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Ozone-induced cell death and stomatal responses in different *Arabidopsis thaliana* ecotypes and mutants.

Master thesis

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ABBREVIATIONS

ABA	abscisic acid
abi	*abscisic acid insensitive*
bp	base pair
$[Ca^{2+}]_{cyt}$	cytosolic calcium concentration
Col	Columbia
Cvi	Cape Verde island
Est	Estonia
ET	ethylene
GSH	glutathione
$HO_2^{\bullet}$	hydroperoxyl radical
H_2O_2	hydrogen peroxide
HR	hypersensitive response
JA	jasmonic acid
lcd	*low cell density*
L*er*	Landsberg *erecta*
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
$O_2^{\bullet -}$	superoxide anion radical
O_3	ozone
oji	*ozone sensitive and jasmonic acid insensitive*
PCD	programmed cell death
PFD	photon flux density
rcd	*radical induced cell death*
ROS	reactive oxygen species
SA	salicylic acid
SOD	superoxide dismutase
UV	ultraviolet
vtc	*vitamin C deficient*
Ws	Wassilewskija
WT	wild type

INTRODUCTION

Different environmental stressors induce plant defence reactions. Ozone (O_3) is a widespread phytotoxic air pollutant in troposphere and it is converted into reactive oxygen species (ROS) in cell walls of affected plant tissue. Application of ozone to plant tissues thus creates first an O_3-dependent oxidative burst, followed by subsequent plant cell dependent oxidative burst. Therefore, O_3 is widely recognized as a tool to study oxidative stress and cell death.

Arabidopsis thaliana is one of the most widely employed species for genetic studies due to advanced cultivation technologies (Noren et al., 2004), short generation time and availability of its full genome sequence (*www.arabidopsis.org*). A multitude of mutants has been produced where some important physiological function is genetically modified. Several mutants with changed stomatal functioning have been described and complex models of signal transduction networks leading to these changes are elaborated (Schroeder et al., 2001; Mäser et al., 2004). Some ecotypes/mutants are shown to have increased (Overmyer et al., 2000; Rao et al., 2002; Kanna et al., 2003; Ahlfors et al., 2004; Joo et al., 2005) or decreased (Tamaoki et al., 2003) sensitivity to ozone.

Although the importance of stomata in regulating ozone entry into the leaf interior is now widely recognized, comparative data on stomatal responses to ozone fumigation in intact leaves of different *Arabidopsis* lines are scarce. This is due to the problems in clamping an attached leaf in a compact prostrate rosette into a commonly bulky leaf cuvette without evoking disturbances in stomatal behaviour. In addition, data from few (mostly biggest) leaves are poorly representative on the organism level due to the different physiological age of leaves in the rosette.

Therefore an eight-chamber whole-rosette ozone fumigation system was constructed at Tartu University, which enables to monitor rosette O_3, CO_2 and H_2O exchange in different Arabidopsis mutants/ecotypes simultaneously.

In this study the O_3-induced stomatal responses were assayed in *Arabidopsis* genotypes Col-0, L*er,* Ws-2, Est1, Cvi and mutants *rcd1, rcd2, rcd3, abi1, abi2, vtc1, oji1*. In addition, O_3-induced tissue damage, production of reactive oxygen species in stomatal guard cells and whole tissue, and finally, ABA induced stomatal closure was assayed.

1. LITERATURE REVIEW

1.1 Ozone - protector in the stratosphere, pollutant in the troposphere and a good tool to study oxidative stress

Schoenbein & Soret discovered and determined the structure of the trimolecular allotrope of oxygen – ozone (O_3), in the middle of the 19th century. The name is derived from the Greek word for "smelly" (Hogsett et al., 1985).

In contrast to stratospheric O_3, which protects plants from harmful UV-B radiation, tropospheric O_3 is an oxidant, which harms both natural and agricultural species (Kley et al., 1999). It was reported for the first time in 1958 by Richards et al. in California, that ozone causes damage on grape leaves. Nowadays O_3 is the most widespread air pollutant in the industrial areas. The damage depends on the concentration and duration of O_3 exposure (Kangasjärvi et al., 1994).

Background concentrations of O_3 have doubled in the last century (Campbell, 1996). Paradoxically, O_3 content in stratosphere has decreased causing 'O_3 holes'. Increased O_3 concentration in troposphere is caused mainly by traffic and industry. The main compounds involved in O_3 formation are different species of oxidized nitrogen (NO_x), which react with oxygen using sunlight – as a result, O_3 is formed.

In Scandinavia, the most common tropospheric O_3 concentrations during summertime are around 20–40 nL L^{-1} (Skärby et al., 1994).

The critical and phytotoxic O_3 levels for vegetation are highly dependent on plant species. In addition, inherent genotypic variation within the plant species has an influence. In Nordic countries, the suggested critical long-term O_3 concentration is expressed as cumulative exposure over the threshold concentration of 40 nL L^{-1} (Skärby et al., 1994).

Ozone causes oxidative stress in plants. The duration and concentration of O_3 exposure affects plant responses. High short-term O_3 concentrations (200 - 400 nL L^{-1}) frequently cause foliar lesion also in the O_3-tolerant Arabidopsis ecotypes (Overmyer et al., 2000). In O_3-sensitive species, such as silver birch and barley visible lesions can be formed at O_3 concentrations of 80 nL L^{-1}. Lower but chronic O_3 concentrations (around 50 nL L^{-1}) may accelerate senescence without causing visible foliar cell death. It has been shown, that the increase in tropospheric O_3 concentration causes yield reduction in many crop plants, which has led to significant economic losses (Krupa et al., 1994). Natural communities, including forest stands are also damaged by

this pollutant (Hogsett et al., 1997). The elevation of tropospheric O_3 concentration is still a continuing problem to be solved.

The question of what determines O_3-sensitivity or tolerance in various plant species or genotypes has been one of the central topics in O_3 research. First of all, the rate of O_3 influx into the plant leaf may determine subsequent O_3 responses. Thus, the number and size of stomata and the stage of their opening, i.e. stomatal conductance, affect O_3 uptake (Rich et al., 1970). Humidity influences stomatal conductance. For example, high air humidity may affect O_3 sensitivity through increased stomatal opening and higher O_3 influx into the plant. Studies have also shown that drought stress may protect from O_3-induced foliar damage possibly due to decrease in stomatal conductance and reduced influx of O_3 into plant cells. It has been shown repeatedly, that O_3 causes stomatal closure (Robinson et al., 1998). Briefly, it could be said that stomata are the first barrier against O_3.

Having penetrated stomata, O_3 reaches leaf interior and diffuses in apoplast. Laisk, Moldau and Kull (1989) established that O_3 concentration in intercellular spaces is extremely low irrespective of the concentration applied (up to 1500 nL/L^{-1}). This means that O_3 is absorbed and rapidly decomposed in the cell walls or plasmalemma and suggests that there is a large pool of antioxidants in the apoplastic space of plants. Chameides (1989) supposed that a great part of O_3 entering the leaf could be absorbed by ascorbic acid in apoplast. Then hypothesis was posed that O_3 sensitivity of plants may depend on their ascorbic acid content. Latter findings have shown that, for example in cereals crops, like wheat and barley, O_3 removed by ascorbic acid is only 10-20 % (Kollist et al., 2000). It is mainly due to the fact that wheat and barley have thin cell walls (0.1-0.12 μm) and thus the effective pathway for the reaction between O_3 and ascorbate is short. As a result, the formation of ROS from O_3 degradation at high O_3 concentrations could easily exceed the antioxidant capacity of the apoplast and the redox balance of the cellular environment will be perturbed. This “redox shift” is triggering plant intrinsic burst of reactive oxygen species that is the functional component of signal transduction pathways leading to processes like hypersensitive response and/or systemically acquired resistance.

The oxidative burst is common to a large number of other pathogenic stresses in plants. For example: flooding, drought, high light, ionising radiation, freezing, heat shock, chilling, heavy metals, high salinity, wounding, air pollutants and pathogen attack can all induce plant ROS production. Therefore the ozonation of plants is established as a good tool to exert artificial oxidative stress in plants and thereby to discover genetic mechanisms activated by ROS.

1.2 Oxidative stress – plant response to biotic and abiotic stressors

1.2.1 Oxidative stress and oxidative burst

Oxidative stress can be defined as the imbalance of ROS production and the antioxidant capacity of the cell, which leads to accumulation of different ROS and potentially to cell death. ROS can be generated by plant itself or by external factors, such as O_3 which has entered the leaf.

Different stressors, both biotic and abiotic can cause enzymatically mediated elevation of ROS both in plant and animal cells (Mahalingam et al., 2003).

It is known that ROS production in different cell compartments influences expression of a number of different genes. This indicates that cells have their own strategies to use ROS as biological signals to drive diverse genetic programs. It is presumed that ROS can interact selectively with target molecules, which detect elevations in ROS concentrations affecting the activity of transcription factors, second messengers (such as Ca^{2+} and kinases) or enzymes involved in biochemical pathways. The cellular redox status can be sensed by abundant redox-sensitive molecules such as thioredoxin and glutathione, which transmit the signal forward. The ultimate targets in all these cases are either the thiol groups of cysteine residues or the iron-sulphur clusters in the catalytic centres of enzymes (Laloi et al., 2004).

The site of ROS formation and the oxygen species produced, depend on the stress in question. For example, at chilling temperatures, the chloroplast electron transport chain is especially vulnerable, whereas during pathogen attack, ROS concentrate in the apoplastic space. In addition, ROS act as signal mediators in programmed cell death (PCD) and hypersensitive response (HR), which is localized cell death (Lamb and Dixon, 1997), in ABA signal transduction and in the senescence of plant organs (Pei et al., 2000).

Active and controlled production of ROS in cells is called oxidative burst. For example, plant-pathogen interactions bring about oxidative burst, where pathogen can be killed directly. Alternatively, oxidative burst may limit the spread of pathogen by mediating local cell death in infected plant cells. The oxidative burst is common also to abiotic stresses in plants, for example flooding, drought, high light, ionising radiation, freezing, heat shock, chilling, heavy metals, high salinity, wounding, air pollutants (Bowler and Fluhr, 2000).

The oxidative burst induced either by pathogens or O_3 can occur in two phases (Schraudner et al., 1998) where the kinetics of this response depends on the type of plant-pathogen interaction or the O_3-sensitivity of the plant. The first peak occurs quickly, rising and falling sharply, typically within the first two hours of the attack. Phase two of the oxidative burst peaks at 12-24

hours and then slowly recedes. The second phase is released by avirulent pathogens, which evokes hypersensitive response. In virulent plant-pathogen interaction only the first, initial burst can be observed (Mahalingham and Fedoroff, 2003). ROS production triggers signal transduction pathways in adjacent cells and activates genes encoding defence proteins and hormonal responses (Lamb and Dixon, 1997).

Transmembrane NADPH oxidase can generate oxidative burst in the cell wall, which mediates electron transport from NADPH to molecular oxygen, generating superoxide thereby. In addition, peroxidases and amine oxidases can mediate oxidative burst in the cell wall (Laloi et al., 2004).

Ozone causes oxidative stress, when having entered the plant leaf. In apoplast O_3 degrades into different ROS, which in turn induce active endogenous ROS production in the plant. In addition, the content of autofluorescent phenolic compounds is elevated, pathogenesis related proteins are expressed, micro- and macro-scale cell death emerges bringing about systematic acquired resistance (Overmyer et al., 2003). Consequently, processes induced by ozone are similar to processes induced by pathogens (Overmyer et al., 2000; 2003).

Reactive oxygen species are produced also in the course of plant natural development and metabolism. For example, as a by-product in mitochondria and chloroplast electron transport, β-oxidation of lipids and in cell wall lignification (Noctor and Foyer, 1998). Natural leaf senescence and formation of ROS have been associated also with oxidative stress (Thompson et al., 1987).

Because under stress conditions ROS formation is accelerated, plants have evolved an efficient network to remove and detoxify excess ROS. There are both, enzymatic and non-enzymatic ways. Enzymatic antioxidant systems such as superoxide dismutase (SOD) and catalase eliminate superoxide and hydrogen peroxide, respectively. Also ascorbate peroxidase eliminates hydrogen peroxide. In addition, there are several low-molecular-weight antioxidants, such as ascorbic acid (vitamin C), glutathione (GSH) and α-tocopherol (vitamin E), which remove excess ROS non-enzymatically (Noctor and Foyer, 1998).

Although ROS production accompanies nearly all plant stresses, the molecular mechanisms of transcriptional changes and of signal transduction are not clear yet (Mahalingham and Fedoroff, 2003). In order to understand the genetic network, which underlies the stress and defence reactions of the plant, it is necessary to identify and characterize genes, which take part in the stress and pathogenesis activated physiological defence mechanisms.

1.2.2 Reactive oxygen species

Oxygen in its stable O_2 form is a necessity for all aerobic organisms and it is involved in many biochemical reactions in cells.

If an O_2 molecule receives excess energy, one of its electrons can change the spin and this leads to the formation of singlet oxygen ($^1O_2$). $^1O_2$ can react with several cellular components, such as DNA, RNA, lipids, sterols and especially proteins (Davies, 2003).

The first reduction product of O_2 is superoxide anion ($O_2^{\bullet -}$), which exists in solution with its conjugate acid, hydroperoxyl radical ($HO_2^{\bullet}$). $O_2^{\bullet -}$ and $HO_2^{\bullet}$ dismutate to hydrogen peroxide (H_2O_2) (Alscher et al., 2002). $O_2^{\bullet -}$ causes lipid peroxidation. The amount of $HO_2^{\bullet}$ is small when compared to $O_2^{\bullet -}$. However, $HO_2^{\bullet}$ is a neutral molecule, thus it is able to cross biological membranes freely and it is proposed that it might have a greater biological significance than thought so far (De Grey, 2002).

Hydrogen peroxide is a relatively long-lived neutral molecule and therefore it can diffuse away from the site of formation and cross lipid membranes. At least partly, the diffusibility of H_2O_2 is likely to be the result of transport through aquaporins. H_2O_2 is potentially harmful to the cell because it can oxidize thiol groups in proteins and in the presence of an oxidizable metal, form hydroxyl radicals (Lamb and Dixon, 1997). Hydroxyl radical is extremely reactive and there is no enzymatic mechanism for its removal (Vranova et al., 2002). Hydrogen peroxide is removed by catalase and several peroxidases.

Since ROS can lead to disruption of cellular functions, there are both enzymatic and non-enzymatic mechanisms to keep their levels under control (Noctor and Foyer, 1998).

1.2.3 Hormonal regulation of ROS-induced cell death

The concept of hormonal regulation of ROS-induced cell death is to be formed. In contrast to the multitude of animal peptide hormones, plant hormones are usually rather small molecules and so far only relatively few of them have been identified. The number of these signalling compounds is increasing all the time. In addition, the sequencing of the Arabidopsis genome also revealed large amounts of putative peptide signalling molecules (Lindsey et al., 2002).

It is becoming more evident that hormonal participation in cell death is not linear. Different hormones act by suppressing each other at different stages or enhancing the process (Overmyer et al., 2003). In recent years it has been revealed that the three core plant hormones that regulate plant responses to pathogens, including the extent of cell death in HR, are ethylene (ET), salicylic acid (SA) and jasmonic acid (JA). The array of responses evoked by each hormone is

diverse and also the interactions between different hormones might be different, depending on the subset of responses being studied. (Overmyer et al., 2000). SA and ET seem to enhance cell death, whereas JA seems to have a role in the containment of the process (Fig. 1).

Salicylic acid

Salicylic acid (SA, 2-hydroxybenzoic acid) is a phenylpropanoid that is ubiquitously found in plants. SA has a wide range of effects in plants. It is involved in disease resistance, it is known in plant signaling for its role in systemic acquired resistance (SAR) and it has also a direct role in cell death regulation during hypersensitive response. SA favours cell death as there is an intimate association between SA-accumulation and cell death (Rao et al., 2002).

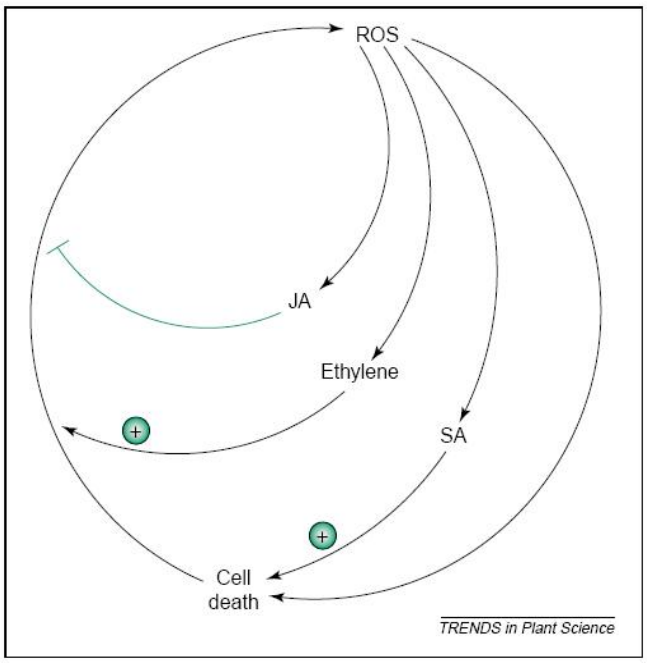


Fig. 1. Hormonal regulation of oxidative cell death.. The concentration of reactive oxygen species (ROS) increases with salicylic acid (SA) concentration and induces cell death. Ethylene (ET) is necessary for amplifying ROS production and propagation of cell death. Jasmonic acid (JA) is negative regulator of the cycle, suppressing the production of ET.
Fig. copied on the premises of authors from article: Overmyer, Brosche and Kangasjärvi (2003) Trends in Plant Science 8:335-341.

There is a clear evidence of cell death signals such as ROS ($O_2^{\bullet -}$, H_2O_2) and NO being involved in the regulation of key steps in SA biosynthesis during pathogen infection (Delledonne et al., 2001; Durner et al., 1997).

Plants exposed to O_3 accumulate SA, whereby the rate of SA accumulation is correlated with the rate of damage.

Rao et al. (2000) showed, that treating plants with SA before O_3 exposure increases cell death remarkably. Simultaneously it has been shown that SA inhibits the last stage of ethylene biosynthesis pathway. This means that at some conditions SA accumulation can contribute to the restriction of damage spread (Rao et al., 2002), as it has been shown repeatedly that ethylene is needed to keep oxidative burst in action.

Ethylene

Gaseous olefin ethylene (ET) is a classical plant hormone, which has vital roles in plant growth and development, such as seed germination, cell elongation, cell fate, sex determination, growth, foliar senescence, fruit ripening and leaf abscission. ET's role as a stress hormone is of great importance. ET is associated with wounding, pathogen attack, anaerobiosis, heavy metals, and

oxidative stress such as O_3 (Wang et al., 2002). It can auto-induce and is also induced by other hormones and signal molecules such as auxins, cytokinins and Ca^{2+}. Generally ethylene levels in plants are extremely low, but in addition to developmental cues, many stress situations induce rapid ET synthesis (Wang et al., 2002). Overmyer et al. (2000) showed that ET has a role in the regulation of ROS production in the O_3 induced oxidative cell death. Ethylene participates also in the regulation of programmed cell death, for example in the senescence of leaves and flowers. ET biosynthesis genes are in large multigene families, where different genes or gene clusters react to different developmental and environmental influences releasing ET production (Wang et al., 2002). Collectively, ethylene is a positive regulator of ROS production enhancing the propagation of cell death.

Jasmonic acid

Jasmonic acid (JA) is plant signaling compound with roles in both stress and development (Turner et al., 2002). A wide range of biotic and abiotic stresses induces JA, including O_3. However it is best known for its protective role in wound response (Berger, 2001). Analyses of gene expression with JA induced marker genes and treatment with JA indicates that JA may be a factor related to restriction of ROS-dependent lesion (Overmyer et al., 2000; Rao et al., 2000). Experiments with O_3-sensitive mutants indicated that JA plays a role in cell death inhibition. Treatment with JA after O_3 exposure inhibited lesion spread (Overmyer et al., 2000). In addition, it has been shown that JA protects cells from O_3 induced cell death spread by directly influencing ET signalling (Tuominen et al., 2004). JA suppresses ET-mediated oxidative cell death. It is thought that JA mediated inhibition of cell death may be related to regulation of ET receptors. Analysis of pathway-specific gene expression revealed that, similarly, ET could suppress the JA pathway. It is predicted that both the temporal and spatial kinetics of ET and JA accumulation fine-tune the hormonal interactions and the spreading cell death induced by O_3 (Tuominen et al., 2004). Molecular mechanisms of ET mediated suppression of JA pathway are not known.

Abscisic acid

Although the role of abscisic acid (ABA) in the regulation of cell death is not yet well established it is clear that it is also directly involved in the regulation of plant response to various stresses. ABA is also necessary for plant development and is involved in seed maturation. ABA prevents plants also from untimely germination by regulating seed dormancy together with gibberellins (Finkelstein et al., 2002). In addition to its roles in development, ABA is well known as a stress hormone, especially in the case of abiotic stresses. It seems that signalling and tolerance for such stresses as cold, drought, salt and UV-B radiation are at least partly dependent on ABA (Xiong et al., 2002). Many of the ABA-related responses require changes in gene

expression. ABA levels rise significantly in response to stress and return to normal after the stress has passed.

ABA signal transduction has been studied extensively and by far the most important model in this respect are stomatal guard cells (Grill and Himmelbach, 1998). Their control is of great importance to the plant and the fast response of guard cells to different stimuli is convenient to monitor.

ABA is synthesized from carotenoids through xantophylls and the early stage of its biosynthesis takes place in plastids. The pathway for ABA biosynthesis continues in the cytosol. ABA is a weak acid, the relative acidity of different cellular compartments has an influence on its localization and possibly on signalling. Diurnal oscillations in ABA concentration might exist.

A number of different signalling compounds have been characterized, but many aspects of ABA signal transduction, such as the receptor itself, remain unknown. It is likely that ABA is recognized through several receptors that don't have to be "traditional " peptide receptors. The interactions between ABA and other plant hormones have gained a lot of interest. The pathways of gibberellins, ethylene, cytokinin, jasmonate and auxin signaling are known to have points of convergence with the ABA pathway (Finkelstein et al., 2002).

Unlikely many other responses evoked by ABA, stomatal closure is a fast process and does not need changes in gene expression (Schroeder et al., 2001). Still, biological studies have found many common second messengers both, in fast and slow processes. This indicates that cellular signaling is quite similar in all ABA regulated processes (Finkelstein et al., 2002; Correia et al., 1997).

1.3 Model system *Arabidopsis thaliana*

1. 3. 1 *Arabidopsis thaliana*

Arabidopsis thaliana was discovered by Johannes Thal (hence, thaliana) in the Harz mountains in the sixteenth century, though he called it Pilosella siliquosa, (and it has gone through a number of name changes since). The earliest report of a mutant was in 1873 (by A. Braun). F. Laibach first summarized the potential of *Arabidopsis thaliana* as a model organism for genetics in 1943 - he did some work on it much earlier though, publishing its correct chromosome number in 1907 (http://www.arabidopsis.org/info/aboutarabidopsis.jsp).

Arabidopsis thaliana, (thale cress) belongs to *Brassicaceae* family and is widely spread all over the world being found also in Estonia (Krall et al., 1999).

For now it has become the most widely studied model system in plant biology. A large public effort has been made to promote, coordinate, and standardize the use of *Arabidopsis* as a model system (Meinke and Koorneef, 1997; Meinke et al., 1998). It was originally chosen because of its favourable basic characteristics as a model organism. *Arabidopsis* is quick to reproduce moving from one generation to the next in 6 – 10 weeks. Its small size enables to grow large population sizes required for mutant screens and other large-scale experiments. The genome consists of only five chromosomes with little repetitive DNA and is believed to be among the smallest in size (~125 Mbp) within the angiosperms. Perhaps the greatest advantage of working with *Arabidopsis* is the genome project, which has resulted in the availability of the entire *Arabidopsis* genome sequence in the year 2000 (Meinke and Koorneef, 1997; Meinke et al., 1998; http://www.arabidopsis.org/info/aboutarabidopsis.html).

The study of *Arabidopsis* is also favoured by the simplicity of *Arabidopsis* genetics, easy transformation and the rich resources of existing mutants and cloned genes.

1.3.2 *Arabidopsis thaliana* ecotypes and mutants

Over 750 natural accessions of *Arabidopsis thaliana* have been collected from around the world and are available from the two major seed stock centers, Arabidopsis Biological Resource Centre (ABRC, http://www.biosci.ohio-state.edu/~plantbio/Facilities /abrc/abrchome.htm) and The Nottingham Arabidopsis Stock Centre (NASC, http://arabidopsis.info/). These accessions are quite variable in terms of form and development (e.g. leaf shape, hairiness) and physiology (e.g. flowering time, disease resistance). Researchers around the world are using these differences in natural accessions to uncover the complex genetic interactions such as those underlying plant responses to environment and evolution of morphological traits (http://www.arabidopsis.org/info/ aboutarabidopsis.html).

Also a wide range of *Arabidopsis* mutants exists and there are different ways of obtaining the mutation. One way of obtaining the mutation is by inserting foreign DNA into the gene of interest and disrupting its function through that.

The mutation can be obtained also chemically by using ethylmethanesulfonate (EMS) or by X-ray irradiation when single nucleotide is mutated. Cloning loci with single base changes induced by EMS or with deletions from gamma-irradiation can be tedious. Nonetheless, these latter mutagens offer advantages which include: non-biased mutagenic saturation of the entire genome and the generation of novel mutant phenotypes including dominant, gain of function protein forms by amino acid alterations or deletions. Genes altered by chemical or physical mutagens can be localized by map-based ping and thus positional cloning. To map a gene, one must

analyse the linkage between the mutant phenotype and markers whose physical position in the *Arabidopsis* genome are known (http://www.arabidopsis.org).

1.4 *Arabidopsis thaliana*, ozone and stomata

Since O_3 entry through the leaf cuticle is negligible (Kerstiens and Lendzian, 1989), stomata play a fundamental role in determining the flux of O_3 into the apoplastic space. Their sensitivity to external stimuli is an important factor in the overall plant sensitivity/tolerance to ozone. If the stomatal aperture is wider, and consequently the flux of gaseous substances is higher in one plant when compared with another, the dose of O_3 delivered to the intercellular space is also larger.

Stomatal closure in response to O_3 is regarded as a protective mechanism and has been documented several decades ago (Hill and Littlefield, 1969).

Moldau et al. (1990) showed that O_3 induced stomatal closure already within the first 12 min of O_3 exposure and concluded that this was caused by a direct effect of O_3 on guard cells rather than by dysfunction of mesophyll photosynthesis since mesophyll conductance to CO_2 remained unchanged during the 4-h exposure period. However, the regulation of stomata by ABA in response to O_3 is also involved (Ahlfors et al., 2004).

It is evident that the study aiming to answer why one *Arabidopsis* genotype is more or less sensitive to ozone than the other should also include the study about stomatal opening during O_3 treatment. However, the roles of stomata and leaf internal factors in modifying stomatal O_3-sensitivity are not yet sufficiently addressed, particularly in the case of O_3-sensitive *Arabidopsis* mutants and ecotypes.

Ahlfors et al. (2004) have shown that ozone sensitive mutant *rcd1* (Overmyer et al., 2000) has 40% higher stomatal conductance than Col-0 wild type. 2 h of O_3 decreased conductance in Col-0 by 40% but only by 20% in *rcd1-1*. After 4 h, *rcd1* had conductance similar to Col-0. Consequently, *rcd1* ozone sensitivity is caused by a greater ozone influx into the leaf. There is no data about the function of stomata during ozonation of other ozone sensitive mutants like *vtc1* (Conklin et al., 1999), *oji1* (Kanna et al., 2003), *lcd1* (Barth and Conklin, 2003) and ecotypes Cvi and Ws-2 (Rao and Davis, 1999; Joo et al., 2005).

1.4.1 Stomata

Stomata are small pores on the surfaces of leaves and stems, bounded by a pair of guard cells, that control the exchange of gases — most importantly water vapour and CO_2 — between the

interior of the leaf and the atmosphere. They make major contributions to the ability of the plant to control its water relations and to gain carbon, thus critically affecting whole plant growth and physiology. Guard cells respond cell-autonomously to well-known plant physiological signals, including red and blue light, CO_2, plant pathogens, the hormones abscisic acid, auxin, cytokinin and gibberellins, and other environmental signals (Schroeder et al., 2001).

They adapt to local and global changes on all timescales from minutes to millennia. Stomatal morphology, distribution and behaviour respond to a spectrum of signals, from intracellular signalling to global climatic change (Hetherington and Woodward, 2003). Leaf gas exchange is regulated by controlling the aperture of the stomatal pore and the number of stomata that form on the epidermis. Environmental signals control stomatal aperture and development (Schroeder et al., 2001). The acquisition of stomata and an impervious leaf cuticle are considered to be key elements in the evolution of advanced terrestrial plants (Raven, 2002). It is suggested that stomata appeared in terrestrial land plants over 400 million years ago. Correlations between changes in global environmental conditions and stomatal evolution can be demonstrated. Guard cell signalling research holds much promise at addressing major environmental and agricultural problems of the twenty-first century.

Stomata range in size from about 10 to 80 μm in length and occur at densities between 5 and 1,000 mm^{-2} of epidermis. Stomata exert major controls on both the water and carbon cycles of the world.

Currently rather little is known about the signalling pathways by which environmental signals control stomatal development. Generally guard cell signalling is thought to be robust, as other pathways can compensate the loss of certain signalling components (Hetherington and Woodward, 2003).

Stomatal guard cells have become a highly developed model system for characterizing early signal transduction mechanisms in plants and for elucidating how individual signalling mechanisms can interact within a network in a single cell. Their cytoplasm is separated from surrounding cells and single cell manipulations are possible. (Schroeder et al., 2001).

1.4.1.1 The regulation of stomatal opening and closure

Opening and closing of stomatal pores is mediated by turgor and volume changes in guard cells. During stomatal opening guard cells accumulate potassium, anions, and sucrose. Osmotic water

uptake leads to guard cell swelling and stomatal opening. Stomatal closing is mediated by potassium and anion efflux from guard cells, sucrose removal, and metabolism of malate to osmotically inactive starch.

O_3 can affect the stomatal function through changes in photosynthesis, ABA signalling, by generating an 'artificial' ROS burst directly in the guard cells, or by inducing ethylene emission, which will then lead to stomatal closure. O_3 affects the stomata with several mechanisms that are both dependent and independent of ABA. It was discovered recently that ABA signalling utilizes ROS (Pei et al., 2000; Murata et al., 2001; Zhang et al., 2001) Therefore, O_3 – or the ROS formed from the degradation of O_3 in the apoplast – could directly have relevance in the regulation of stomatal function.

It is suggested that ROS production is a general feature of ABA signalling in plants (Kwak et al., 2003). It has also been shown that O_3 can affect the aperture of stomata by directly affecting the K^+ fluxes in the guard cells (Torsethaugen et al., 1999). However, only a few reports have addressed the effect of O_3 on ion fluxes across the plasma membrane in general (Castillo and Heath, 1990; McAinsh et al., 1996; Clayton et al., 1999; Torsethaugen et al., 1999). Thus, the mechanisms by which O_3 affects the stomatal function still requires further study.

Various biotic (pathogens) and abiotic stimuli (water deficit, aerial pollutants, cold shock) induce stomatal closing, which requires ion efflux from guard cells which is triggered by ABA. In brief, ABA induces cytosolic Ca^{2+} increases (McAinsh et al., 1990) (Fig. 2), which in turn, inhibit plasma membrane proton pumps and K^+_{in} channels and activate two types (S- and R-type) of plasma membrane anion channels that mediate anion release from guard cells. This leads to loss in guard cell turgor and stomatal closing.

Most ions diffusing across the plasma membrane of guard cells need first to be released into the cytosol from guard cell vacuoles.

ABA-insensitive *Arabidopsis* mutants *abi1-1* and *abi2-1* show greatly reduced ABA-induced $[Ca^{2+}]_{cyt}$ elevations, thus representing good tools to study stomatal movements.

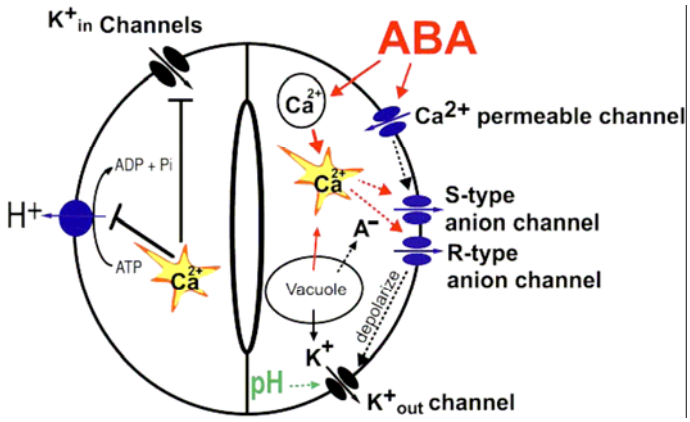


Fig. 2. A guard cell model, illustrating the proposed functions of ion channels in ABA signalling and stomatal closing. The right cell of the stomata shows ion channels and regulators that mediate ABA-induced stomatal closing. The left cell shows the parallel effects of ABA-induced $[Ca^{2+}]$cyt increases that inhibit stomatal opening mechanisms. *Figure is copied from Schroeder et al., 2001*.

2. AIM OF THE STUDY

The aim of present thesis was:

1. to study O_3 sensitivity of different *Arabidopsis thaliana* ecotypes and mutants

2. to facilitate side-by-side comparisons of stomatal responses during ozone exposure in different *Arabidopsis* ecotypes and mutants by the means of an eight-chamber whole-rosette ozone fumigation system.

3. MATERIALS AND METHODS

3.1. Plant material

The model organism *Arabidopsis thaliana* was used in this study. Numerous ecotypes and mutants available make it a convenient species for molecular genetic studies.

3.1.1 Genotypes used in the study

Col-0 (Columbia) is the most used *Arabidopsis* ecotype. Columbia is also the ecotype that was sequenced in the Arabidopsis Genome Initiative. It was initially collected by George Redei from Germany. It is named according to University of Columbia, Missouri, USA. However, for now it has been growing only in a greenhouse conditions for more than a fifty years and thus is considered as "laboratory" ecotype. Col-0 is wild type for *rcd* and *vtc* mutants and for all hormone mutants except ABA mutants that are mostly in L*er* background.

L*er* (Landsberg *erecta*) originates from Landsberg, Germany. The Landsberg *erecta* line was selected from the population of Landsberg seed that had been irradiated. It was called Landsberg *erecta* by Will Feenstra (http://seeds.nottingham.ac.uk). It carries mutation in *erecta* gene, which is a putative receptor of protein kinase, which confers for compact inflorescence, blunt fruits, and short petioles (Torii et al., 1996). Although L*er* is used as ecotype, it is also a mutant. Last year it was also shown that *erecta* gene is involved in pathogen response. L*er* is probably the second most used background line.

Ws-2 (Wassilewskija) originates from Dnepr shores in Russia and is also widely used ecotype. It is wild type for O_3 sensitive mutant *oji1* (Kanna et al., 2003).

Cvi-0 (Cape Verde island) originates from Cape Verde Island and is known to be hypersensitive to O_3 (Rao and Davis, 1999; Rao et al., 2000). According to Rao and Davis Cvi-0 is a hyper accumulator of SA. Secondly, Cvi-0 is insensitive to JA.

Est1 originates from the shores of Piusa River, Estonia. According to Tamaoki et al. (2003) it is O_3 tolerant. Recombinant inbred lines for Est-1 will soon be available (http://www.naturalvariation.org/).

***rcd* (*r*adical induced *c*ell *d*eath) mutants are** isolated by Jaakko Kangasjärvi from the screen for O_3 sensitive mutants (Overmyer et al., 2000). Briefly, approximately 14,000 individual M2 plants, grown from ethyl methanesulfonate–mutagenized Columbia (Col-0) seed, were exposed for 3 days to 250 nL L^{-1} O_3 (8 hr/day). Fifty-six individuals displaying O_3-induced lesions on

rosette leaves were identified. In the subsequent screenings, four lines represented independent loci and consistent O_3-sensitive phenotypes. (Overmyer et al., 2000). Out of these four lines *rcd1, rcd2* (allelic to *lcd-1*)*, rcd3* were used in this study.

rcd1 (radical-induced cell death1). Ozone sensitivity is displayed as spreading lesions that start from leaf margins and progress towards the midvein. The mutant is also more sensitive to superoxide but not to hydrogen peroxide. In fact, if compared with its wildtype, *rcd1* is sensitive to apoplastic ROS, such as O_3 treatment but tolerant to chloroplastic ROS, such as paraquat treatment (Overmyer et al., 2000; Ahlfors et al., 2004).

In addition, *rcd1* has distinctive leaf and developmental phenotypes: The rosette is smaller and more erect than in the wild type and the edges of the leaf blade are wavy. The mutant *rcd1* plant has also a shorter life cycle than the wild type (Ahlfors et al., 2004).

The *rcd1* mutation was mapped to the gene At1g32230 where it disrupts an intron splice site resulting in a truncated protein. RCD1 belongs to the (ADP-ribosyl)transferase domain–containing subfamily of the WWE protein–protein interaction domain protein family. The results suggest that RCD1 could act as an integrative node in hormonal signaling and in the regulation of several stress-responsive genes (Ahlfors et al., 2004).

rcd2 (radical-induced cell death 2). Is allelic to *lcd1*, which was also identified in an another screen for O_3 sensitive mutants by Robert Last and Patricia Conklin from Boyce-Thompson Institute, USA (Conklin et al., 1996). The mutation causing *rcd2* phenotype is in gene At2g37860. LCD1 is suggested to regulate normal leaf development and response to O_3 and virulent pathogens (Barth and Conklin, 2003).

rcd3 (radical-induced cell death 3). The location of the mutation is not yet identified. Map-based cloning of RCD3 is underway in prof Kangasjärvi's group. According to rough mapping the mutation is located in the northern end of the first chromosome.

abi1 and ***abi2***

The mutants *abi1-1* and *abi2-1* (Koornneef et al., 1984) are highly ABA insensitive in seed germination as well as in vegetative responses, including stomatal closure and gene expression, their phenotypes are mostly overlapping. The genes have been cloned and they encode homologous serine/threonine phosphatases of the group 2C (Leung et al., 1997). Genes mutated in *abi1* and in *abi2* are At4g26080 and At5g57050, respectively.

vtc1 (*vit*amin *C* deficient*1*) was also isolated by Robert Last and Patricia Conklin (Conklin et al 1996).

oji1 (*o*zone sensitive and *j*asmonic acid *i*nsensitive) was isolated by Kanna et al. (2003). Its phenotype is O_3 sensitive and jasmonate semi-insensitive. Stomatal response to O_3 is unknown. This mutant is particularly interesting since it is insensitive to JA. Recently it was shown that JA is also involved in the stomatal closure (Suhita et al., 2004).

3.2 Plant growth

3.2.1 Tartu, mode 1

Seeds of different *Arabidopsis* ecotypes and mutants were placed between wetted blotting papers on Petri dishes and kept in darkness at 4^0C for 2 d to synchronize germination. Plastic pots (10×10×6 cm) were filled with a mix of vermiculite and peat (1:1 v/v). A 10×10 cm glass plate (0.25 cm thick) with a drilled conical hole (∅ 3 mm) in the center was placed on top of the pot (Photo 1). The pot corners remained open providing free air movement over the substrate.

About 10 mm^3 of 1% agar gel with KNOP nutrient solution was spread into the hole and two to three seeds were plunged to the gel-substrate boundary. For one week the hole was covered with a small Petri dish to maintain high humidity. Then the dish and less viable seedlings were removed. Plants were exposed to uniform day/night temperature of 23±2^0C and relative humidity of 65±4% in a growth room. Seedlings received 12 h light (PPFD at rosette-height 150 μmol $m^{-2}s^{-1}$) from a battery of fluorescent lamps (Powerstar HOI-T 400W/D; Osram). Plants were sub-irrigated with tap water.

Plants were selected for experiments when they were 3-4 weeks old and not yet bolting.

3.2.2 Tartu, mode 2

Plants were planted as described in 3.2.1 and grown under 12 h photoperiod, 23^0C/19^0C day/night temperature and 70%/90% relative humidity in controlled environment growth chambers (AR-22L, Percival Scientific, Incl., Iowa, USA). The average photon flux density (PFD) during the light period was 200 μmol $m^{-2}s^{-1}$. Three-week-old plants were used in the study.

3.2.3 Helsinki

Plants were grown under 12 h photoperiod, 23 ^{0}C/19 ^{0}C day/night temperature and 70%/90% relative humidity in controlled environment growth chambers (Bio 1300, Weiss Umwelttechnic, GMBH, Germany). The average photon flux density (PFD) during the light period was 200 μmol $m^{-2}s^{-1}$. The sowing and potting medium used was 1:1 mixture of vermiculite and medium peat (Type B2, Kekkilä, Espoo, Finland). Seeds were sown at high density, kept at 4^{0}C for 2 days in order to synchronize the germination and one-week-old seedlings were transplanted to 9 x 9 cm square pots at 5 plants per pot. Plants were sub-irrigated every third day with tap water. Three-week-old plants were used in the study (Overmyer et al., 2000; Tuominen et al., 2000).

3.3 Ozone exposure

3.3.1 The eight-chamber whole-rosette ozone fumigation system

In order to study the stomatal behaviour of different genotypes during the ozonation and to elucidate, whether O_3 sensitivity of some genotypes is caused by their more open stomata during the ozonation, an eight-chamber whole-rosette ozone fumigation system was used (see 3.3.1.1 and 3.3.1.2 below). This apparatus was built in Tartu University at the Department of Plant Physiology especially for monitoring gas exchange of *Arabidopsis thaliana* (Photo 2).

Photo 1. To separate shoot gas exchange from soil and roots, plants were grown through a hole in a glass plate.

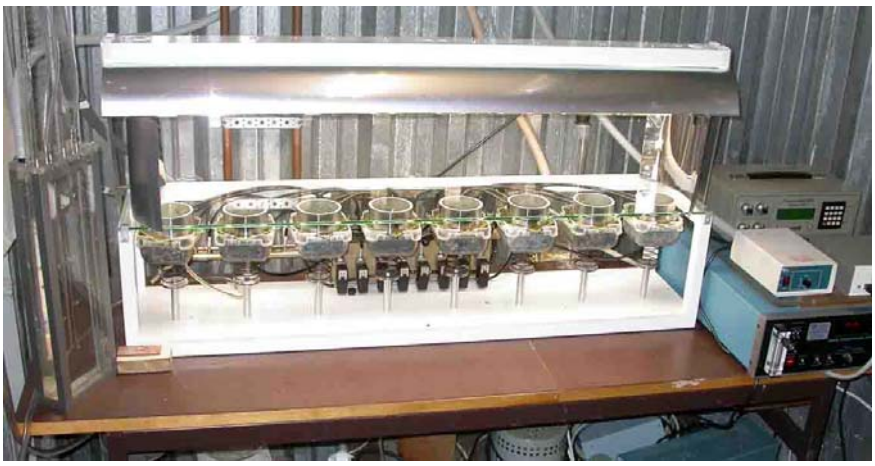


Photo 2. The eight-chamber whole-rosette ozone fumigation system.

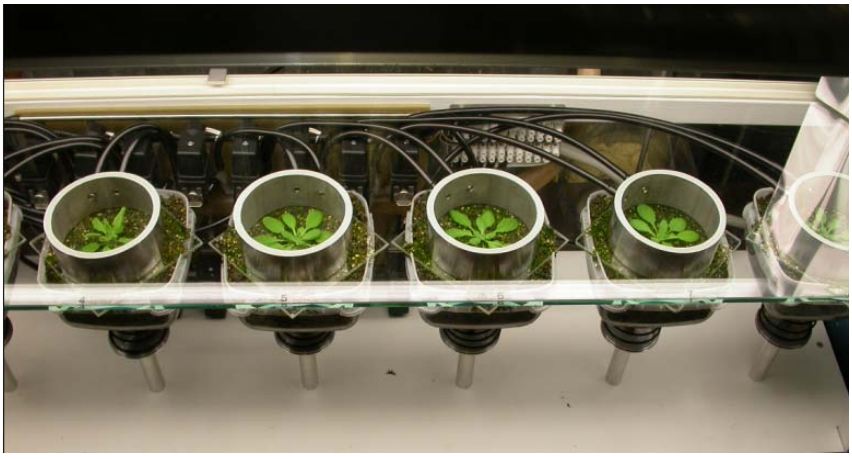


Photo 3. Eight different plants can be inserted into stainless steel cylinders through its lower open end and treated with ozone. Their O_3, CO_2 and H_2O exchange can be followed online simultanously.

3.3.1.1 Ozone exposure vessels

The main exposure module of the measurement system is a set of eight similar stainless steel cylinders (inner diameter 7.8 cm, wall thickness 0.5 cm, height 3.5 cm) sealed to the lower surface of a 0.6 cm thick rectangular (25×120 cm) glass plate with an epoxy seal (ECCOBOND® 286; Emerson & Cuming, Belgium N. V.). The plant was inserted into a cylinder through its lower open end (Photo 3).

Each vessel has a small air entrance port (∅ 0.09 cm) and a larger output port (∅ 0.4 cm). The ports are oriented to minimize air 'shortcut' between the ports. A temperature-matched NTC resistor (type ACC-001, Rhopoint Ltd, Surrey, England) is mounted close to the outlet port to monitor air temperature in a vessel. The whole assembly of vessels is uniformly (±2.5%) illuminated from above by two fluorescent tubes (TDL 36W/840 T8; Philips) at intensities of 110 μmol $m^{-2}s^{-1}$ (Table 1) above the plant.

3.3.1.2 Gas supply-measurement system

Air from outside the laboratory is forced into the gas system by means of a pump through a humidity stabilizer and buffering volume. Air is subsequently divided into eight equal streams. To each stream ozone may be supplied through a capillary from a generator (model 500M; Fisher, Meckenheim, Germany) fed with pure oxygen. Thereafter each stream is divided into two equal sub-streams. One is forced (at an inflow rate of 18.3 cm^3/s) into the entrance port of a vessel; the other is used for reference. Ozone concentration can be selected for each vessel individually by adjusting pressure difference on its ozone supply capillary by means of a tube in an overflow pressure stabilizer. Although the cuvette response time (volume: air inflow rate) is 9.1 s (Table 1), ozone concentrations reach stable values after about 30 s, due to initial rapid absorption on vessel walls.

The outlet air and inlet reference air pass to computer-controlled gas-switching stainless-steel solenoid valves (ASCO/JOUCOMATIC, Scherpenzeel, The Netherlands), which allow to suck samples sequentially from each vessel and its reference through a water vapor/CO_2 analyzer (model 6262; Li-Cor, Lincoln, Neb., USA) and an ozone analyzer (model 1008-RS; Dasibi Environmental Corporation, Glendale, Calif., USA). Individual readings during samplings were taken at 9 s intervals and were currently displayed. The total sampling time was 55 s (at a rate of 16.5 cm^3/s). The mean value of last three individual readings was stored as the recorded data point. Thus each vessel was sampled every 15 min.

Another mode of computer-controlled data acquisition was applied to get time patterns with higher time resolution (one-cell program). In this case the switching between vessels was blocked and the data points from a vessel were recorded at two min intervals. Thereafter it was possible to apply this mode to another vessel, or to use the eight-vessel mode. Essentially in the one-vessel mode the full synchronization is lost.

3.3.1.3 Data processing

Raw data acquired by gas analyzers was converted to flux rates and calculated per unit projective rosette area. The latter was obtained by photographing rosettes with a digital camera (model Coolpix 4500; Nikon). The images were contrasted by package Adobe Photoshop 8 and the area was calculated using the PINDALA10 package.

Stomatal conductance for water vapor (g_s) was calculated according to van Caemmerer and Farquhar (1981), except that cuticular conductance (g_c) was distinguished in the overall leaf gas phase conductance (Moldau and Bichele, 2002). To get the boundary layer conductance (g_a), evaporation of water from saturated filter paper replicas was measured as described by Donahue et al. (1997). The g_s was calculated according to the formula $g_s = (g_c - g_r)/(g_c \times g_r)$, with $g_r = g_o - g_a$, where g_o is the overall leaf gas phase conductance for water vapor (Moldau and Bichele, 2002). The g_s determined so, is an 'effective' value physically averaged over all leaves in the rosette.

Operational data of the system, including rosette heat exchange coefficients are listed in Table1.

Ozone uptake through the boundary layer + stomata (U_s) was calculated employing formula $U_s = 0.62 \times (g_a \times g_s) \times [O_3] / (g_a + g_s)$ where 0.62, the ratio of ozone and water vapor diffusivities in air, converts conductances to O_3 (Moldau and Bichele, 2002) and $[O_3]$ denotes O_3 concentration in cuvette.

Table 1

Parameter	Value
Cuvette volume	167 cm^3
air input rate	18.3 cm^3/s
response time	9.1 s
air relative humidity	60±4%
CO_2 concentration	outside air (360-400 ppm)
O_3 concentration	adjustable (up to 500 ppb)
Rosette boundary layer conductance	1560±300 mmol H_2O/m^2s
heat exchange coefficient	57.4 $W/m^2\ ^0C$
convective	45.7 $W/m^2\ ^0C$
radiative	11.7 $W/m^2\ ^0C$
photosynthetic photon flux density	110 μmol quanta/m^2s
shortwave radiation density	30 W/m^2 (= 120 μmol quanta/m^2s)
Air sampling rate	16.5 cm^3/s
time	55 s
frequency	15 min or 2 min

3.3.1.4 Auxiliary data

All measurements were made in a temperature-controlled room kept at 23 ± 2^0C. Before O_3 exposure plants were allowed to acclimatize and reach steady-state values of transpiration and CO_2 exchange. Air temperature in illuminated vessels was about two degrees higher than the room temperature. Air humidity over rosettes with open stomata ranged from 66 to 85 % and from 63 to 65%, when the stomata were closed.

3.3.2 Ozone exposure conditions in Helsinki

Plants were exposed to ozone in the same Bio 1300 growth chambers and under the same growth conditions that were used for growing the plants (see 3.2.3). For experiments we used 22-day-old plants. Ozone treatments (300 nL L^{-1}) were started 2 h after the beginning of the light period.

3.4 Cell damage assessment by Evan´s Blue

Plants were grown as described in 2.2.3. Plants were exposed to 200 nL L^{-} of O_3 for 10 and 90 minutes. Thereafter one to three leaves were infiltrated with Evan's Blue solution and inspected immediately by microscope at 10X magnification. Result was documented by digital camera (Coolpix 4500, Nikon).

3.5 Tissue damage quantification by ion leakage

Extent of tissue damage was quantitated by measuring ion leakage as described in Overmyer et al. (2000). Plants were collected 1 h after the end of O_3 treatment. Briefly, plants were cut right below the rosette and submerged in fresh Milli-Q water in 50 ml centrifuge tubes. The tubes were shaken on a rotary shaker at 200 rpm for one hour and the conductivity (mS/cm) of the solution was measured (Mattler Toledo, MC 226 Conductivity Meter). For analysing the total ions, the sample tubes were frozen at –20°C, melted, shaken as before and the conductivity was measured again. Results are expressed in percentage of total ions (electrolyte leakage = (first conductivity/second conductivity) x 100%).

3.6 Reactive oxygen species (ROS) measurements

To quantitate ROS production we used fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA,Molecular Probes, Invitrogen). Oxidation of the nonfluorescent H_2DCFDA, to the highly fluorescent 2',7'-dichlorofluorescein (DCF) is commonly used to detect the generation of reactive oxygen intermediates (Joo et al., 2005)

3.6.1 ROS assays

Plants were exposed to 150 nL L^{-} of O_3 for 10 and 44 minutes in the exposure vessels described in 2.3.1. The studied plant was cut down from the root and immediately frozen in liquid nitrogen. ROS assays were carried out as described in Joo et al. (2005) with slight modifications. Frozen plant tissue was hand ground in liquid nitrogen; the powder was weighed and 20 mg of the powder was immediately taken up in 400 ml 10 mM Tris-HCl buffer, pH 7.3. The extract was centrifuged at 15,000 rpm for 6 min at 1^0C. 2 X 100 µl of supernatant of each sample was pipeted to 96 well plates. The pellet was used later for chlorophyll content measurements. Because there are indications that the H_2DCFDA is not completely specific for ROS, we added catalase (300 units/mL) to one half of the sample. ROS production was assayed by adding 100 mM H2DCFDA in DMSO to a final concentration of 10 µM and measuring fluorescence using a

VersaFluor fluorometer (96 well plate reader VICTOR, France). Each sample was measured after every two minutes for 40 times.

We then subtracted the catalase insensitive background from each experimental value. To measure chlorophyll content, the above described pellet was taken up in 2 ml of 80% acetone, mixed thoroughly and centrifuged at 15,000 rpm for 6 min. The chlorophyll content was calculated according to absorbance at 663 and 646 nm measured by spectrophotometer (UV-2100, Shimadzu, Japan).

From the obtained fluorescence value the catalase value was subtracted, multiplied by the dilution coefficient (dilution coefficient = (buffer volume + plant material in grams)/plant material in grams)), divided by the chlorophyll content and expressed as relative fluorescence units (RFU).

3.6.2 Visualization of ROS production in stomata by confocal microscopy

Fluorescence microscopic observations were performed as described in Joo et al. (2005) with slight modifications. Plants were grown as described in 3.2.3. Reactive oxygen species (ROS) production in guard cells was analysed by observing H_2DCF-DA fluorescence with confocal microscopy. Three-week-old plants were exposed to 300 nL L^{-1} O_3 for the 20 min (ozonation as in 3.3.2). Leaves were then transferred to 100 μM H_2DCF-DA in 10mM Tris-HCl, pH 7.2, infiltrated and kept in dark for 5 min. Excess H_2DCF-DA was removed by washing with the same buffer. Samples were observed with Leica SP2 AOBS scanning confocal microscope, with the following settings: excitation, 488 nm; emission, 520-530 nm. 10X objective magnification was used.

3.7 ABA treatment

In order to elucidate ABA sensitivity of the mutant *rcd3*, treatment with ABA was carried out. Plants were grown as described in 3.2.3. Two to three leaves from different plants of each genotype were floated under light for 45 minutes adaxial side upwards in Petri dishes containing 0, 1, 5, 25, 50 μM of ABA. Leaves were then dried from excess water using tissue paper and weighed immediately. Dry leaves were incubated in light in order to keep the stomata open and the loss of initial weight was determined after 30 and 60 min. Results are expressed in percentage of loss of weight from controls of the same genotype (WLC%). First, percentage of loss of weight (WL%) was calculated as follows ((weight after floating – weight after incubation)/weight after floating) x 100. Secondly, WLC% was calculated (WL% x 100)/WL% of control.

4. RESULTS

4.1 Ozone-induced tissue damage quantified by ion leakage

In order to quantify tissue damage caused by 300 nL L^{-1} of O_3 for 4 hours, ion leakage measurements were carried out. The following ecotypes: Col-0, L*er*, Ws-2, Cvi-0, Est-1 and mutants: *abi1*, *abi2*, *rcd1*, *rcd2*, *rcd3*, *oji1*, *vtc1-1* were used (Fig. 3).

Ozone tolerant ecotype Col-0 had the lowest rate of tissue damage (3.2%). Ecotype Est1 and mutant *vtc1-1* had also fairly little damage - 6.3% and 6.1%, respectively. Ecotypes L*er* and Ws-2 can be regarded as ozone sensitive with damage rates of 17.6% and 12%. Cvi-0 is known to be hypersensitive to O_3 (Rao and Davis, 1999; Rao et al., 2000). In this study it had the highest rate of damage among the studied ecotypes (30.6%).

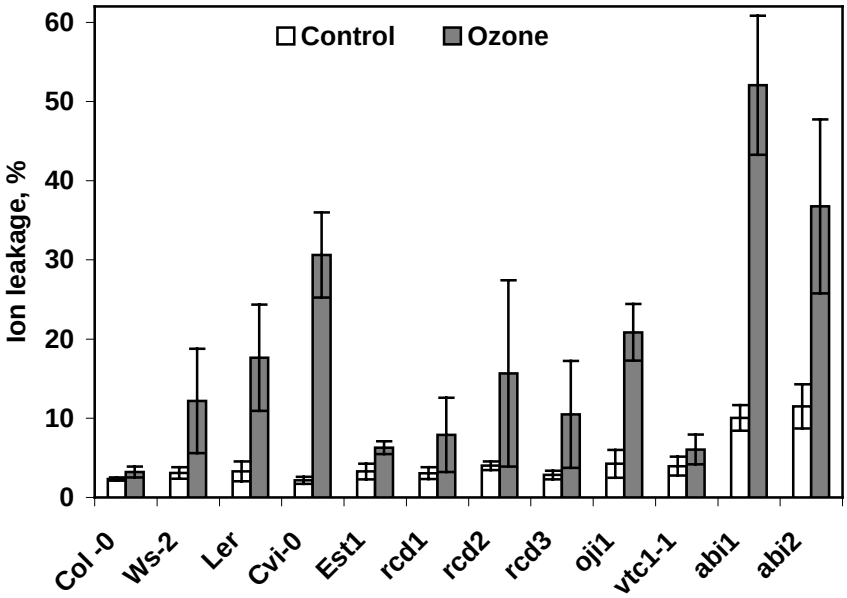


Fig. 3. O_3-induced tissue damage after four-hour treatment with 300 nL L^{-1} of O_3. Error bars indicate standard deviation (n = 5).

Ozone sensitive mutant *rcd1* (Overmyer et al., 2000) had more than twice damage (8%) when compared to the wild type Col-0. Mutants *rcd2* and *rcd3* seem to be even more O_3 sensitive with damage rates of 10.5% and 15.7%, respectively.

Also O_3-sensitive mutant *oji1* had a relatively high rate of damage (21%). ABA insensitive mutants *abi1* and *abi2* with L*er* background are clearly the most sensitive mutants used in this study: damage 52% and 36.7%, respectively.

4.2 Stomatal conductance of different mutants/ecotypes

In order to elucidate whether ozone sensitivity or tolerance of the studied genotypes is related to their stomatal conductance, the eight-chamber whole-rosette ozone fumigation system was used (Fig. 4). Due to limited growth space, not all the genotypes were used in this study. Data is shown for genotypes where at least 4 repeats were carried out.

Wild-type Col-0 is known to be O_3 tolerant (Overmyer et al., 2000) and in this study it had lowest stomatal conductance of 300 mmol m^{-2} s^{-1} as well as lowest O_3-induced tissue damage (Fig 3). The mutant *rcd3* had twice as high stomatal conductance of 620 mmol m^{-2} s^{-1} when compared to its wild type Col-0. Wild type L*er* had even higher stomatal conductance of 670 mmol m^{-2} s^{-1} than *rcd3*. As anticipated, ABA insensitive mutants *abi1* and *abi2* with L*er* backround showed highest conductances of 1550 mmol m^{-2} s^{-1} and 920 mmol m^{-2} s^{-1}, respectively.

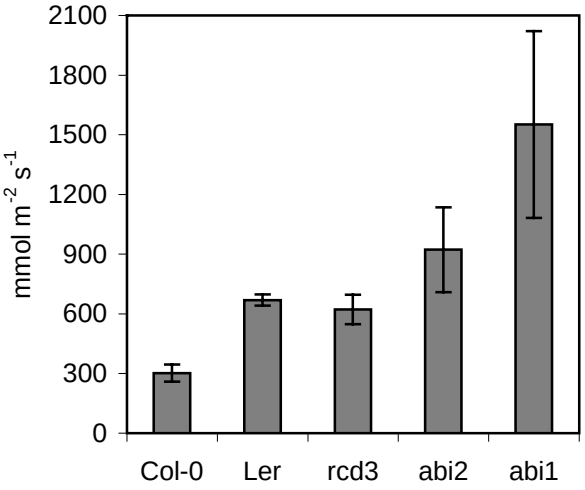


Fig. 4. Stomatal conductance of 22-day-old Col-0, L*er*, *rcd3*, *abi2*, *abi1*. Error bars indicate standard deviation (n = 4).

4.3 Diurnal course of transpiration in *rcd3, rcd1, abi1, abi2* Col-0 and L*er*

Fig. 5 reproduces diurnal patterns of transpiration rates for mutants *rcd3*, *rcd1*, *abi1*, *abi2* and for corresponding wild types Col-0 and L*er*. In all plants transpiration rates approached fairly stable values to the middle of the light period. A decline in transpiration was observed during the second half of the light period in *rcd1,* Col-0 and to a lesser extent in L*er*. This decline was very small in *rcd3* and entirely absent in *abi1* and *abi2*.

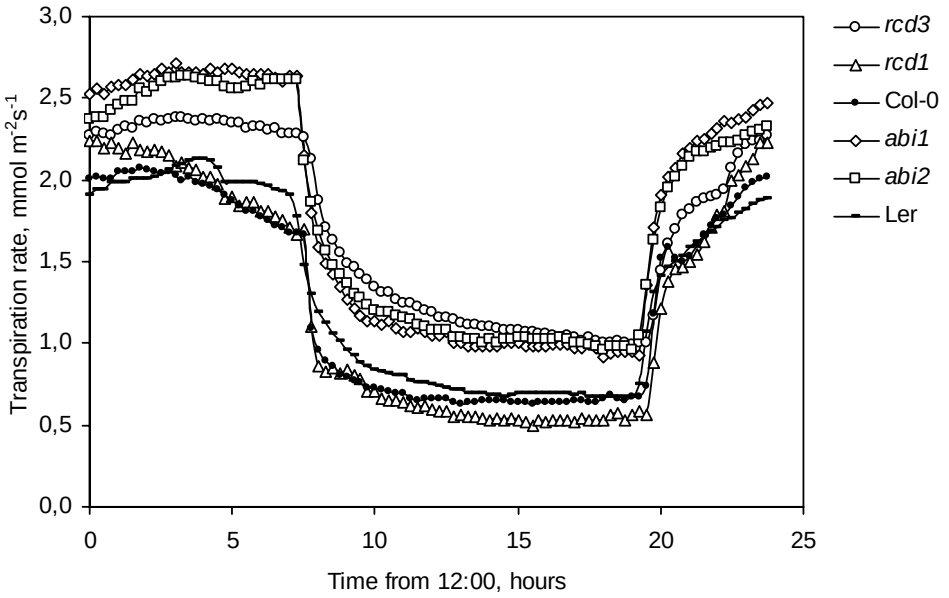


Fig. 5. Diurnal patterns of transpiration rates for radical induced cell death mutants *rcd3*, *rcd1*, for abscisic acid-insensitive mutants *abi1*, *abi2* and for corresponding wild types Col-0 and L*er*.

4.4 Ozone-induced stomatal responses in Col-0, L*er*, Ws-2, *rcd1*, *rcd2*, *rcd3*, *abi1*, *abi2*

The 8-chamber ozone fumigation system was used to study the patterns of stomatal conductances during ozone exposure.

Fig. 6A illustrates time patterns of stomatal conductances (g_s) in an experiment where *abi1*, *abi2* mutants and L*er* were simultaneously exposed to 125-140 nL L^{-1} ozone in the middle of the light period. Plants in other three vessels served as corresponding controls. In L*er* a transient decrease in g_s was observed 15 min after the ozone onset. Interestingly, this decrease was absent in *abi1* and *abi2*. A rapid sustained decrease of g_s followed about 45 min after ozone onset in *abi1* and *abi2*, at that time visual damage started to appear as well (see Fig. 10). In L*er* the signs of sustained decrease appeared only about 2 h of ozone exposure.

In treated plants ozone concentrations around leaves ($[O_3]$) were close to each other in this experiment (Fig. 6B). Therefore the time patters of stomatal ozone uptake rate (U_s) were largely determined by patterns of g_s (Fig. 6C). Cumulative stomatal ozone uptake ($U_{\Sigma s}$) was initially higher in *abi1* and *abi2* than in L*er* (Fig. 6D) owing to their higher g_s and U_s; during the sustained decrease in g_s in mutants, their $U_{\Sigma s}$ approached plateau.

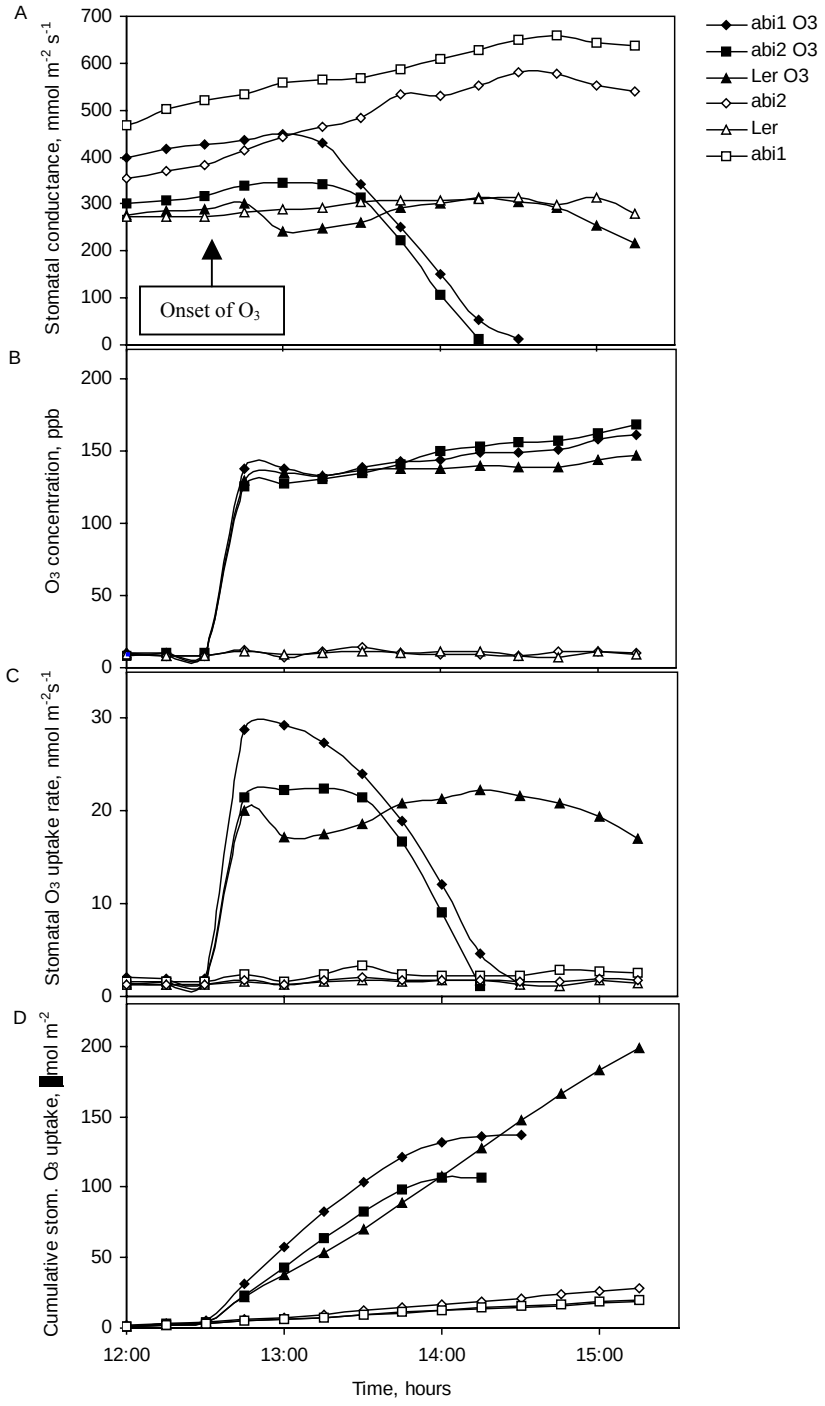


Fig. 6. Stomatal conductance (A), O_3 concentration around the leaves (B) ozone uptake through the stomata (C) and cumulative O_3 uptake in *abi1*, *abi2* and Ler .

Transient stomatal closure 15 min after the ozone onset was observed also in other studied ecotypes: Ws-2 (Fig. 7), Col-0 (Fig. 8A) and Cvi (data not shown). This closure was present also in Col-0 based ozone sensitive (Overmyer et al., 2000; Ahlfors et al 2004) mutants *rcd1* (Fig. 7) and *rcd2* (data not shown), as well as in Ws-2 based ozone sensitive but jasmonate insensitive (Kanna et al., 2003) mutant o*ji1* (Fig. 7).

Interestingly, this transient closure was absent in *rcd3* over a range of g_s and $[O_3]$ (Fig. 8A). On the contrary, this closure was present in Col-0 over a wide range of $[O_3]$ (Fig. 8B).

To follow the transient closure at higher time resolution, the single-cuvette mode of data acquisition was applied. Fig. 9A shows that in Col-0 the initial decrease in g_s was actually very rapid: the minimum was reached within 8-10 min of ozone exposure.

It took about 45 minutes to restore the initial g_s. After about 1.5 h the signs of the final sustained decrease could be seen.

An experiment with *rcd3* (Fig. 9B) confirms the data of Fig. 8A that the ozone-induced transient stomatal closure is lacking in this mutant; it was not observed even under $[O_3]$ = 460 nL L^{-1}. Under this high $[O_3]$ the final decrease in g_s commenced already after 25 min of ozone onset and was extremely rapid (data not shown).

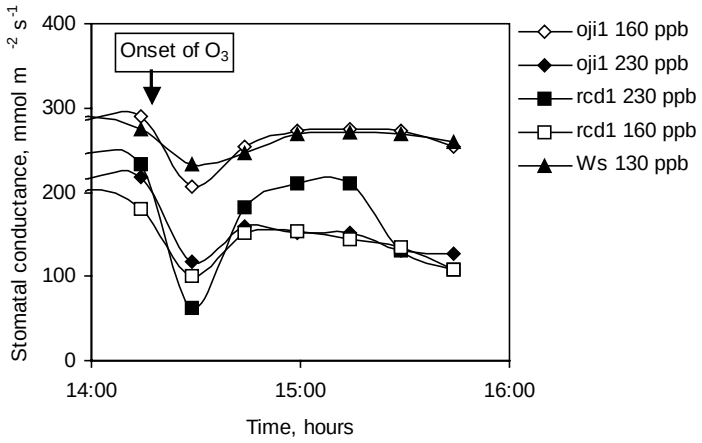


Fig. 7. O_3-induced transient stomatal closure in *oji1*, *rcd1* and Ws-2.

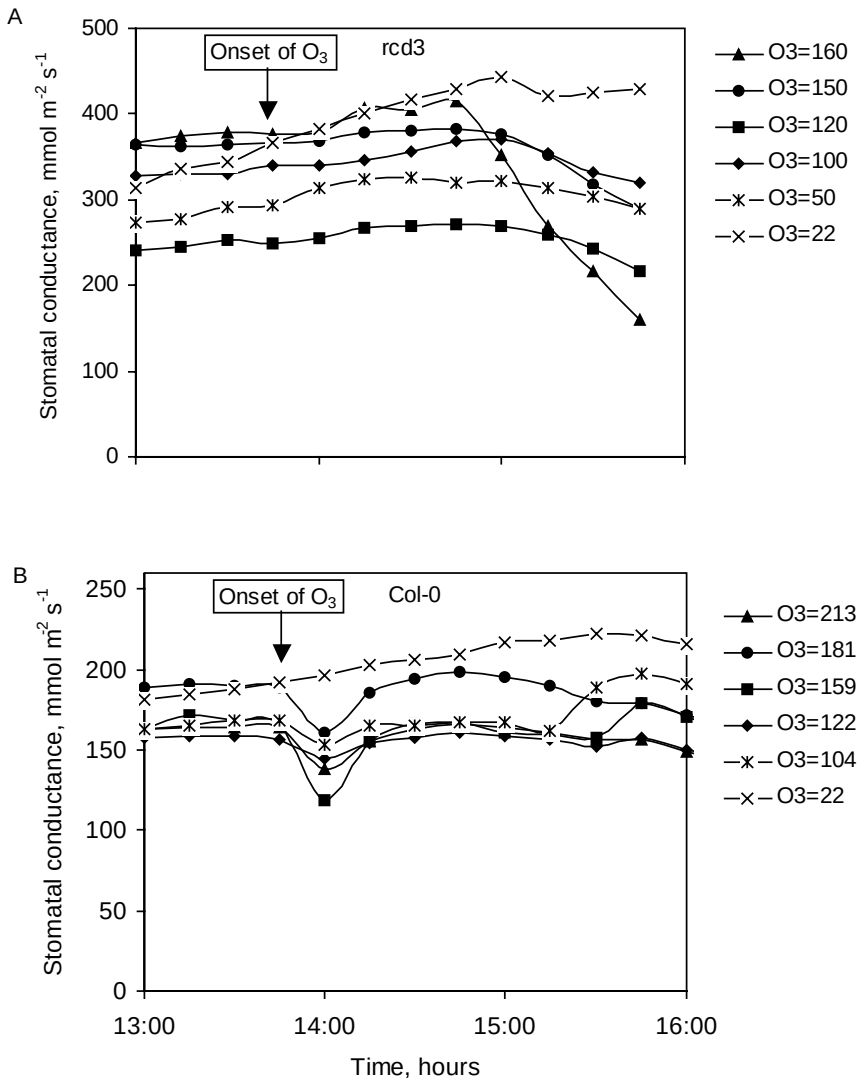


Fig. 8. Stomatal responses of *rcd3* (A) and Col-0 (B) at different O_3 concentrations (O_3 =190-350 ppb). Note that the transient closure was observed in Col-0 over a range of [O_3]. In contrast, it was entirely absent in *rcd3*.

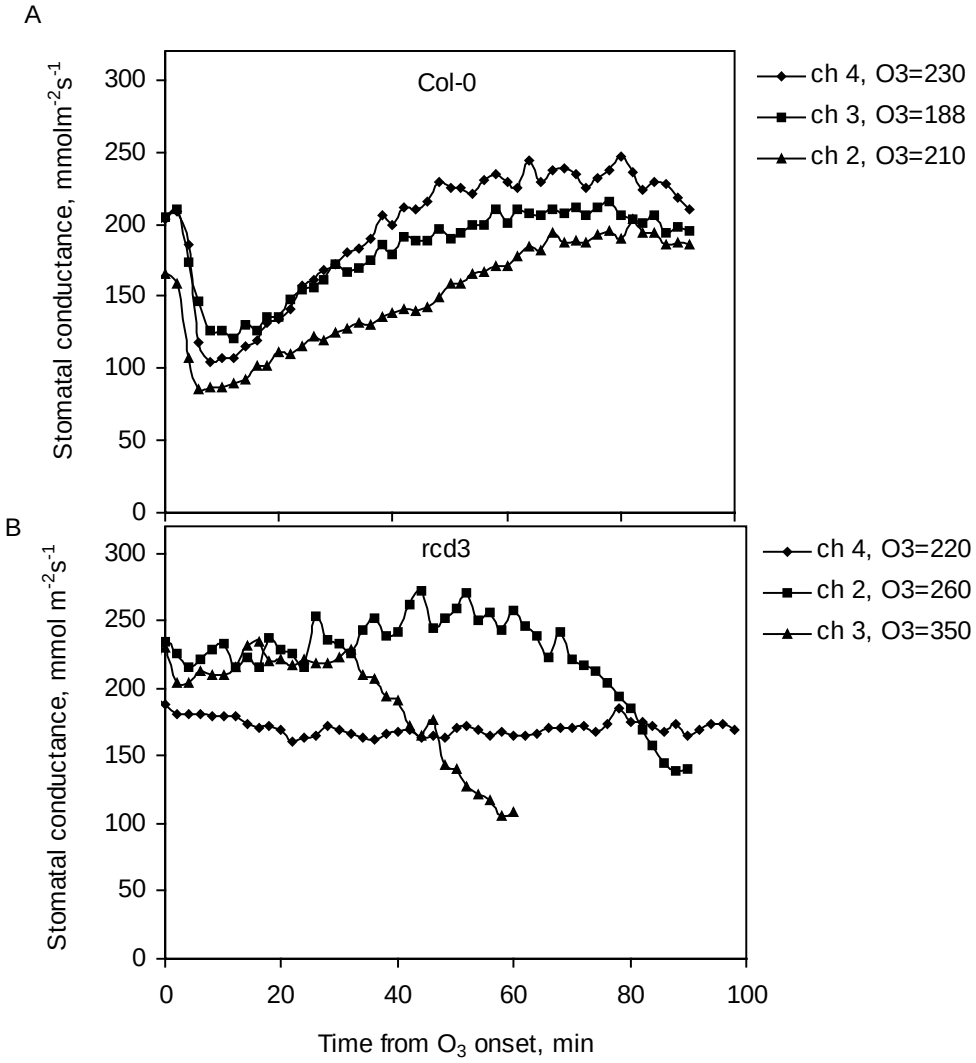


Fig. 9. Stomatal responses of Col-0 (A) and *rcd3* (B) during fumigation with different O_3 concentrations measured at higher time resolution.

4.5. Ozone-induced damage in Col-0, L*er*, *rcd1*, *rcd3*, *abi1*, *abi2*

Ozone exposure for 2h at the concentration of 150 nL L^{-1} did not cause visual damage in Col-0 plants (Fig. 10). However, in *rcd1* and *rcd3* large-scale damage could be seen already 1.5 h after the start of ozone exposure. Also in L*er* wild type plants 1.5 h of ozone exposure caused visual damages. Damage was most severe in *abi1* and *abi2* where large-scale lesions developed already after 40 minutes of O_3 (150 nL L^{-1}) exposure.

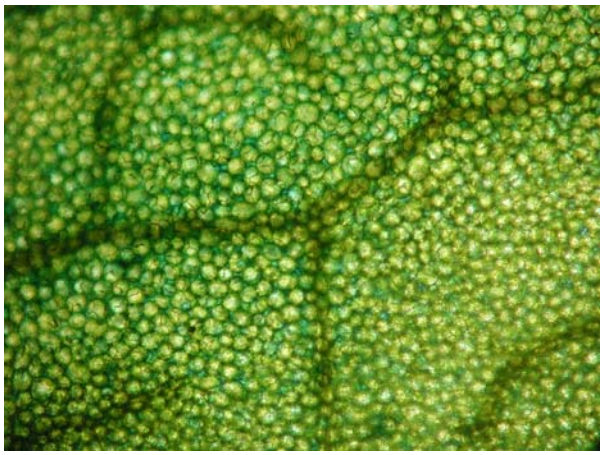
4.6 Ozone-induced cell damage identification by Evan's Blue in Col-0

As shown above, ozone exposure caused transient stomatal closure within 10-15 min of the start of ozonation in most of the studied genotypes. Approximately 1.5 h from the start of ozonation final sustained decrease in stomatal conductance was observed. In order to elucidate whether there is cell damage at either of the timepoints, cell damage was assayed by Evan's Blue staining. The dye enters cells through broken membranes and dark blue damaged cells can be visualized by microscopy. Infiltrated healthy tissue has light blue intracellular spaces. In Col-0 wild type plants damaged cells could not be seen at the first (10 min) timepoint (Fig. 11B). At the time of the sustained stomatal closure (90 min from the start of O_3 exposure) micro-scale damage could be observed (Fig. 11C). At either timepoint no visual damage was detected.

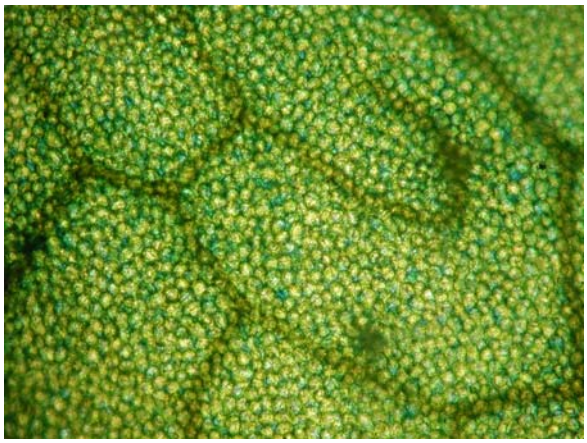
A	**Col-0**	***rcd1***	***rcd3***
			
B	**Col-0 O_3**	***rcd1* O_3**	***rcd3* O_3**
			
C	**L*er***	***abi1***	***abi2***
			
D	**L*er* O_3**	***abi1* O_3**	***abi2* O_3**
			

Fig. 10. Pictures of Col-0, L*er*, *rcd1*, *rcd3*, *abi1*, *abi1* 22 days old plants before (A, C) and after treatment with 150 nl L^{-1} of O_3 for 90 min.(B, D).

A



B



C

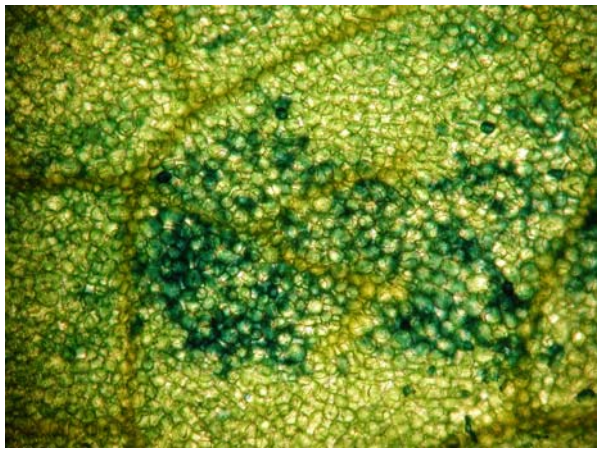


Fig. 11. Damage assessment in Col-0 by Evan's Blue staining at 10 (B) and 90 (C) minutes from the start of ozone exposure and in control (A). Experiment was repeated 4 times with similar results. 10 X objective magnification was used.

4.7 ROS production in Col-0, *rcd3*, L*er* and *abi2* at 10 and 44 min from the start of ozonation

To elucidate, whether there is difference in ROS production between the genotypes having (Col-0, L*er*) and not having (*rcd3, abi2*) the O_3-induced transient stomatal closure, the formation of H_2O_2 as catalase-inhibitable fluorescence generated upon incubation of plant extracts with the fluorescent dye 2,7-dichlorofluorescin diacetate (H_2DCF-DA) was measured (Joo et al., 2005). Plants were exposed to 150 nL L^{-1} of O_3 for 10 (time of the transient closure) and 44 min (time of recovery in conductance). Fig. 12 shows a typical time pattern of fluorescence (FU) formation after adding the dye. It is also shown that catalase inhibits fluorescence formation. Initially the formation of FU is exponential, the curve approaches plateau approximately 80 minutes after adding the dye.

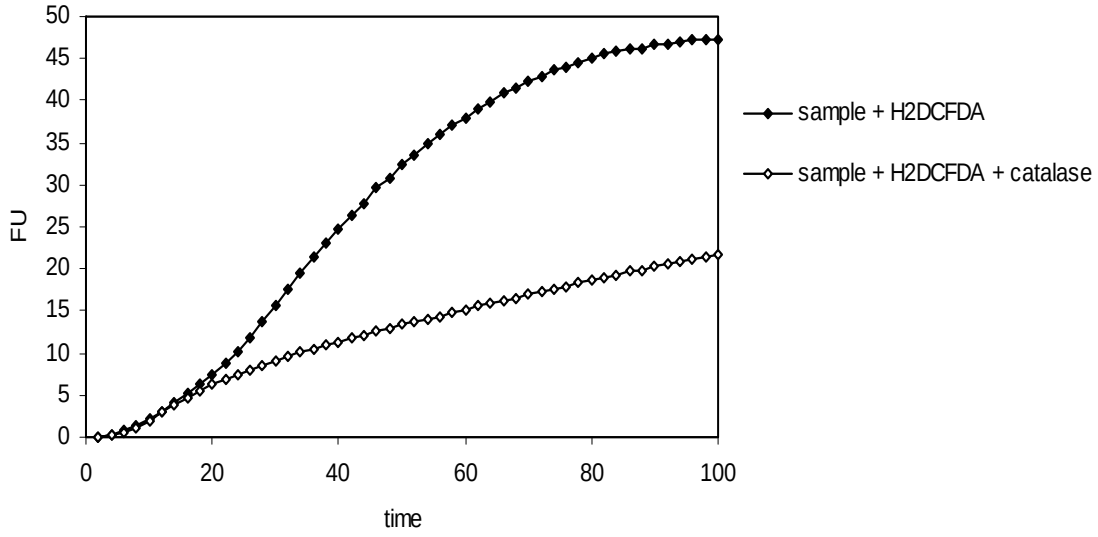


Fig. 12. Typical time patterns of fluorescence (FU) formation after adding H_2DCFDA and H_2DCFDA + catalase to the sample. Time is in minutes.

In all studied genotypes ROS content tended to be higher at 10 min time point than at 44 min time point (Fig. 13). Col-0 and *rcd3* had higher RFU rates than L*er* and *abi2*. Due to limited growth space in this series, the number of controls was two for each genotype; thus the numbers obtained varied twofold in some cases. Whether the ROS content at the 10 min time point may be higher than without ozone, remains to be elucidated with a higher number of repeats.

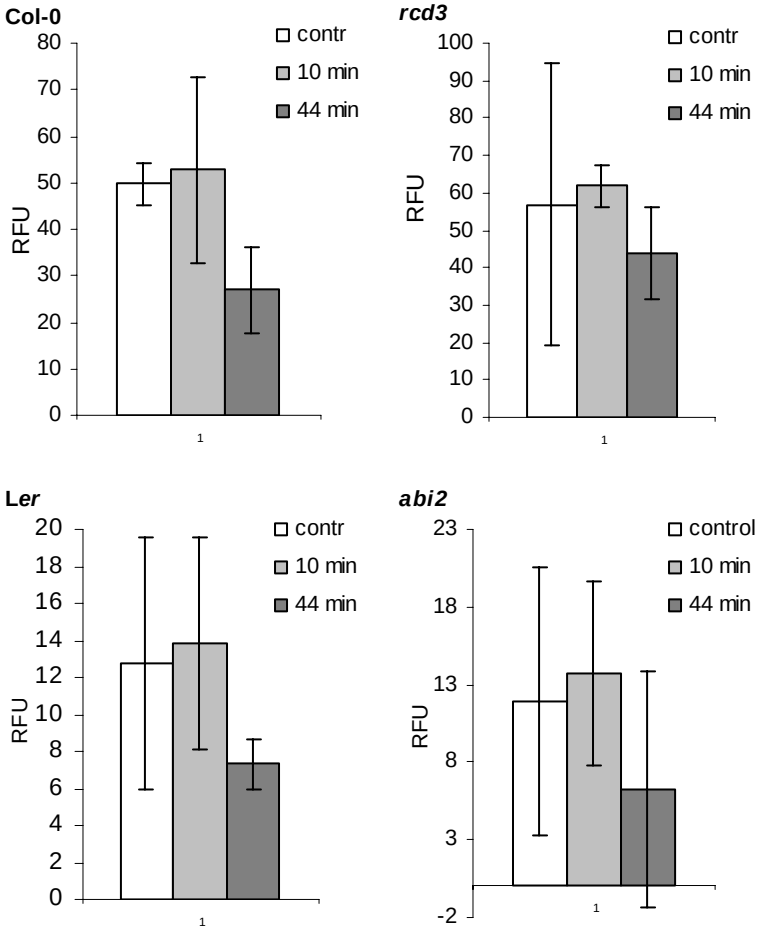


Fig. 13. The production of ROS in Col-0 , *rcd3* , L*er* and *abi2* at 10 and 44 min from the start of ozone exposure at 150 nL L^{-1}. Error bars indicate standard deviation (n = 4 for O_3 treated and n = 2 for control samples).

4.8 Ozone-induced ROS production in the guard cells of Col-0, *rcd3*, *rcd1*, *abi2*, and L*er*, visualized by confocal microscopy

Lee et al. (1999) and Pei et al. (2000) have successfully followed H_2DCF-DA fluorescence formation using confocal microscopy in order to detect ROS production in intact guard cells. Here we decided to use similar approach to analyse whether there is O_3-induced ROS production after 20 minutes of O_3 exposure in guard cells of *rcd1, rcd3* and *abi2* and in respective wild types Col-0 and L*er*.

In the guard cells of Col-0, L*er* and *rcd1* ROS production could be visualized already 20 minutes after the onset of O_3 treatment (Fig. 14. B, C, G). Guard cells of control plants did not show any H_2DCF-DA fluorescence (Fig. 14 A, D). Also in O_3-treated *abi2* no ROS production was detected (Fig. 16 H). It has been reported earlier that one functional step in the signal transduction pathway activated by ABA and leading to stomatal closure is production of ROS and this phenomenon is absent in *abi2* mutant (Murata et al., 2001).

Interestingly, this rapid O_3-indued ROS production was also absent in the guard cells of *rcd3* (Fig. 16 E, F).

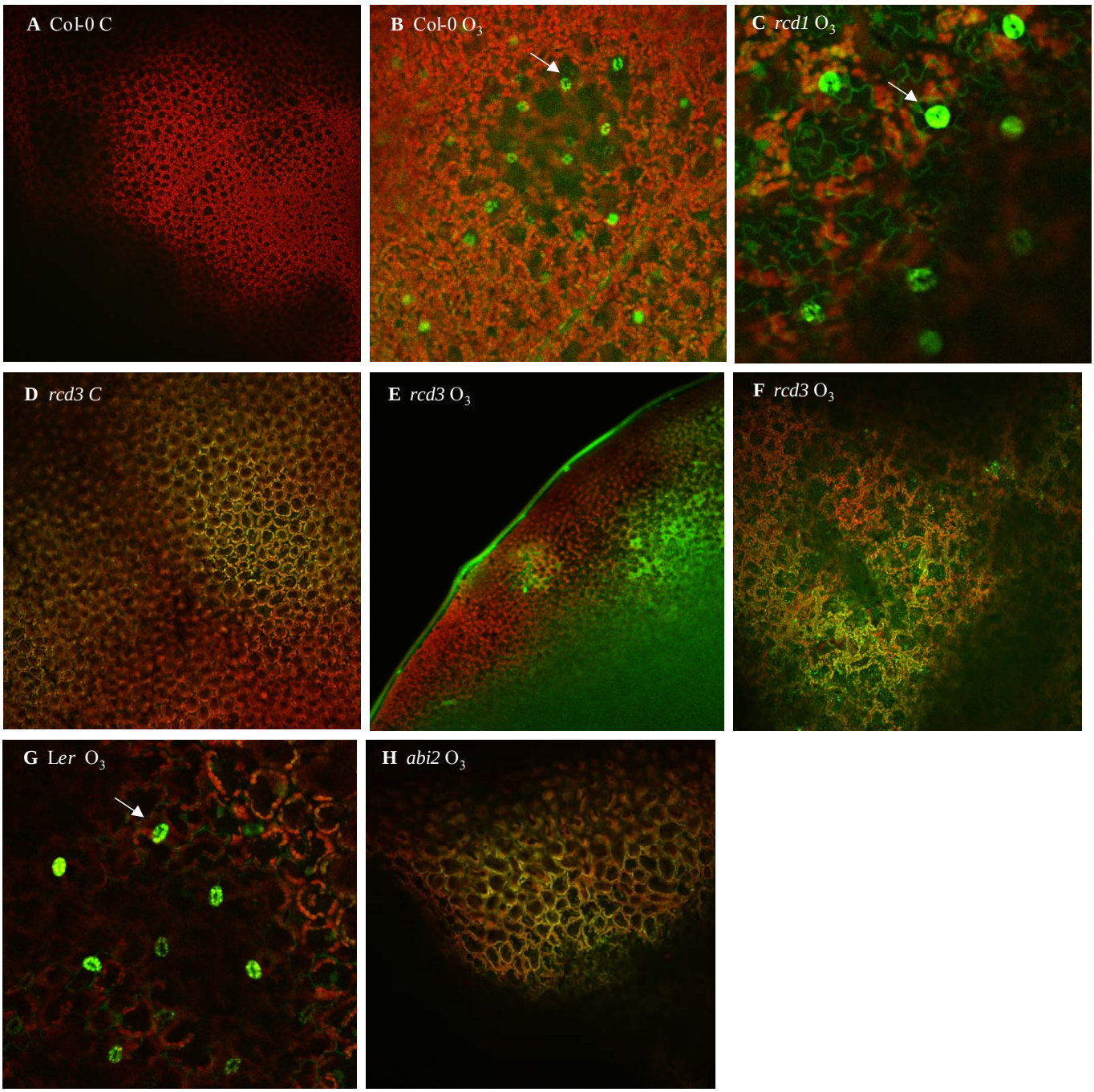


Fig. 14. Confocal Microscopic Imaging of ozone induced ROS production in the guard cells of Col-0, *rcd3*, *rcd1*, L*er* and *abi2*. Three-week-old plants were exposed to 350 nL L^{-1} O_3 for 20 min. Green colour indicates ROS production visualized as H_2DCFDA fluorescence and red colour indicates chlorophyll fluorescence. White arrowheads indicate stomatal guard cells. 10 X objective magnification was used.

4.9 Water loss and ABA induced stomatal closure in Col-0, Ler, *abi2* and *rcd3*

ABA induces cytosolic Ca^{2+} increase, which finally leads to stomatal closure. ABA-insensitive *Arabidopsis* mutant *abi2* has greatly reduced ABA-induced $[Ca^{2+}]_{cyt}$ elevations; thus *abi2* is unable to close their stomates in response to ABA treatment. To elucidate whether *rcd3* has the same phenotype we performed water loss experiments as described by Leung et al. (1997).

Fig. 15 shows water loss (WL) from untreated excised rosette leaves. Data are shown as percentage of initial fresh weight lost after 30 and 60 minutes. WL was highest in *abi2* and lowest in Col-0, which is in accordance with the stomatal conductance data presented above (Fig. 4). L*er* had slightly higher WL than Col-0, although its conductance was almost twice as high as that of Col-0. Although *rcd3* had similar stomatal opening as L*er*, its WL was remarkably higher; being closer to *abi2* which had nearly 50% higher conductance than *rcd3* (see Fig. 4). Collectively, our data are in good agreement with the previous results presented by Leung et al. (1997) who showed that stomatal response to excision is impaired in *abi2*. Our data reveal the same phenotype for *rcd3*.

Fig. 15. Water loss (WL) of excised leaves of Col-0, L*er*, *rcd3* and *abi2*. Three young rosette leaves were excised and their total fresh weight was measured after 30 and 60 minutes of incubation at ambient laboratory conditions. Water loss is expressed as percentage of initial fresh weight. . Error bars indicate standard deviation (n = 3). Differences between Col-0 and *rcd3* and between L*er* and *abi2* were significant ($P < 0.05$).

In order to elucidate ABA sensitivity/insensitivity of the guard cells of *rcd3,* WL of excised leaves floated in different ABA solutions for 45 minutes was measured. Results are presented in Fig. 16 as percentage of water loss compared with corresponding control i.e. leaves floated in distilled water (WLC%, see 3.7). Five different concentrations (0.1μM, 1μM, 5μM, 25μM, 50μM) of ABA were used and measurements were carried out at two different timepoints. Col-0 and L*er* were used as positive controls whereas *abi2* served as a negative control.

Floating leaves in 0.1μM ABA solution did not have an effect on stomatal closure in any of the studied genotypes. In Col-0 no effect was detected also at 1μM concentration and 5 μM ABA solution had the strongest influence on stomatal closure, whereas 25μM ABA concentration had even a slightly smaller effect than 5μM and 50μM can be considered already inhibiting. Interestingly, in L*er* 50μM concentration was not yet inhibiting and induced overall strongest effect on stomatal closure.

In *abi2*, treatment with ABA did not induce remarkable stomatal closure at any of the ABA concentrations applied. On the contrary in *rcd3* the strongest effect on WLC% was detected at 50μM ABA concentration and 1μM, 5μM and 25μM of ABA had smaller but still significant effect on stomatal closure indicating that *rcd3* stomata can sense ABA.

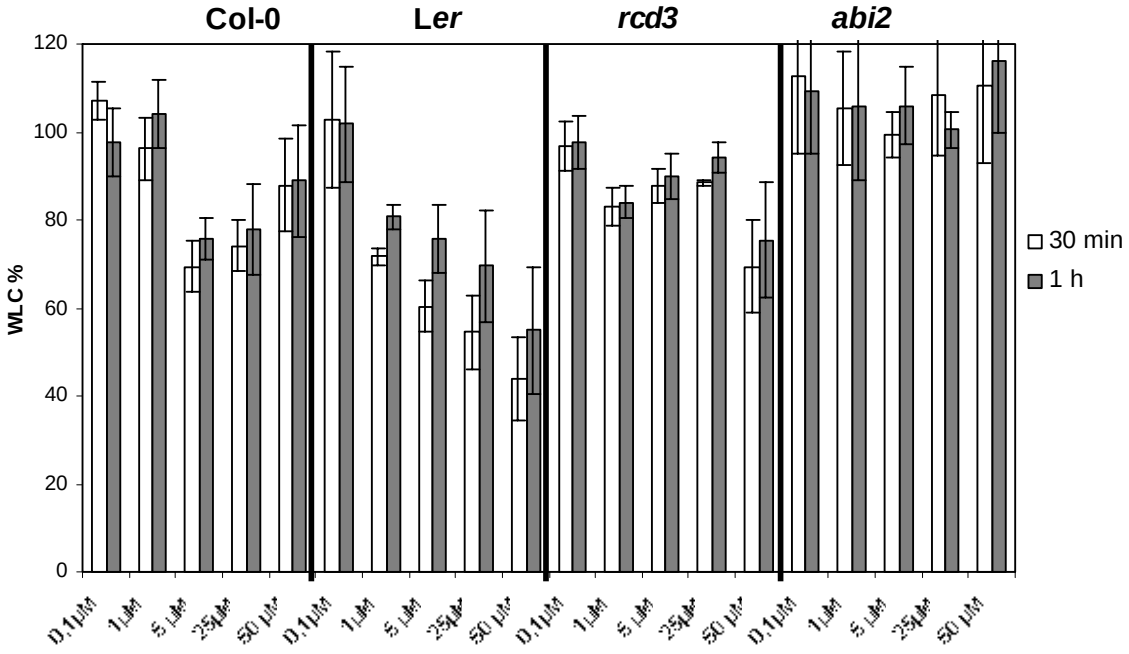


Fig. 16. ABA-induced reduction of water loss (WLC%) in Col-0, L*er*, *rcd3*, and *abi2*. Three young excised rosette leaves were floated for 45 minutes in different concentrations of ABA and their total fresh weight was measured after 30 and 60 minutes of incubation at ambient laboratory conditions. Results are expressed as percentage of water loss compared with corresponding control. Error bars indicate standard deviation (n = 3).

5. DISCUSSION

The main objective of this work was to carry out side-by-side comparisons of stomatal responses and their relation to ozone sensitivity in different *Arabidopsis* ecotypes/mutants.

Firstly, O_3 sensitivity of ecotypes Col-0, L*er*, Ws-2, Cvi-0, Est-1 and mutants *abi1*, *abi2*, *rcd1*, *rcd2*, *rcd3*, *oji1*, *vtc1-1* was studied (Fig. 3). According to ion leakage measurements Col-0 was O_3 tolerant, which is in accordance with previous studies (Overmyer et al., 2000); it is also widely used as an O_3 insensitive control. Among the genotypes, where stomatal conductance measurements were applied (Fig. 4), it had the overall lowest stomatal conductance, thus also lowest O_3 uptake dose.

L*er* had five times more damage than Col-0 as revealed from ion leakage, but its stomatal conductance was only twice as high as that of Col-0. This indicates that there should be also other factors besides stomatal O_3 uptake dose which play role in determining plant O_3 sensitivity or tolerance. L*er* carries mutation in *erecta* gene, which has recently shown to be involved in pathogen response and this might be the reason for increased O_3 sensitivity of L*er* since a number of O_3-sensitive mutants like *rcd1* and *lcd1* (allelic to *rcd2*) have also shown to be sensitive to pathogens (Overmyer et al., 2000; Barth and Conklin, 2003).

Est1 has been reported to be fairly O_3 tolerant (Tamaoki et al., 2003); this is in agreement with our data in Fig.3 where it is the second O_3 tolerant ecotype.

Cvi is known to be hypersensitive to O_3 (Rao and Davis, 1999; Rao et al., 2000) and in the present study it had also the highest rate of damage among the studied ecotypes. It is a hyper accumulator of SA, which is needed for the development of O_3-induced cell death; in addition, it is insensitive to JA, which counteracts lesion propagation. These facts explain well enough its O_3 sensitivity.

All *rcd* mutants are sensitive to ozone since Jaakko Kangasjärvi isolated them from the screen for O_3 sensitive mutants. In present study *rcd2*, which is allelic to *lcd1* (Barth and Conklin 2003), showed highest O_3 sensitivity among *rcd* mutants. According to Barth and Conklin (2003), it has lower-cell-density, owing to what more oxygen radicals can attack a single cell, which results in pronounced damage when compared to the wild type. So a higher dose accounts for its O_3 sensitivity. Studies with similar O_3 doses per cell in *lcd1* and its wild type could elucidate whether *LCD1* gene is responsible also for other processes in relation to defence reactions. According to preliminary results (not presented in this study) it has also higher stomatal conductance when compared to the corresponding wild type.

Ozone exposure caused visible damages on *rcd1* (Fig. 10). Overmyer et al. (2000) have shown that in *rcd1*, the initial amount of ROS formation from O_3 is not the only factor involved in the initiation of lesion propagation. Ethylene evolution, which is needed for the development of O_3-induced cell death, was three- to fivefold higher in *rcd1* than in Col-0; in contrast to Col-0 ethylene evolution in *rcd1* continued after the period of O_3 exposure. Ethylene production is regarded as one of the fastest responses of plants to O_3 (Tuomainen *et al*., 1997; Vahala *et al*., 1998; Overmyer *et al*., 2000); in addition, the amount of ethylene produced and the extent of cell death are well correlated (Langebartels *et al*., 1991; Tuomainen *et al*., 1997; Tamaoki *et al*., 2003). According to Ahlfors et al. (2004), *rcd1* has constitutively 30% more open stomata than Col-0 thus resulting in a higher O_3 dose, which plays an additional role in its O_3 sensitivity. Ahlfors et al. (2004) have established also two downregulated ABA-related genes in *rcd1*, which may be related to its higher stomatal conductance and ozone sensitivity when compared to Col-0. *rcd3* possessed higher damage rates and also higher stomatal conductance than its corresponding wild type. It still remains to be answered, if there are any additional components besides higher conductance, which leads to ozone sensitivity of this mutant.

ABA insensitive mutants (*abi1* and *abi2*) were found to be extremely sensitive to ozone. This was apparent in both, visual observations (Fig. 10) and ion leakage measurements (Fig. 3). The damage appeared rapidly and only a short exposure time was needed to induce it.

Since ABA controls stomatal closure and the *abi* mutants used in this study are known to have impaired stomatal regulation (Koorneef et al., 1982; Koorneef et al., 1984), the simplest explanation for the observed behaviour is that the mutants receive higher O_3 dosage during the exposure. The flux of O_3 probably exceeded the mesophyll antioxidative capacity, leading to large-scale lesion formation. Stomatal conductance measurements (Fig. 4) showed that *abi1* and *abi2* had remarkably higher conductances than the wild type, which clearly supports this conclusion. It has been reported also by Ahlfors et al. (2004) that *abi2* has higher conductance than the corresponding wild type. The possible effect of ABA insensitivity of *abi* mutants leading to ozone sensitivity (other than of stomatal regulation) is not excluded by these results. However, even if hormonally controlled ozone sensitivity existed, it would be masked by the higher stomatal dose. In order to answer this question, similar doses of O_3 should be applied to mutants and wild types.

In the present study O_3 caused high damage rates in *oji1*, which is in accordance with previous experiments carried out by Kanna et al. (2003). This mutant is insensitive to jasmonic acid, which counteracts lesion propagation. It is noteworthy that JA is involved also in the stomatal closure (Suhita et al., 2004). However, the fact that rapid O_3-induced stomatal closure is also

present in *oji1* (Fig. 7) indicates that protein coded by OJI1 gene is not involved in the regulation of ROS dependent stomatal closure as ABI1, ABI2 and RCD3.

In conclusion it could be said that both, higher O_3 dose and alterations in hormonal regulation influence the O_3 sensitivity of the studied genotypes.

Stomatal behavior during ozone exposure of different ecotypes and mutants was studied. A simple system where leaf rosettes of single intact plants are enclosed in eight separate cuvettes (Pictures 1, 2, 3) in a fully non-invasive manner and where subtle changes in whole-rosette stomatal conductances (g_s) may be monitored in response to acute ozone exposure has been developed. To calculate ozone uptake rates/doses through stomata, cuticular conductance was separated in the overall conductance for water vapor.

Under sudden exposure to ozone, a transient decrease in stomatal conductance (g_s) in *A. thaliana* ecotypes Columbia (Col-0), Wassilewskaja (Ws) and Landsberg *erecta* (L*er*), as well as mutants *rcd1* and *oji1*, was observed (Figs 6, 7). In contrast, this phenomenon was absent in *rcd3*, *abi1* and *abi2* (Fig. 6).

There seems to be no earlier description of transient decrease in g_s for *A. thaliana* under ozone. However, some indications of its occurrence can be found for other species. Hill and Littlefield (1969) observed a rapid transient stomatal closure after exposure of oat plants to 600 ppb ozone. Since in their experiment ozone exposure was discontinued when g_s was just at its minimum, it was possible to interpret the subsequent increase as a recovery after exposure. Moldau et al. (1990) observed a trough in g_s after 15 min of exposure of *Phaseolus vulgaris* shoots to ozone concentrations of 160-400 ppb; thereafter the stomata reopened gradually within about one hour in spite of the continuation of ozone treatment. The effect was ascribed to ozone induced transient membrane leakage of ions and osmotic substances across plasma membrane and associated hydraulic effects.

During recent years substantial progress has been made in the detailed characterization of signaling pathways leading to stomatal closure under stress. In brief, in response to a sudden attack by a stressor the production of reactive oxygen species (ROS) in guard cells increases. This leads to changes in Ca fluxes across membranes, to marked rise in cytosolic [Ca^{2+}], to depolarization of plasma membrane, loss of anions and K^+, resulting in an increase in H_2O efflux and stomatal closure. Positive and negative regulator proteins trigger various segments of this general sequence of events. Functioning of these proteins depends on concentration of ROS and ions formed.

There are several pieces of evidence that the observed ozone-induced transient stomatal closure in our experiments may be an expression of this general sequence of events. Ozone exposure is known to elevate ROS in cell apoplast, both as a result of ozone breakdown and as a product of the ozone-induced early oxidative burst. Superoxide radical anion ($O_2^{\bullet -}$) formed is rapidly converted to H_2O_2 by dismutation and/or using appropriate reductants, e.g., ascorbate (Conklin and Barth 2004). H_2O_2 may readily cross plasmalemma, suggestedly using aquaporin isoforms encoded by numerous specialized genes in *A. thaliana* plasma membrane (Jang et al., 2004). All these events may proceed at high rates. For example, production of ROS in Col-0 guard cells has been detected already 5 min after the onset of 350 ppb ozone, it was quite marked by 15 min but declined after about 1 h of exposure (Joo et al., 2005). In epidermal peels these authors observed enhanced production of ROS even within one minute after the addition of H_2O_2. Finally, the most weighty indirect support to the involvement of this general scheme in the observed g_s depression comes apparently from a recent study by Evans et al. (2005) showing that *A. thaliana* aerial green tissues respond to ozone with a biphasic increase in cytosolic calcium ($[Ca^{2+}]_{cyt}$). The first peak in $[Ca^{2+}]_{cyt}$ was induced within a minute after ozone application followed by slower decrease; thereafter a more gradual and sustained rise in $[Ca^{2+}]_{cyt}$ was observed. A biphasic increase in cytosolic $[Ca^{2+}]_{cyt}$ was shown also in *A. thaliana* ecotype RLD1.1 when elicited with exogenous H_2O_2 (Rentel and Knight, 2004). The necessity of increased cytosolic $[Ca^{2+}]$ for stomatal closure is demonstrated in a number of reports (McAinsh et al., 1996; Pei et al., 2000).

The most intriguing result in our data is the observed lack of the transient depression in L*er* mutants *abi1* and *abi2* and in Col-0 mutant *rcd3*.

The Col-0 based mutant *rcd3* carries a still undefined mutation locus. Since *rcd3* also did not show the transient stomatal closure (Fig. 8) and the diurnal course of transpiration was very similar to that in *abi1* and *abi2* (Fig. 5), it is possible that *rcd3* carries mutation in the signaling pathway for stomatal closure as *abi1* and *abi2*. Work is currently going on to locate mutation in *rcd3*.

What may be the general mechanism and physiological significance of the evidently widespread transient depression in stomatal conductance under ozone attack? Obviously, the first line of defense against invading ozone resides in cell apoplast, containing antioxidants (mainly ascorbate) up to millimolar concentrations. Under an intense ozone pulse the apoplastic pool of antioxidants may be exhausted within 2-3 min (Moldau and Bichele, 2002). The shielding capacity of apoplast, including that in guard cells is lost, ozone flux to plasmalemma increases steeply, inducing ROS generation above its resting levels and subsequent activation of Ca^{2+}

channels and the whole cascade of events ultimately resulting in the loss of ions and water from guard cells. Due to the attendant decrease in stomatal conductance, ozone uptake may decrease to some threshold rate where the constitutive levels of antioxidants are able to cope with ozone and prevent further perturbations in plasmalemma functioning. Simultaneously, the rapid depletion of antioxidants may induce their increased supply from the cytosol during depression, particularly if the pH gradient on plasmalemma decreases (Moldau and Bichele, 2002; Meinhard et al., 2002). The stomatal system turns on recovery where the protective resource in apoplast may be even higher than initially. In addition, during depression and recovery the osmotic and hydraulic properties of the stomatal complex may change so that even an overshoot in stomatal conductance may occur after recovery (Moldau et al., 1990; in present study Fig. 9A).

According to Fig. 6C, minimum stomatal ozone uptake rates during depression were 52-58 % of corresponding rates immediately after ozone onset. Thus at minimum g_s the load on antioxidant system was up to two times less than it were in the absence of the transient depression.

Thus the response in g_s markedly decreased the load on the antioxidant system over the entire depression-recovery period. Apparently, the transient stomatal depression contributes to the avoidance of early injury in vulnerable components of plasma membrane. At the same time, ROS induced in the early phase of stomatal closure, may also initiate the hormone-mediated signaling cascade leading to more sustained transcriptional defense responses like hypersensitive response, programmed cell death and systemic acquired resistance (Sandermann, 2000).

The type of the response depends on the kind as well as on the intensity of the initial ROS formation (Pastori and Foyer 2002; Kacperska, 2004).

Analysis of stomatal behavior during O_3 exposure generally showed a sustained decrease in stomatal conductance 90 to 120 minutes from the start of exposure. *abi* mutants showed a very rapid decrease already 45 minutes after the onset of O_3 (Fig. 6A). At the same time, large-scale damages could be observed. It is likely, that this extremely rapid reduction in conductance in *abi1* and *abi2* was associated with reduction of the healthy tissue (Fig. 10).

At the time of the sustained decrease in Col-0 no visible damages were observed. However, microscopic examination of Col-0 tissue revealed individual cells and small clusters of cells that had died as a result of O_3 exposure (Fig. 11C). The microscale injury was not associated with the sustained decrease in stomatal conductance.

As discussed by Kangasjärvi et al. (2005) when O_3 concentrations exceed the antioxidant capacity of the apoplast, the redox balance of the cellular environment will be perturbed. This 'redox shift' activates signal transduction, which results in hypersensitive response. SA-

dependent (Örvar et al., 1997; Rao and Davis, 1999) cell death, continues until some other component, antagonistic to lesion propagation, counteracts the enlargement of the lesion. In Col-0 this "other" component was present (for example JA) to inhibit lesion propagation. It remains to be answered, whether the function of this "other" component is impaired in *abi1, abi2* and *rcd3*, since similar doses were not applied above.

It has been shown also earlier that O_3 induces death of a small number of cells, visible at the microscopic level in O_3 resistant plants (Overmyer et al., 2000).

As discussed above, O_3 caused transient stomatal closure within 10 min of the exposure in most of the studied genotypes (Figs 6, 8, 9). This phenomenon is absent in *rcd3*, *abi1* and *abi2*. Ozone-induced ROS production causes elevation in $[Ca^{2+}]_{cyt}$ which leads to anion channel activation and stomatal closure (Clayton et al., 1999).

Higher tissue ROS content was detected in all studied genotypes at 10 minutes from the start of O_3 exposure when compared to the 44-minute time point (Fig. 13). Since there was no difference in tissue ROS production between ecotypes having (Col-0, L*er*) and mutants not having (*rcd3*, *abi2*) the transient stomatal closure, it is likely that the mutation in *rcd3* and *abi2* causes only changes in the behaviour of guard cells, but does not affect ROS signalling in whole tissue level.

Differences in the ROS level of the stomatal guard cells could not be detected by this method, since they make up only 0.01% of the whole plant tissue. However, leaf tissue of Col-0 and *rcd3* had higher ROS content than that of L*er* and *abi2*. This phenomenon can not be explained by higher O_3 dose due to more open stomata, since Col-0 and *rcd3* had lower stomatal conductance than L*er* and *abi2*. Is this peculiarity related to ozone sensitivity remains to be answered. Also it is a future challenge to elucidate whether ozone exposure causes a transient increase in ROS production in the whole tissue and whether it reaches its normal level already after 44 minutes as a result of defense reactions. The alternative is that there is no O_3-induced transient increase in ROS and it content decreases monotonously after some time due to activation of antioxidative defense response.

It is still mostly unresolved how O_3 affects the stomatal function. The mechanisms suggested span from changes in photosynthesis to abscisic acid (ABA) signalling, generation of an 'artificial' ROS burst directly in the guard cells, and ethylene emission, which all affect stomatal aperture. It has been shown that ABA-dependent closure of stomata is ROS-mediated (Pei et al., 2000; Murata et al., 2001; Zhang et al., 2001). Therefore also O_3 – or the ROS formed from the degradation of O_3 in the apoplast – could have direct effect on stomatal function. Generation of

ROS is required for elevation in $[Ca^{2+}]_{cyt}$ in guard cells, which in turn triggers the closure of the stomata (Clayton et al., 1999).

Production of ROS was detected by confocal microscopy in the guard cells of Col-0, L*er* and *rcd1* at the time of the O_3 induced rapid stomatal closure (Fig. 14). In contrary, guard cells of *abi2* did not show ROS production. Murata et al. (2001) showed earlier that although ABA induces guard cell ROS production in *abi2* it does not induce stomatal closure and thus ABI2 functions downstream of ABA induced ROS production. ABI2 is 2C type protein phosphatase (PP2C). Since PP2C is shown to regulate stomatal signaling networks downstream of H_2O_2 production and immediately upstream of Ca^{2+}-permeable channel activation (Schroeder et al., 2001), it is plausible that the transient stomatal closure in response to ozone attack is absent in *abi2*. However, it seems to be not plausible for *abi1* since this mutant was shown to be able to close its stomata in response to H_2O_2 but not to ABA (Murata et al., 2001). Also, guard cells of *rcd3* did not show O_3-induced ROS production, showing that ROS signaling plays a vital role in inducing rapid stomatal closure. According to present studies *rcd3* is not ABA insensitive (Fig. 16). Intriguing is the fact that according to Murata et al. (2001) H_2O_2 clearly activates Ca fluxes in *abi1* guard cells, thus inducing stomatal closure but in our study, O_3 was not able to induce fast stomatal responses in *abi1* (Fig. 6).

This intriguing phenomenon is present also in *rcd3*. Does this mean that there is some additional factor needed, to induce O_3 dependent ROS production thus resulting in stomatal closure? It would be of great interest to study the influence of H_2O_2 on stomatal guard cells of *rcd3*. In any case, it is likely that map-based cloning of RCD3 will reveal an yet unidentified regulator for ROS induced stomatal closure.

5.1 Conclusions and future challenge

Although the main objective of this study – to provide side-by-side comparisons of stomatal conductances and its relation to ozone sensitivity of different Arabidopsis genotypes, was not entirely fulfilled due to nonuniform growth conditions, it provided very interesting insights for stomatal behavior during ozone exposure and signaling mechanisms involved.

We have obtained the first evidence of the lack of a common ozone-induced transient initial decrease in stomatal conductance in *abi1* and *abi2*, carrying known mutations in genes encoding regulator protein phosphatases of type 2C. The absence of this transient decrease in stomatal conductance also in ozone sensitive mutant *rcd3*, containing yet unidentified mutation locus, points that map-based cloning of RCD3 might reveal a new component for signal transduction pathway leading to stomatal closure.

Future challenge is to employ described eight-chamber ozone fumigation system to unmask new components for the guard cell signaling network.

It would be of great interest to estimate experimentally the effect of transient stomatal closure on plant responses by changing ozone concentration around leaves so that ozone uptake rate remains constant. Using this mode of action, ecotypes and mutants having different initial stomatal conductances and time patterns of behavior under ozone, may be compared at strictly equal ozone uptake rates. This approach actually compensates modulations caused by stomata, allowing direct comparison of sensitivities in the liquid phase of cells. Application of the apparatus in this mode is currently underway.

SUMMARY

Stomata play a fundamental role in determining plant sensitivity to ozone. However, data on O_3-induced stomatal regulation in different *Arabidopsis* O_3-sensitive mutants and ecotypes is extremely scarce. To fill this gap an eight-chamber whole-rosette ozone fumigation system was constructed. This system enables simultaneous monitoring of *Arabidopsis* rosette O_3, CO_2 and H_2O exchange.

Stomatal conductance (g_s) of O_3-sensitive mutants *rcd1, rcd2, rcd3* and ecotypes Col-0 and L*er*, as well as abscisic-acid insensitive mutants *abi1* and *abi2* was analysed. This revealed that the initial values of g_s were higher in *abi1* and *abi2* than in L*er*. Similarly, g_s was higher in *rcd1, rcd2, rcd3* than in Col-0. The rates of damage could be correlated with stomatal opening.

Closer inspection of stomatal opening right after the onset of O_3 exposure revealed that there was a rapid transient depression of g_s in Col-0, L*er*, Cvi and Ws as well as in mutants *rcd1, rcd2* and *oji1*. The maximum depression was reached already within 6-8 min of ozone exposure. Interestingly, this phenomenon was absent in *rcd3*, *abi1* and *abi2*. The presence or absence of this transient depression in g_s appeared to be not dependent on O_3 concentration as a wide range of concentrations (75 – 450 ppb) was studied.

About two hours after the onset of O_3-treatment the stomata started to close ultimately in all cases, whereas the closure rates and injury symptoms were different in different mutants/ecotypes.

The first evidence of the lack of a common ozone-induced transient initial decrease in stomatal conductance in *abi1* and *abi2*, carrying known mutations in genes encoding regulator protein phosphatases of type 2C, has been obtained.

The absence of this transient decrease in ozone sensitive mutant *rcd3* containing yet unidentified mutation, points that map-based cloning of RCD3 might reveal a new component for the signal transduction pathway leading to stomatal closure.

To address mutual relationships between ozone- and ABA-induced stomatal responses and induction of the oxidative burst ROS production in leaf tissue during the transient depression in g_s and also after the reopening in *rcd3* and *abi2* and corresponding wild types was analysed. No difference between the mutant and corresponding wild type in ROS production due to ozone exposure in whole plant tissue was detected.

ROS production was visualized in the stomatal guard cells of Col-0, L*er* and r*cd1*, during rapid O_3-induced transient stomatal depression by confocal microscopy. At the same time, guard cells of r*cd3* and *abi2* did not show ROS production.

The most intriguing result of this study is the fact that transient O_3-induced stomatal depression is absent in L*er* mutants *abi1* and *abi2* and in Col-0 mutant *rcd3*.

KOKKUVÕTE

Õhulõhed mängivad fundamentaalset rolli taimede osoonitundlikkuse määramisel. Vaatamata sellele on andmed *Arabidopsis thaliana* osoonitundlike mutantide ja ökotüüpide õhulõhede motoorika kohta väga puudulikud. Selle lünga täitmiseks konstrueeriti vastav 8-kambriline aparatuur, mis võimaldab võrrelda ökotüüpide/mutantide intaktsete leherosettide gaasivahetust osoneerimise käigus.

Käesolevas töös analüüsiti järgmiste osoonitundlike mutantide: *rcd1, rcd2, rcd3* ja ökotüüpide: Col-0 ja L*er*, samuti abtsiishappele insensitiivsete mutantide *abi1* ja *abi2* õhulõhede juhtivust (g_s). *abi1* ja *abi2* õhulõhed olid enam avatud võrreldes L*er*-ga. Samuti olid *rcd1, rcd2* ja *rcd3* õhulõhed enam avatud kui Col-0. Kahjustuse määrad korreleeruvad õhulõhede avatusega.

Lähem õhulõhede motoorika uurimine osoneerimise käigus näitas, et kohe peale taimede eksponeerimist osoonile toimub kiire langus g_s väärtustes kõikidel uuritud ökotüüpidel (Col-0, L*er*, Cvi ja Ws) ja järgmistel mutantidel: *rcd1, rcd2* ja *oji1*. Langus jõudis maksimumini juba 6 – 8 minutit peale ekspositsiooni algust. Selle kiire sulgumise olemasolu ei sõltunud osooni kontsentratsioonist selle laias vahemikus (75 – 450 ppb). Järgneva tunni jooksul g_s kasvas endisele tasemele sõltumata jätkuvast osoneerimisest. Huvitav on asjaolu, et kiire langus ja vastav taastumine õhulõhede juhtivustes puudus mutantidel *rcd3*, *abi1* ja *abi2*.

Umbes kaks tundi peale osooni ekspositsiooni algust sulgusid kõikide uuritud genotüüpide/mutantide õhulõhed, kusjuures kahjustuse määrad olid erinevad.

Seega, käesolev uurimus näitas esmakordselt, muidu teadaoleva, osooni poolt indutseeritava kiire õhulõhede sulgumise puudumist *abi1* ja *abi2* mutantides, mis kannavad mutatsiooni fosfataas 2C tüüpi regulaatorvalke kodeerivates geenides.

Kiire õhulõhede sulgumise puudumine ka osoonitundlikul mutandil *rcd3*, mis kannab seni lokaliseerimata mutatsiooni võib päevavalgele tuua uue komponendi õhulõhede sulgumist reguleerivas signaalirajas.

Uurimaks vastastikust seost osooni ja ABA poolt indutseeritud õhulõhede reaktsioonides ning oksüdatiivse purske indutseerimises, analüüsiti ROS-de esinemist taimekoes õhulõhede maksimaalse suletuse hetkel ning pärast taasavanemist *rcd3* ja *abi2* mutantidel ning vastavatel metsiktüüpidel. ROS-ide esinemise erinevust mutantide ja vastavate metsiktüüpi taimede vahel ei täheldatud.

ROS-de produktsioon visualiseeriti Col-0, L*er* ja r*cd1* sulgrakkudes kiire osooni poolt indutseeritud õhulõhede sulgumise ajahetkel konfokaalmikroskoobiga. Samal ajahetkel ei täheldatud ROS-de produktsiooni r*cd3* ja *abi2* sulgrakkudes.

Käesoleva uurimuse kõige uudsemaks ja huvitavamaks tulemuseks võib pidada fakti, et kiire osooni poolt indutseeritud õhulõhede sulgumine puudub Ler-i mutantidel *abi1* ja *abi2* ja Col-0 mutandil *rcd3*.

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PRESENTATION 1

What can we learn by monitoring rapid O_3-induced guard cell responses in Arabidopsis?

(Abstract of a poster presentation at the 4th NorFa Workshop "The use of Arabidopsis thaliana as a model for economically important plants." 12-14.11.2004, Norway)

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Although the importance of stomata in regulating ozone entry into the leaf interior is widely recognized, comparative data on stomatal responses to ozone fumigation in different Arabidopsis genotypes/mutants are scarce. This is due to technical complications in measuring gas exchange in this species, having small vulnerable leaves and a very short stem. We have constructed an eight-chamber whole-rosette ozone fumigation system which enables to monitore rosette O_3, CO_2 and H_2O exchange in different Arabidopsis mutants/ecotypes simultaneously. From emergence the plant grows through a small hole in a glass plate partly covering the substrate vessel; during fumigations and measurements this plate forms the air-tight bottom plate of the rosette chamber. A computer program allows to calculate gas exchange parameters online including average stomatal conductance of the rosette. Thus ozone concentration in each chamber may be adjusted according to the current stomatal conductance so that stomatal ozone uptake rates in all chambers are similar. In general, stomatal conductances in mutants were markedly higher than in corresponding wildtypes. Comparative analysis of stomatal behaviour in O_3-sensitive mutants *rcd1*, *rcd2*, *rcd3*, ecotypes Col-0, L*er* and WS-2 as well as abscisic- and salicylic acid insensitive mutants *abi1*, *abi2* and *npr1*, *sid2*, respectively, exposed to 100 - 150 ppb for 4 h, showed negligible transient decrease in stomatal conductance in *abi1*, *abi2* as well as in *rcd3*. At the same time, in Col-0 and L*er* wildtypes the onset of O_3-treatment induced rapid stomatal closure within the half hour of O_3 exposure followed by subsequent reopening. About two hours after the onset of O_3-treatment the stomata started to close ultimately in all cases, the closure rates and injury symptoms (visual and estimated using thiobarbituric acid) being different in different mutants/ecotypes. The concentration of ABA (measured in *rcd1*, *rcd2*, *rcd3* and Col-0) was not induced at least within 3 h of O_3 exposure, after 8 h a pronounced rise in ABA was detected. Mutual relationships between ozone- and ABA-induced stomatal responses and induction of the oxidative burst are discussed.

PRESENTATION 2

What can we learn by monitoring rapid O_3-induced guard cell responses in Arabidopsis?

(Abstract of an oral presentation at the 16th International Conference on Arabidopsis research. 15-19.06.2005 Madison, USA)

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The importance of stomata in regulating ozone entry into the leaf interior is widely recognized. Also a number of Arabidopsis O_3 sensitive mutants and ecotypes have been isolated. However, comparative data for their stomatal responses to ozone and the role of stomata in modifying their ozone sensitivity are scarce. This is probably due to technical complications with measuring gas exchange in this species. We have constructed an eight-chamber whole-rosette ozone fumigation system which enables to monitore rosette O_3, CO_2 and H_2O exchange in different Arabidopsis mutants/ecotypes simultaneously.

We analysed stomatal conductance (g_s) of O_3-sensitive mutants *rcd1, rcd2, rcd3* and ecotypes Col-0, L*er* and Ws as well as abscisic-acid insensitive mutants *abi1, abi2*. This revealed that the initial values of g_s were higher in *abi1* and *abi2* than in L*er*. Similarily, g_s was higher in *rcd1, rcd2, rcd3* than in Col-0.

Closer inspection of stomatal opening right after the onset of O_3 exposure revealed that there was a rapid transient depression of g_s in Col-0, L*er* and Ws as well as in mutants *rcd1, rcd2*. The maximum of the initial depression was reached already within 6-8 min of ozone exposure. The depression was associated with induction of the reactive oxygen species (ROS) in guard cells Interestingly, the depression was absent in *rcd3*, *abi1* and *abi2* The presence or absence of this transient depression in g_s appeared to be independent of O_3 concentration over a wide range of ozone concentrations (75 – 450 ppb).

About two hours after the onset of O_3-treatment the stomata started to close ultimately in all cases, the closure rates and injury symptoms being different in different mutants/ecotypes.

The concentration of ABA (measured in *rcd1*, *rcd2*, *rcd3* and Col-0) was not induced within the whole 3 h of O_3 exposure indicating that initial stomatal closure was not induced by ABA. Only after 8 h a rise in ABA was detected in wild type as well in all *rcd* mutants.

To address mutual relationships between ozone- and ABA-induced stomatal responses and induction of the oxidative burst we analysed ROS production in whole leaf tissue at the time of the transient depression in g_s and also after the reopening in *rcd3* and *abi2* and corresponding wild types. There were no differences in ROS production between ecotypes/mutants investigated, indicating that the presence/absence of the transient stomatal depression is a specificity of ROS induction in guard cells.