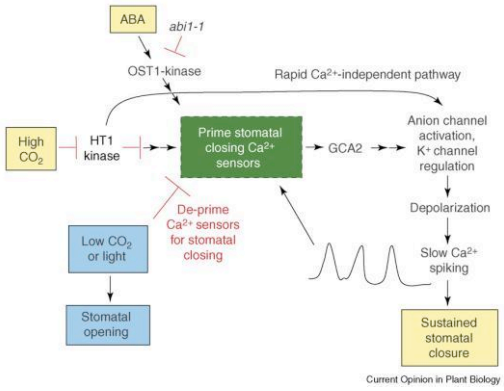


Figure 1: Simplified model of CO2 signal transduction mechanisms in guard cells. Israelsson *et al.*, 2006.

stomatal pore (Mott, 1988). Israelsson and colleagues (2006) recently reviewed the last breakthrough in CO_2 signaling mechanism, especially the role of the HT1 kinase. They also differentiated Ca^{2+} -dependent and independent pathways (Figure 1).

Ozone is a major air pollutant that generates increased reactive oxygen species (ROS) production in cells termed "oxidative burst". ROS are not only harmful, oxidative molecules but also act as signaling molecules triggering stomatal closure via the calcium signature (Evans *et al.*, 2005).

Stomatal aperture (diameter of the pore) is regulated by the water status of the guard cells. The more turgescent the guard cells, the more open the stomatal pore. Mechanically, because the cell walls of the guard cell forming the pore are thicker, the water pressure expands the guard cells on the opposite side of it (Figure 2).

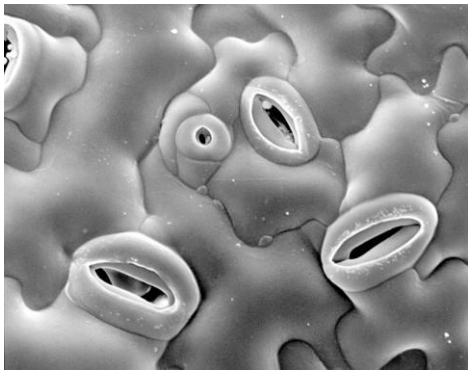


Figure 2: Open stomata from *Arabidopsis thaliana*. Cryoscanning electron microscopy picture by F. Sack.

As movement of ions and solutes across the guard cell plasma membrane drive the osmotic potential and concomitant water uptake and release from the guard cells, ion channel regulation are of great importance for this process. The major ions responsible for the stomatal opening and closure are potassium (K^+), calcium (Ca^{2+}), and the counter-ions chloride (Cl^-) and malate^{2-}(Fan *et al.*, 2004).
Ion channels can be monitored by the plasma membrane electric gradient. Allen and colleagues (2001) brought to light the singular calcium signature required for stomatal functioning. In the case of stomatal closure, rising cytosolic calcium concentration ($[Ca^{2+}]_{in}$) activates outward rectifying anion channels. The subsequent depolarization of the membrane induces the activation of outward rectifying K^+ channels. Ion balance is maintained due to accompanying efflux of counter-ions. As a consequence, water leaves the guard cell and the stomate closes. Increased $[Ca^{2+}]_{in}$ also acts on H^+-ATPase pump, inhibiting proton (H^+) efflux which is responsible for the plasma membrane hyperpolarisation (Figure 3) (Schroeder *et al.*, 2001 ; Fan *et al.*, 2004).

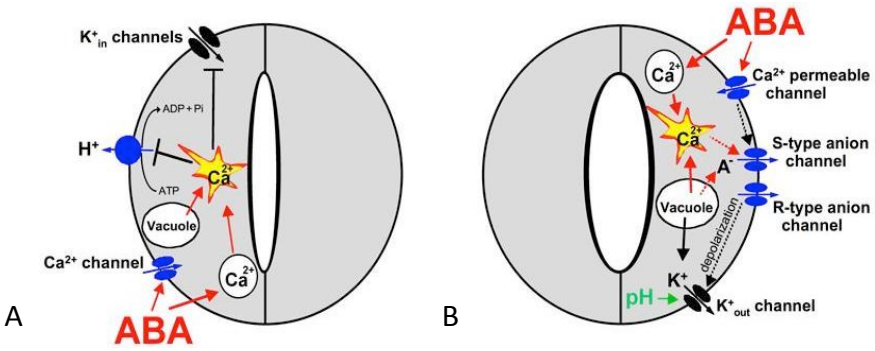


Figure 3: Simplified model for the role of ion channels and pumps in ABA-induced stomatal movements. ABA inhibits stomatal opening (A) and triggers stomatal closing (B). Schroeder *et al.*, 2001. http://www.biology.ucsd. /schroeder/clickablegc2/overview.html

Signals leading to stomatal opening and closure are transduced through complementary and regulated cascades termed "the guard cell signaling". These cascades are under a tight hormonal control. Abscisic acid (ABA) plays a major role (Wilkinson & Davies, 2002) acting both in promoting stomatal closure and inhibiting stomatal opening (Figures 1 and 3) (Schroeder *et al.*, 2001). When linked to membrane receptors like GPCR-type G proteins (Pandey *et al.,* 2009), ABA induces the activation of calcium inward channels and the release of calcium from an intracellular pool (Sanders *et al.,* 1999). Webb and colleagues (2001) pointed out the critical role of Ca^{2+} in the ABA-induced inhibition of stomatal opening. This is mediated by hydrogen peroxide, a reactive oxygen species (ROS) (Pei *et al.*, 2000). Trouverie and colleagues (2008) highlighted that anion channel activation and proton pumping inhibition induced by ABA are both dependant of reactive oxygen species (ROS).

Guard cell ABA-turgor signaling has been shown to act through a complex, network involving many intermediates (Li *et al.,* 2006). Membrane depolarization, anion efflux and actin cytoskeleton reorganization have been predicted to be three essential components of stomatal closure. Suppression of one of these components completely blocks ABA-induced closure (Li *et al.,* 2006).

Key proteins involved in the processes of stomatal opening/closure can be discovered either with the use of inhibitory compounds or with genetic manipulation. Arabidopsis, the model organism in plant biology, is very convenient as a tool for finding and studying both proteins and responsible genes that are involved in guard cell signaling. Screening methods of mutant libraries have already provided several interesting mutants such as *ost1*, *ht1* or *slac1*. For example, OST1 is a protein kinase involved in the ABA-signaling pathway upstream of ROS production (Mustilli *et al.,* 2002). HT1 kinase activity is important in modulating the CO_2 signaling pathway that mediates stomatal movements (Hashimoto *et al.,* 2006). The slow anion channel-associated protein (SLAC1) is a plasma membrane protein required for stomatal closure in response to multiple abiotic constraints as the mutant *slac1* has impaired anion currents through S-type anion channels (Vahisalu *et al.,* 2008). SLAC1 has recently characterized as a chloride channel (Hedrich, 2009) (Figure 4).

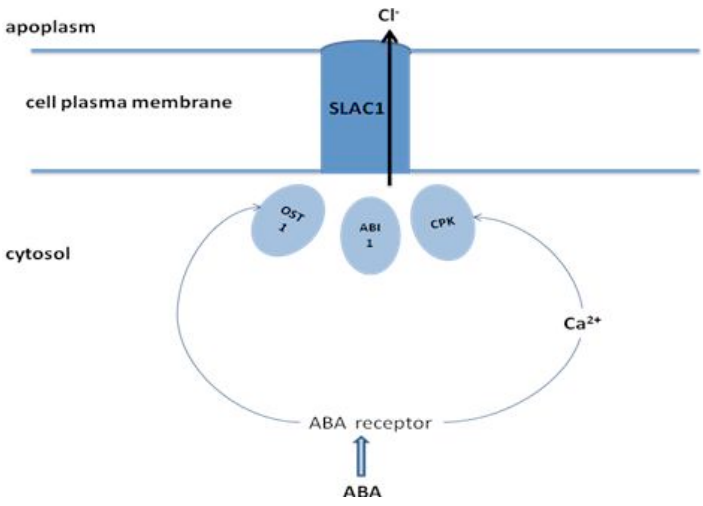


Figure 4 :edrich's theory about the activation of the slow-anion channel associated protein 1, which is a plasma membrane chloride channel. Inspired from Heidrich R., 2009.

It is likely that key elements controlling plant transpirational water loss and stomatal function are still to be discovered. Mutants have been proved to be a successful tool to round out existing pathways (Kangasjärvi *et al.*, 2005). We pursued 3 objectives: (1) selecting new mutants affected in regulating transpiration; (2)

characterizing their responses to ozone, high CO_2 concentration and changes in air humidity and (3) identifying the mutated genes responsible for the high transpiration phenotype.

Two screening methods, of which one new, were applied and stomatal conductance was measured with a whole plant gas-exchange system available in the hosting laboratory (Kollist *et al.*, 2007). Stomatal conductance refers to the ability for plant to allow gas exchange with the atmosphere through the stomata. We optimized the molecular technique TAIL-PCR to our conditions in order to identify candidate mutated genes.

2. MATERIAL AND METHODS

2.1. Plant material and growth conditions

Experiments were performed on two sets of *Arabidopsis thaliana* mutant. *A. thaliana* T-DNA insertion lines* containing 35S promoter and a selection marker were ordered from the European Arabidopsis Stock Center (http://arabidopsis.info/CollectionInfo?id=59, Weigel *et al.*, 2000). *Arabidopsis thaliana* ozone-sensitive lines (*ozs*) are ethyl methane sulfonate (EMS) mutants* isolated for their ozone sensitivity (Overmyer *et al.*, 2000) and provided by Jaakko Kangasjarvi at the Department of Biological and Environmental Sciences, University of Helsinki. Both sets of *A. thaliana* mutants were in *Columbia (Col-0)* background. The *ozs* lines also carried the *gl1* (*glabrous*) mutation as a visual marker.

Dry seeds were stored at room temperature. Seed stratification consists of breaking dormancy and to achieve this seeds were soaking in water at 4°C for at least 2 days. When required, seeds were sterilized as follows: (1) dry seeds were incubated in 750 μL sterilizing solution (10 mL bleach (Procter & Gamble) with 5 μL Tween 20 (Scharlau)) for 3 min after mixing; (2) 2 rinsing cycles with sterile water after centrifugation 8000 rpm for 1 min; (3) sterilized seeds were kept in 1 mL of sterile water.

Plants were grown *in vitro* on plant medium plates containing 2.15g of Murashige-Skoog (MS) medium (Duchefa Biochemie), 10g of sucrose and 5g of phytoagar (Duchefa Biochemie) per liter, set to pH 5.75 with KOH and kept in the long-day growth room (14h light/10h darkness, 100 ± 15 μmol photons m^{-2} s^{-1}, 23°C ± 2°C, air humidity ranged from 30 to 40% in winter and from 50 to 60% in summer time with an ambient CO_2 concentration.). Plants grown in soil were kept in a short-day growth room (conditions same as in long-day room except that light/darkness period was 12h/12h) or in a Percival growth chamber. Soil composition was 4:3:3 (v:v:v) peat:vermiculite:tap water. 250 ± 5 g of soil was used for plants dedicated to gas exchange measurements.
Conditions in Percival controlled chambers for most experiments were as follows : 12h/12h day/light photoperiod, 120 μmol photons m^{-2} s^{-1}, 23°C ± 1°C (day) and 18°C ± 1°C (night), the relative humidity was around 65% and the CO_2 concentration was ambient.

2.2.Screening

High transpiration cools down leaves surface. This cooling effect was noticed to induce a condensed vapor spot on the inner side of a filled Petri dish. *slac1-3*, a slow-anion channel mutant impaired in stomatal closure (Vahisalu *et al*., 2008) was used as a positive control. Seeds were first sown in tray and the seedlings grown in a growth-room for approximately one month. Two trays of 135 wells were used per batch. Two leaves per plant were cut off and placed abaxial side down on the lids of Petri dishes filled with 17°C green-coloured water (for increased visibility). The presence/absence of a condensed vapor spot was monitored after 20 min by moving the leaves. (Appendix)
Leaves causing the formation of vapor spots were monitored for visual signs of dehydration after 1h (i.e. if they looked shriveled). Individuals whose leaves gave a vapor spot, were transferred into bigger pots for seed collection and water loss measurement which was done when total leaf area reached approximately 10 cm^2 (after approximately another 2 weeks of growth).

Water loss was calculated according to the following equation: 

with *FWt0* : fresh weight of excised leaves and *Wt90* : weight after 90 min while leaves were left adaxial side down on the bench.

2.3.Toluidine blue coloration test

The test consisted in soaking excised leaved in the toluidine blue dye for 2 min and rinsed in tap-water (adapted from Tanaka *et al.,* 2004). The dye cannot penetrate the epidermal cells in wild type plant because of the cuticle barrier indicating that stained leaves have an impaired cuticle.

2.4.Selection of transgenic plants

Resistance to the antibiotic kanamycin is conferred by a bacterial gene encoding the enzyme neomycin phosphotransferase (NPT). BASTA is the common name for the herbicide phosphinothricin (glufosinateammonium). Resistance to BASTA is conferred by the bacterial bialophos resistance gene (BAR) encoding the enzyme phosphothricin acetyl transferase (PAT) (Weigel & Glazebrook, 2002).

Sterilized seeds were spread on plates containing plant medium with kanamycin (50 μM final concentration) or BASTA (20 μM final concentration).

2.5.Genotyping

DNA extraction was made by following the DNeasy kit mini protocol (Purification of Total DNA from Plant Tissue, DNeasy Plant Handbook, Qiagen, July 2006) with some adaptations. Steps 1 to 6 were replaced by one single step consisting of mashing the biological material and 50 μL Buffer AP1 with a plastic pestle mounted on an electric drill. This allowed the material to be sufficiently mashed and homogeneous to get a relatively good DNA

concentration at the end. Buffer AP1 was added up to 400 μL in the next step. Two additional spins with tubes in different positions were made at step 17 after having discarded the flow-through. These spins ensured that no residual ethanol, which would still be along the tube, was carried over during elution. At step 18, elution was made with only 50 μL Buffer AE and step 19 (which consists of a step 18 repeat) was not carried out in order to have a higher concentration of DNA.

Primer pair used for genotyping was LBb1.3 (5'- ATT TTG CCG ATT TCG GAA C -3')-Slac1GTRP (5'- GAC CAT TTC TTT GCC TGT TTG -3') (http://signal.salk.edu/tdnaprimers.2.html).

Cycling parameters were as followed: (1) 98°C for 3 min; (2) 31 cycles of 98°C for 10 s, 50°C for 10 s and 72°C for 1.5 min; (3) 72°C for 5 min.

For PCR amplification either DreamTaq (Fermentas) or FirePol (Solis Biodyne) were used.

2.6.Stomatal response to abiotic constraints

Stomatal conductance was measured with a whole-rosette gas exchange system (Kollist *et al.*, 2007). The higher the stomatal conductance, the higher the transpiration through stomata meaning they are open. Leaf areas, optimally between 8 and 15 cm^2, were calculated with the Image J software from pictures of each plant.

Ozonation : ozone-sensitive lines (*ozs*) were subjected to a 3-minutes ozone pulse of 400 ppb ± 50. Stomatal conductance for at least 2 replicates was recorded. Values are expressed relatively to the conductance measured at T_0, *ie* 3 minutes before the fumigation.

Elevated CO_2 : 800 ppm ± 20 were applied on selected *ozs* lines for 37 minutes.

Changes in air humidity : air relative humidity was dropped from 70% to 40% in the cuvette for 37 minutes.

2.7.Gene identification

Molecular techniques were used and optimized in order to identify the mutated gene(s) of selected mutants. The working hypothesis was that the T-DNA insert was located close to the candidate gene nucleotide sequence in the plant genome.

DNA extraction was performed the same way as described in the genotyping procedure. The Thermal Asymmetric Interlaced–Polymerase Chain Reaction or TAIL-PCR procedure was adapted from Sessions *et al.*, 2002. In the amplification rounds, different annealing temperatures were used which better suited our primers. Cycling parameters for the first round were as followed: (1) 94°C for 2 min and 95°C for 1 min; (2) 4 cycles of 94°C for 30 s, 54°C for 1 min and 72°C for 2.5 min; (3) 2 cycles of 94°C for 30 s, 25°C for 3 min (R=0.8°C.s-1),and 72°C for 2.5 min (R=0.5°C.s-1); (4) 15 cycles of 94°C for 10 s, 56°C for 1 min, 72°C for 2.5 min, 94°C for 10 s, 56°C for 1 min, 72°C for 2.5 min, 94°C for 10 s, 44°C for 1 min, and 72°C for 2.5 min; and (5) 72°C for 7 min. Cycling parameters for the second round were as follows: (1) 94°C for 3 min; (2) 5 cycles of 94°C for 10 s, 60°C for 1 min, and 72°C for 2.5 min; (3) 15 cycles of 94°C for 10 s, 60°C for 1 min, 72°C for 2.5 min, 94°C for 10 s, 60°C for 1 min, 72°C for 2.5 min, 94°C for 10 s, 44°C for 1 min, and 72°C for 2.5 min;

(4) 5 cycles of 94°C for 10 s, 44°C for 1 min, and 72°C for 3 min; and (5) 72°C for 7 min.(Eppendorf® Mastercycler gradient).

Primer pairs used for the first round were LB1 (5′- TCC TGC TTT AAT GAG ATA TGC GAG AC -3′) as the T-DNA specific primer and AD1, AD2, AD3 (5′- NGT CGA SWG ANA WGA A -3′ 5′- TGW GNA GSA NCA SAG A -3′ 5′- AGW GNA GWA NCA WAG G -3′ respectively) as arbitrary degenerated primers. For the second round, the three AD primers were the same but LB2 (5′- GTC CGC TCT ACC GAA AGT TAC GGG CA -3′) was used instead of LB1. LB primers were at a final concentration of 0.5 μM and AD primers at 3 μM.

After the first round, PCR products were diluted 100 times and used as DNA templates for the second round. PCR products from the second round were purified following the Quiagen Bench Protocol: QIAquick PCR purification Microcentrifuge Protocol (QIAquick Spin Handbook, Qiagen, November 2006).

For the third and final TAIL-PCR round a new strategy was developed. PCR products below and above 500 kb were extracted with a Qiagen gel purification kit and cloned into a vector following pGEM-T Easy kit protocol (Promega Quick Protocol, 2005). Transformed cells were selected with the antibiotic carbenicillin and plasmids were extracted with GeneJET™ Plasmid Miniprep Kit (Fermentas Quick Protocol).

PCR was then conducted using insert specific primer LB7 (5′- CTT TCA TTT TAT AAT AAC GCT GCG G -3′) and the plasmid specific primer T7 (5′-TAA TAC GAC TCA CTA TAG GG -3′). Specific PCR products were then gel-extracted and cloned for a second time. Plasmids were sequenced with the primer LB7 or the plasmid specific primer pQE30b (5′- CGG ATA ACA ATT TCA CAC A -3′). Sequences were blasted for a nucleotide-nucleotide search in the ncbi data base.

2.8.Statistical analysis

Fisher LSD test was used for water loss measurement data analysis (Statistica software).

T-Test was used to compare ozs7 and WT Col gl in response to an ozone pulse (R software).

3. RESULTS

3.1. Selection of T-DNA mutants

Previously, from approximately 12000 T-DNA transformed seedlings, five *Arabidopsis thaliana* mutants had been selected for unusually high transpiration using an unpublished screening test that relied on the physical leaf cooling effect of transpiration (Appendix). Later, they were also tested for the water-loss within 90 minutes. The putative mutants had been named *cb1* to *cb5* for *cool breath 1 to 5*.

Propagated seeds of the lines, except for *cb1* that died early during bolting, were tested again confirming the previous results. To ascertain that these mutants did originate from the original batches, propagated seeds were grown on medium containing the selection chemical BASTA or the antibiotic kanamycin. The positive control mutant *slac1-3* carries the kanamycin resistance gene and proper T-DNA mutants were expected to carry the BASTA resistance gene. Since only *cb5* individuals were viable on BASTA medium (Figure 5), it led us to the hypothesis that the screening trays might have been contaminated with the positive control, *slac1-3*.

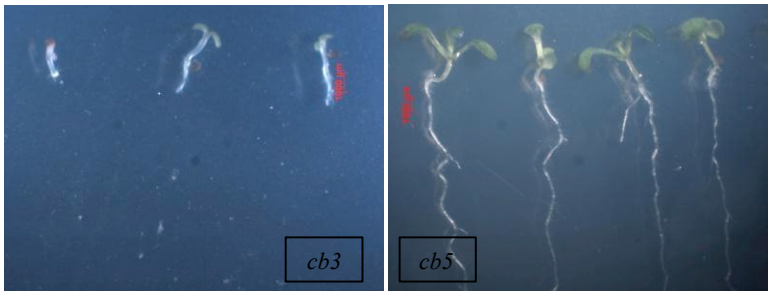


Figure 5: Selection of *cool breath* mutants on BASTA medium. *cb3* on (left panel) is sensitive to BASTA as *slac1-3, cb2 and cb4* (not shown)while *cb5* displayed the resistance to the antibiotic (right panel).

Two pairs of *slac1-3* specific primers were established that amplifies a specific fragment from the mutant. These primers were used on genomic DNA from *cb1-cb5*. Amplification occurred for *cb2*, *cb3* (not shown) and *cb4* but not for either *cb1* or *cb5* (Figure 6). Thus, together with the antibiotic resistance results, we concluded that *cb2*, *cb3* and *cb4* were actually *slac1-3* mutants.

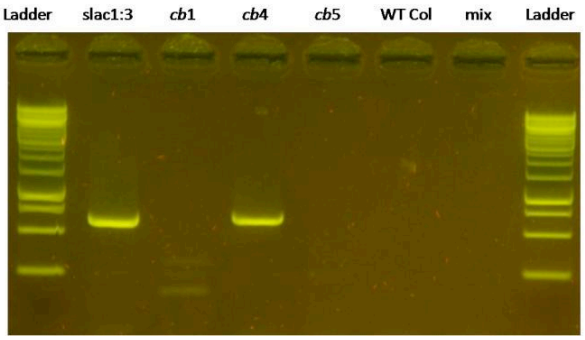


Figure 6: Genotyping of *cool breath* T-DNA mutants. Mix corresponds to the control without DNA template. Primers LBb1.3 and slac1GTRP.

3.2.Characterization of T-DNA selected mutants

3.2.1. Phenotypic description

cb1 had a delayed bolting phenotype and as a consequence we were not able to collect the progeny for further study. However, two rounds of genomic DNA were extracted from rosette leaves in order to identify putative mutated genes by TAIL-PCR.

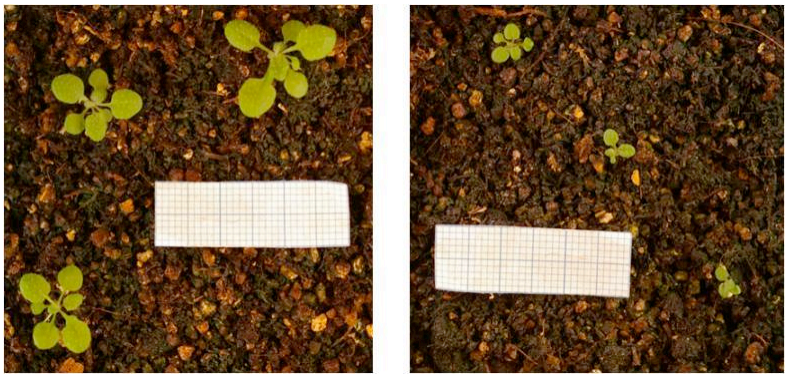


Figure 7 : 22-days old WT Columbia and *cb5* grown in the short day-room.

After 2-3 weeks *cb5* was smaller than WT (Figure 7) and later appearing rosette leaves were curled-up and appeared to be wrinkled as compared to WT Col-0 or *slac1-3* leaves. Some of these leaves also displayed dead tissue in the leaf margins.

Rosette leaves of *cb5* were sometimes fused, and were also very drought sensitive when relative air humidity was below 70% (i.e. normal). This was especially apparent during later growth stages (Figure 8).



8: Abnormal phenotype of the *cb5* mutant. Two-months old plant kept under a lid.

cb5 was shown to carry a recessive mutation as crossings between *cb5* and WT Col individuals did not display a vapor spot, drought-sensitivity and visual phenotype of *cb5* at any stage of the development. Furthermore, segregation for the antibiotic resistance (Figure 9) seemed to follow the 25%/75% ratio corresponding to ¼ WT homozygotes with no mutated alleles, ½ heterozygotes and ¼ homozygotes with mutated alleles.

Two plants out of 9 replanted resistant individuals later displayed a clear *cb5*-like phenotype confirming that the mutation was recovered in the following generation, as expected from a recessive mutant.

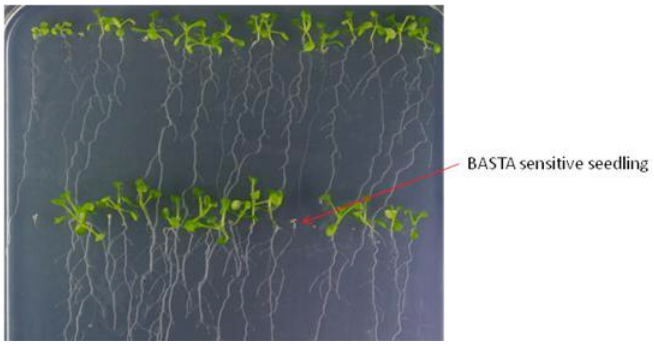


Figure 9: Crossings between *cb5* and WT Col-0 individuals grown on BASTA medium.

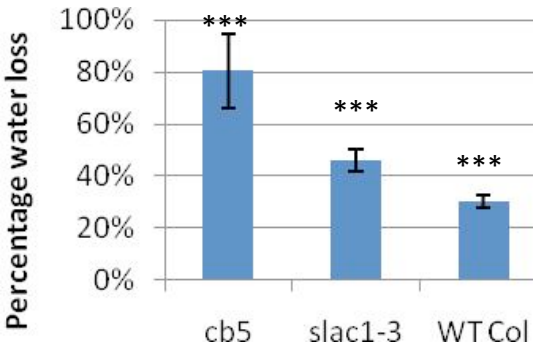


Figure 10: Percentage of water loss after 90 minutes of ozone-sensitive cut-off leaves. Error bars are standard deviations (n=4). Fisher LSD test ($p<0.005$) ***: highly significant.

The percentage of water loss for *cb5* was significantly higher ($p<0.05$) than for the negative (WT Col-0) and positive (*slac1-3*) controls (Figure 10) and stomatal conductance could not be measured with a porometer as extreme values over 1000mmol.m^{-2}.s^{-1}, were recorded. This is well above the calibration range (around 1500 mmol.m^{-2}.s^{-1}, data not shown).

It has been shown that mutants having defects in the cuticle have similar visual phenotype as *cb5*. To test the intactness of *cb5* cuticle we decided to apply the simple method recently described by Tanaka and colleagues (2004).

A coloration test with toluidine blue evidenced the permeability of the epidermis as most of the *cb5* leaves were stained whereas control leaves were not (Figure 11).

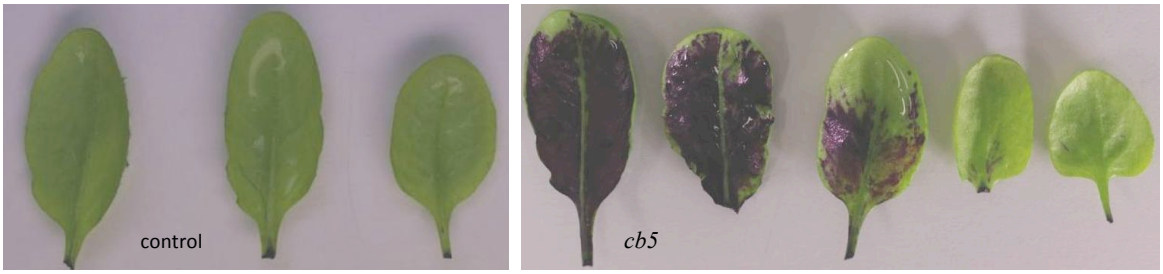


Figure 11: Abaxial surface of *cb5* leaves stained with toluidine blue for 2 min. Control in the left panel.

However, dicotyledons, early rosette leaves and cauline leaves were not stained in *cb5*, suggesting that the phenotype is only visible in rosette leaves at later stages of growth. Interestingly, recently developing rosette leaves and the apical meristem displayed a strong staining when whole 25 days-old plants were analyzed (data not shown).

Also, three 25 days-old small *cb5* plants showed a fast ozone-induced stomatal closure or Rapid Transient Stomatal Closure (RTSC) (Vahisalu *et al.*, 2009) (data not shown) whereas three other 30 days-old bigger plants responded differently from each other to ozone (figure 12). One explanation for such contradictory results could be that the mutant phenotype depends on the developmental state of the rosette leaves.

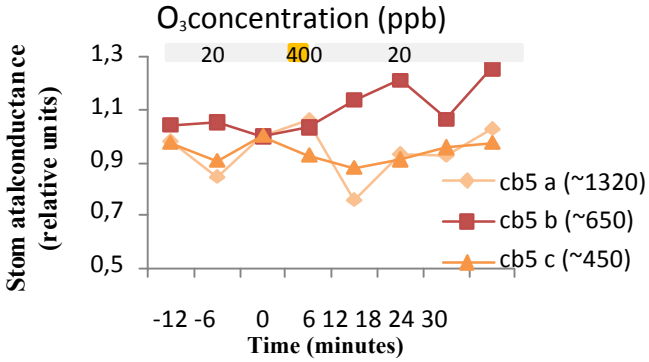


Figure 12: Time courses of stomatal response of 3 30-days old *cb5* replicates to a 3-minute ozone pulse of about 400ppb. Values are expressed relatively to the conductance measured at T_0. Values in brackets are absolute stomatal conductance in mmol.m^{-2}.s^{-1}.

3.2.2. Gene identification

We hypothesized that a T-DNA insert disturbed the normal expression of a gene, thus leading to a high transpiration phenotype. Identifying the potential mutated gene consisted of rescuing the flanking sequence of the T-DNA insert with the help of appropriate primers. For this purpose, the TAIL-PCR method was successfully adapted to the laboratory conditions and improved from Session and colleagues (2002). At least two potential candidate genes were identified for being responsible of the high water loss phenotypes for both *cb1* and *cb5*. For *cb1*, SLAH1 (AT1G62280) and a putative disease resistance gene (AT5G43740) were identified. Sequences from *cb5* corresponded to a FAD-dependent oxidoreductase family protein (AT3G56840) and a putative c-myb-like transcription factor (MYB3R3) mRNA (AT3G09370).

The SLAH1 gene and the putative disease resistance gene are located on chromosomes 1 and 5, respectively. The FAD-dependent oxidoreductase family protein and the putative transcription factor are coded by genes situated both on chromosome 3. T-DNA insertions were located in exons except for the transcription factor, for which the insert was in the promoter region of the MYB3R3 gene. The SLAH1 protein is a homologue of the slow-anion channel 1 (SLAC1). Neither of the other proteins have been functionally characterized yet. These discoveries seem to validate the new strategy used in the laboratory to identify mutated genes.

3.3. Characterization of ozone-sensitive lines under abiotic constraints

Ozone-sensitive mutants (*ozs*) were screened for two parameters: (1) percentage of water loss and (2) stomatal response to a 3-minutes ozone pulse of about 400ppb.

3.3.1. Water loss measurements

Among the 43 *ozs* lines screened for water loss, none were found to exhibit a phenotype similar to the positive control *slac1-1 gl1* (Figure 13).

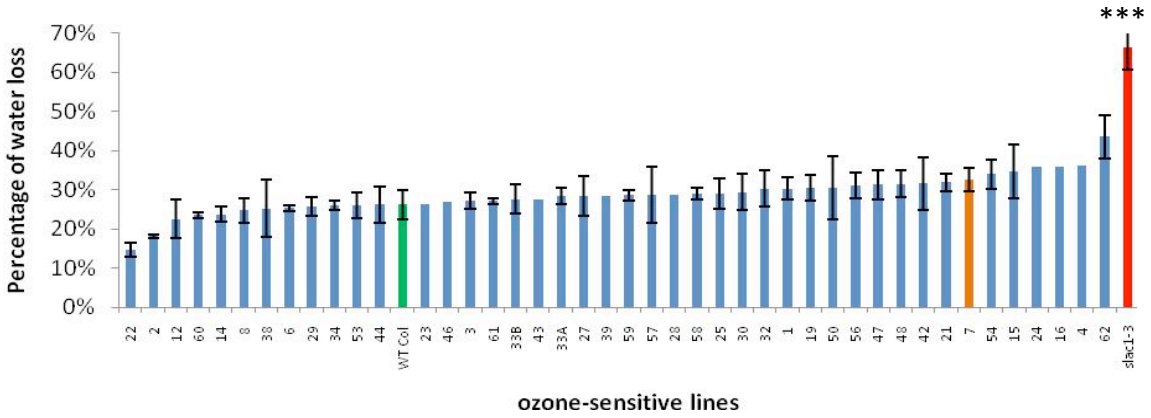


Figure 13: Percentage of water loss after 90 minutes of ozone-sensitive cut-off leaves. Error bars are standard deviations when n>2 (n=3). Fisher LSD test p< 0.05). ***: highly significant.

Instead, *ozs2* and *ozs22* were together identified as mutants with significantly lower percentage of water loss (<20 %) compared to other lines (Figure 13) and to WT Col-gl 0.

3.3.2. Stomatal response to an ozone pulse

Stomatal responses to short pulse of ozone were also tested for some *ozs* mutants. Majority of mutants behaved as WT (data not shown). However, *ozs7* showed the same response as the stomatal mutant *slac1-1 gl1*, that is no RTSC (Figure 14). Some results were however contradictory. For example, *ozs2* and *ozs22* did have a RTSC but *ozs2* did not recover by 30 minutes like observed for wild-type and the other lines (data not shown but see WT Col in Figure 14).

Only *ozs2*, *ozs7* and *ozs22* were selected for further characterization under high CO_2 concentration and changes in air humidity.

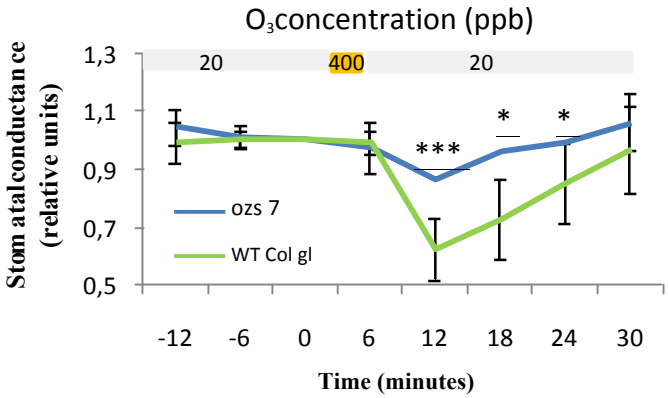


Figure 14: Time courses of stomatal response to single 3 min pulse of ozone (400 ppb) for ozone-sensitive line 7 (*ozs7*) and WT Col. Values are expressed relatively to the conductance measured at T_0. Absolute stomatal conductance ranged from 170 to 540 mmol.m^{-2}.s^{-1} for *ozs7* and from 130 to 600 mmol.m^{-2}.s^{-1} for WT Col gl. N= 7 for *ozs7* and n=3 for WT Col gl. Error bar is standard error. T-test ($p<0.005$) ***: highly significant, *: significant.

3.3.3. Stomatal response to elevated CO_2

ozs7 did not respond to high CO_2, confirming a *slac1* behavior (Figure 15). *ozs2* and *ozs22* responded as wild-type control (data not shown).

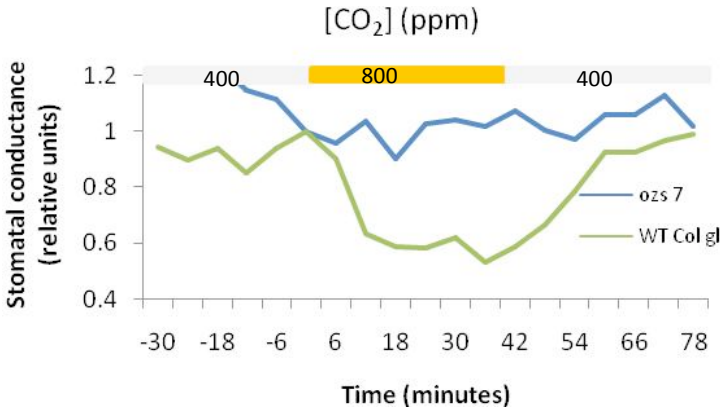


Figure 15: Time courses of stomatal response 800 ppm of CO_2 for 30 min. n=2 for *ozs7* and n=1 for WT Col gl.

3.3.4. Stomatal response to changes in air humidity

It has been shown that *slac1* mutants have also impaired stomatal responses to reduction of air humidity (Vahisalu *et al.* 2008). To address this point in *ozs7* (Figure 15 and Appendix), *ozs2* and *ozs22* (not shown) we tested their stomatal responses to reduction of air humidity from 70% to 35%. All responded as wild-type control.

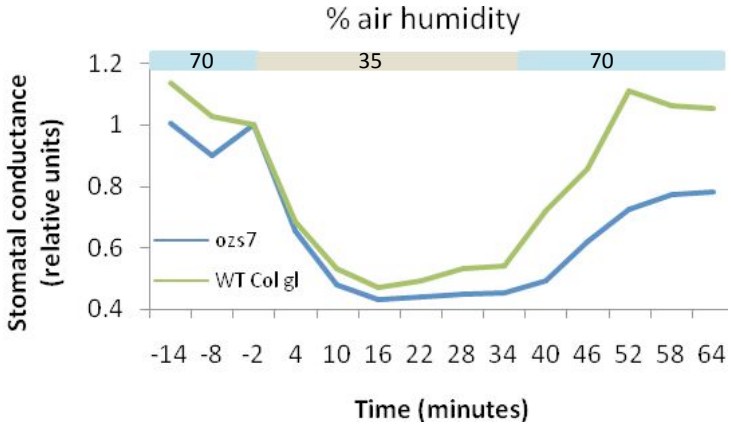


Figure 15: Time courses of stomatal response to changes in air humidity. n=2 for *ozs7* and n=1 for WT Col *gl*.

4. DISCUSSION

Molecular mechanisms of stomata function have been extensively studied, but much remains to be understood (Schroeder *et al.*, 2001, Li *et al.*, 2006). Finding novel mutants in *A. thaliana*, the model organism in plant biology, is an effective way to gain more information about the components regulating the processes of stomatal opening and closure. Infrared thermal imaging is an efficient method to detect stomatal mutants. It relies on the difference of evaporative cooling between the control and the stomatal mutant because high transpiration cools down the leaf surface. For example, a previous thermo-imaging screen of drought stress plants yielded to the discovery of the stomatal mutants *ost1* (Merlot *et al.*, 2002). By applying a new screening method, based on the same cooling property with excised leaves, on a library of activation tagged T-DNA lines (Weigel *et al.*, 2000) we isolated two interesting mutants named *cb1* and *cb5*, displaying a high water loss. Dependent on where activation tagged T-DNA is inserted, it should yield mutants in which particular genes are either over-expressed or knocked out. As T-DNA insertion by *Agrobacterium* is a random process and because the aim was to find new key elements, any particular gene or function were expected to be (re)discovered. Ideally, mutants would visually look like the wild type and would have a phenotype related with stomatal regulation only, especially a defective closure under changing environmental conditions, similarly to the *slac1-3* mutant.

The first discovered mutant was named *cb1* (*cool breath1*). Unfortunately *cb1* did not start flowering and therefore no further phenotypic characterisation of the mutant could be done. However, genomic DNA extraction allowed some genetic characterisation. The SLOW

ANION CHANNEL ASSOCIATED 1-HOMOLOGUE (SLAH1) was identified as a potential gene responsible for the high water loss trait in *cb1*. Negi and colleagues (2008) recently described SLAC1 and three homologues (SLAH1, SLAH2 and SLAH3). They are all plasma membrane proteins but the 3 homologues were specific to in vascular tissues whereas only SLAC1 was located in guard cells. The *cb1* mutant, which had been selected for its high transpiration rate, and water loss, suggests that the responsible gene encodes a protein affecting stomatal regulation. Negi and colleagues (2008) also showed that SLAH1 can complement SLAC1 function using the SLAC1 guard cell specific promoter, which is consistent with our hypothesis that SLAH1 and SLAC1 have the same anion channel function. Another potential gene was identified as a gene coding for a putative disease resistance protein of the CC-NBS-LRR class (coil-coiled nucleotide-binding site leucin rich repeat). NB-LRR proteins functions as immune receptors in plant innate immunity but alternative roles were mentioned in defence and drought signalling (Tameling *et al*., 2007). Beyond, McHale *et al*. (2006) reported the NB-LRR proteins can induce oxidative burst and calcium and ion fluxes, which are common processes to plant defence responses and stomatal regulation. Unfortunately, *cb1* could not be tested for its response to an O_3 pulse, to generate an oxidative stress.

Another interesting mutant with a high water loss, named *cb5,* exhibited a phenotype related to the cuticle (Figure 8). Two genes potentially affected in *cb5* were also identified: a FAD-dependant oxidoreductase and a myb transcription factor. None of these proteins have been previously described to be involved in cuticle-related processes. The mutant phenotype together with the staining results with toluidine blue strongly suggests that *cb5* is a cuticular mutant. Plant cuticle is a waxy covering of plant leaves and stems and all other aerial organs without periderm. The barrier it forms protects plants against abiotic and biotic stresses and also insulates organs from each others. The latter role of cuticle explains the fused leaf phenotype observed in *cb5* and other cuticular mutants like *fiddlehead* (Lolle et al., 1992). Secondly, the cuticle prevents water evaporation through the epidermis and strong staining with toluidine blue dye demonstrated the permeability of the abaxial surface of older *cb5* leaves (Figure 11). Notwithstanding, the impairment of *cb5* cuticle was documented to occur only in rosette leaves developing at a certain age (~ from 25-30 days-old). This has to be further confirmed (Lindgren *et al.,* 2009). In addition, gas exchange data supported this observation since heterogeneous responses to the ozone pulse were recorded (Figure 12). Despite being of the same age, foliar surfaces of the three replicates were very different, ranging exceptionally from 10 to 21 cm^2 when grown at 90% humidity. The absolute stomatal conductances were also very different between plants.

In a 24h time course study, one tiny *cb5* plant with wild type-looking phenotype had the wild type stomatal conductance and response to light and darkness whereas the responses of bigger *cb5* plants of the same age but carrying a visual convoluted phenotype of *cb5* only displayed a transient response. The stomatal conductance of the latter was considerably higher throughout the day (data not shown). Also there was no obvious stomatal closure induced by the onset of normal darkness (data not shown). Higher stomatal conductances of these plants might be explained by the inefficiency of the cuticle to restrict the water loss through the epidermis, whatever the light conditions. More importantly, as conductance was measured with water

vapor fluxes, high conductance values for the *cb5* mutant may not represent the true stomatal conductance if water was able to pass through the epidermis. Hence, the stomatal regulation phenotype might be due to other secondary effects which might be more complex.

These results raised several hypotheses: either there is a developmental switch during the vegetative development of plant leaves where *cb5* mutation is involved, or the leaf expansion itself might require further development of the cuticle and this process is affected in the *cb5* mutant. Scanning electron microscopy images of the mutant epidermis and gas chromatography - mass spectrometry analysis of the cuticle composition could bring new insights about the nature of the impairment and help narrowing down the research to correlate the defect with a FAD-dependant oxidoreductase and/or a myb transcription factor.

When screening populations that have been mutagenized with an insertion element like T-DNA, not all of the new phenotypes will be due to the insertions. Furthermore, insertions may occur at many different loci within the whole genome. As a consequence, isolation of the flanking sequence may not correspond to the gene involved in the stomatal phenotype of isolated mutants.

To overcome these uncertainties, genotyping of the implicated genes has to be performed with genomic DNA from several back-crossed, second generation individuals displaying the phenotypic trait in order to ascertain the linkage between the trait and the insertion element. The mutation is naturally transmitted to the next generation according to segregation rules. The identity of the potential gene is confirmed if the same gene is again identified in the genome of next generation. This additional step might also discriminate the two potential genes picked-up by the TAIL-PCR. A further verification would be a test for complementation of the mutant phenotype, by the wild-type allele of the suspected gene, in transgenic plants (Weigel & Glazebrook, 2002). If complementation occurs, the normal function is at least partially recovered.

An additional approach to find mutants affected in stomatal regulation was carried out with a collection of *ozs* mutants – EMS-mutagenized lines already isolated in a screen for their ozone sensitivity (Overmyer *et al.,* 2000). The control line *slac1-1*, previously named *rcd3* (Overmyer et al. 2008) originated from the same library. This suggested that additional mutants affected in stomatal regulation might be discovered from this batch. Measuring the water loss percentage of cut-off leaves after 90 minutes did not yield any mutants carrying a phenotype similar to *slac1-1*. However, monitoring their stomatal responses to ozone revealed that *ozs7* did not respond to a 3-min ozone pulse of 400 ppb (Lindgren *et al.,* 2009) (Appendix). Stomatal conductance reflects the open/closed average state of the stomata. Ozone was used as a screening tool to identify mutants defective in stomatal closure as *slac*. Indeed, ozone generates an increased production of ROS, which are signaling molecules inducing stomatal closure. It has been documented that 70 ppb of O_3 is sufficient to induce a Rapid Transient Stomatal Closure (RTSC) within 6-10 minutes after the onset of ozone in wild type and a subsequent reopening within the further 30 min (Kollist *et al.,* 2007). However, RTSC has also shown to be absent in the guard cell signaling mutants *abi1*, *abi2*, *ost1* and *slac1* suggesting a role for the ABI1, ABI2 phosphatases, OST1 kinase and SLAC1 anion channel, respectively (Vahisalu *et al.,* 2009). Additionally, *ozs7* did not respond to high

CO_2 concentration. However, based on water loss measurement and response to changes in air humidity, *ozs7* is different from *slac1*. Indeed, *ozs7* stomatal conductance decreased exactly like the wild type control when dry air was supplied whereas *slac1* exhibited a reduced and delayed response (Vahisalu *et al.,* 2008). These data suggests that the *ozs7* mutation is not involved in the regulatory pathway responsible for transducing the changes in air humidity. Lack of high CO_2 and O_3 induced stomatal closure in *ozs7* suggests that the mutated protein is a key element shared by the O_3 and CO_2 signaling pathways. O_3-induced ROS are altering the photosynthetic machinery, which might lead to a decrease in CO_2 assimilation rate and the increase in internal CO_2 concentration as a consequence. If *ozs7* were only insensitive to CO_2 concentration, a stomatal closure would be expected in response to high ozone concentration because of the additional ROS signaling function. As, it is not the case, a hypothesis of exclusive insensitivity to CO_2 concentration is not validated.

5. CONCLUSIONS

Stomatal functioning is regulated by a complex network of signaling pathways transmitting changes in environmental conditions into a controlled transpiration rate. Mutants are used to identify the network components and disentangle their relationships.

In all, three mutants, *cb1, cb5* (T-DNA insertion mutants) and *ozs7* (EMS-mutant) were partially characterized within the frame of this project. *cb5* was shown to be a cuticular mutant. Optimization of the molecular techniques of TAIL-PCR (Sessions *et al.*, 2000) allowed identification of four possible mutated candidate genes in *cb1* and *cb5*. The genes were all implied as being responsible for increased transpiration rates (*cb1* and *cb5*) or an increased sensitivity to ozone (*ozs7*). Establishing the linkage between highly promising genes and the high water loss phenotype is in process, by analyzing the mutants and by other methods. Especially, the SLAC HOMOLOGUE mutants, one of which could be *cb1*, have already been shown to complement the *slac1* mutant (Negi *et al.*, 2008) and will soon be tested for their stomatal response to an ozone pulse. In addition, the EMS-mutant ozs7 showing a *slac1*-like phenotype in response to an ozone pulse and to high CO_2 concentration was found. Genome sequencing is scheduled by our collaborators in Helsinki within the next few months. The three mutants and their particular characteristics might bring new insight into the understanding of stomatal regulation mechanism cuticle formation. General knowledge obtained from *Arabidopsis thaliana* mutants affected in these processes might also be helpful for understanding stomatal regulation of forest tree leaves in the changing environment.

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Appendix

Plant Abiotic Stress-from signaling to development. 2nd meeting of the International Network of Plant Abiotic Stress (INPAS). 14-17 May 2009 Tartu Estonia.

Methods for finding novel stomata mutants in *Arabidopsis thaliana.*

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INTRODUCTION

Molecular mechanisms of stomata function have been extensively studied, but much remains to be understood. Finding novel mutants in *A. thaliana* defective in stomatal functioning is an efficient way to gain more information about the components regulating the processes of stomata opening and closure. We screened a T-DNA activation tagged library using *slac1* (SLOW ANION CHANNEL ASSOCIATED1) [1] as a positive control and are also characterizing EMS-mutagenized ozone-sensitive lines previously selected [2]. Our aim is to find and characterize open stomata mutants and to identify the genes responsible for the phenotype. By applying a new screening method we have found two water-loss mutants named *cb1* (*cool breath1*) and *cb5*. Also, by analyzing putative ozone-sensitive mutants with our unique gas-exchange system we have found one line affected in stomatal closure named *ozs7* (*ozone-sensitive7*).

MATERIAL AND METHODS

❖ 1 month-old 35S insertion lines of *Arabidopsis* (Columbia) were screened. Two leaves per plant were cut off and placed abaxial face down on a Petri dish lid. The dish was filled with 17°C green-coloured water. The presence/absence of a condensated water vapor spot on the inner side of the lid was monitored after 20 minutes. A visible spot of comparable size to the one of *slac1-3* leaves was interpreted as coming from a putative water-loss mutant. After one hour, the visible phenotype of these leaves were examined. Very dehydrated (shrivelled) leaves similar to the control was taken as a further indication of a water-loss mutant. Seedlings that had leaves with both traits were grown further for about two weeks and the water-loss of excised leaves were measured before and after 90 minutes, using WT Columbia and *slac1-3* as controls.

❖ Whole rosettes of ozone-sensitive lines were exposed for 3 min to 400±25 ppb O_3 in a rapid-response gas exchange measurement system [3]. Stomatal conductances for water vapour were calculated on the base of measured transpiration rates.

RESULTS

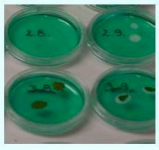


Figure 1. The presence/absence of vapour spots in *A. thaliana* 35S insertion lines. *slac1-3* as a positive control on the right.

Figure 2. Percentage of water loss in excised leaves after 90 min relative to fresh weight (n=4).

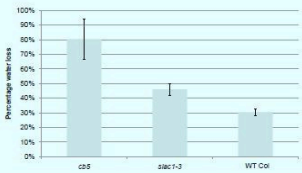




Figure 3.
The 35S insertion mutant *cb5*.

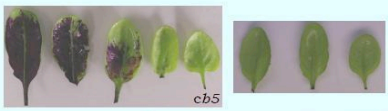


Figure 4. Staining of abaxial epidermal cells of *cb5* (left panel) and of control (right) with toluidine blue [4].

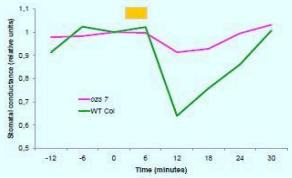
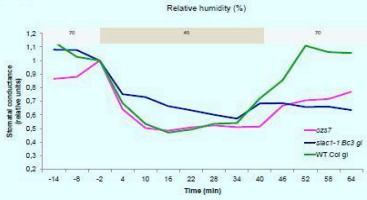


Figure 5. Time course of stomatal changes in response to a 3-min ozone pulse of 400 ppb (orange box) (n=2).

Figure 6. Time course of stomatal changes in response to changes in air humidity (n=3).



1. Two 35S insertion mutants named *cb1* and *cb5* (*cool breath*) have been discovered with a new screening method based on high transpiration rate (Figure 1).
2. *cb5* displayed an even stronger water-loss phenotype than *slac1-3* (Figure 2) and a cuticle mutant phenotype with fused leaves (Figure 3). Interestingly, young rosette leaves and cauline leaves do not display these phenotypes (Figure 4).
3. The EMS mutagenized *ozs 7* showed only weak stomatal response to ozone (Figure 5) but a normal response to changes in air humidity (Figure 6).

CONCLUSIONS

The high transpiration rate and water-loss in *cb5* can be explained by an impaired cuticle as implicated by the toluidine blue stainings. Identifying the mutated gene with the tail-PCR method and spatial and temporal localization of the responsible protein will confirm this assertion.

ozs7 is a good stomatal mutant candidate, having weak response to O_3 but responding normally to changes in air humidity. This suggests that the defective element is not involved in the regulatory pathway responsible for transducing the changes in humidity. The *ozs7* stomatal response phenotype to elevated CO_2 has still to be ascertained.

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Characterizing new *Arabidopsis thaliana* mutants affected in transpiration.

Arabidopsis thaliana mutants are commonly used in plant physiology to understand cellular and molecular mechanisms in general. We focused on stomatal functioning and are aiming at contributing to the knowledge of stomatal regulation.
A new screening method was used to select 5 T-DNA insertion mutants having a high transpiration rate and molecular techniques were optimized to rescue potential mutated genes. One of them, *cb1*, is possibly the already mentioned anion-channel SLAH1 gene (Negi *et al.*, 2008). Phenotypic characterization of *cb5,* consisting of water loss measurements and toluidine blue staining of the epidermis demonstrated a defect in the cuticle of this mutant.
A screen for stomatal mutants was conducted on previously selected EMS-mutagenized lines that were sensitive to ozone in order to identify new mutants. One line, *ozs7*, was selected and showed no response to either ozone or CO_2. However, it did display a wild-type response to changes in air humidity. Further investigations are required to confirm and to make the most out of these discoveries.

Key words : *Arabidopsis thaliana* mutant, transpiration, stomatal conductance, ozone, TAIL-PCR

Caractérisation de nouveaux mutants d'*Arabidopsis thaliana* ayant une forte transpiration.

Les mutants d'*A. thaliana* sont utilisés avec succès en biologie végétale pour comprendre les mécanismes cellulaires et moléculaires des différentes fonctions. Nous avons particulièrement étudié la régulation stomatique, fonction essentielle contrôlant les échanges gazeux entre les feuilles et l'atmosph re, afin de contribuer à la connaissance des mécanismes de cette fonction.
Une nouvelle strat gie de criblage a permis la s lection de 5 mutants d'insertion ayant un taux d' vapotranspiration sup rieur à la normale. L'optimisation des techniques mol culaires (extraction d'ADN et tail-PCR) ont permis l'identification de 4 g nes. Notamment, SLAC HOMOLOGUE 1 (SLAH1) qui est un canal anionique, est potentiellement impliqué dans la régulation stomatique du mutant *cb1*. Un fort pourcentage de perte en eau et une coloration épidermique accréditent la thèse selon laquelle le mutant d'insertion *cb5* serait un mutant cuticulaire.
Un criblage, bas sur la r ponse stomatique à un pic d'ozone, a t effectu en parall le sur une collection de mutants EMS sensibles à l'ozone. Un mutant, *ozs7*, n'a pas r pondu au stimulus ozone, ni à une forte concentration en CO_2. Cependant, ozs7 répond normalement à des changement d'humidit atmosph rique. Une caract risation plus pouss e devrait confirmer ces premiers résultats et tenter d'en tirer des conclusions sur la r gulation stomatique de ce mutant.

Mots clès : mutant *Arabidopsis thaliana*, transpiration, conductance stomatique, ozone, TAIL-PCR