The role of mitochondrial ROS generation and elimination systems in bacterial elicitor induced plant stress response

1. The determination of ascorbate and glutathione from biological samples

Two small molecular weight antioxidants could be recognized in the heart of our project: the ascorbate and the glutathione. The application of appropriate analytical methods can always be crucial. Thus we compared the most common analytical methods for the determination of ascorbate and glutathione to choose the best and to modify them to suit the requirements of our special experimental conditions.

1.1.The determination of ascorbate

The determination of ascorbate is a continuous and returning point of our project. Thus in our study two spectrophotometric and a fluorometric ascorbic acid determination methods have been compared with each other and with the so called etalon HPLC method to find the best one. The OPDA-fluorometric method can be suggested for the determination of samples containing the ascorbate at low concentration because of its low LoD and LoQ values. However, this method can be characterized by the narrowest linear range. Furthermore, the ascorbate content of different fruit and vegetable samples has been a slightly underestimated by this method probably due to the derivatization of ascorbate. Unfortunately, the analytical properties of the OPDA method with spectrophotometric detection have been far lag behind the others. Hence we cannot recommend the application of this method. The 2,2'-bipyridyl method could give a balanced performance all over the tests. Furthermore, the results gained by the 2,2'-bipyridyl method are the closest to the results of the reference HPLC method in the case of fruit and vegetable samples. The specificity issue of the method can be diminished by the application of orthophosphoric acid. Similarly, to the OPDA-fluorometric method the throughput of the 2,2'-bipyridyl method could be significantly increased by detecting the absorbance or the fluorescence with a plate reader.

1.2.The determination of glutathione

The metabolism of ascorbate is always coupled to the metabolism of glutathione (GSH), since the investigation of ascorbate metabolism includes the parallel investigation of GSH metabolism. GSH through its important functions in the antioxidant protection of cells and in the conjugation of drugs and xenobiotics has crucial importance. Since glutathione is most often measured in liver tissue and different cell organelles it is important to choose the method that best suits for the determination of GSH from cell organelles. We made an attempt to compare three commonly applied methods to choose the one that best suits for our investigations. The GSH content of cell organelles isolated from control and BSO-treated liver tissues was determined by the GSH-NEM-HPLC-UV, by the monochlorobimane-GSH-HPLC-

fluorescence method and by the DTNB-GSH recycling assay to find the best for the determination of GSH from cell organelles. Since GSH is most often measured in liver cells, tissues and different cell organelles of liver cells such as mitochondria and endoplasmic reticulum the concentration of GSH was determined from liver homogenate, mitochondrial, microsomal and cytoplasmic fractions isolated from liver of control and BSO treated male Wistar rats. Although NEM is an excellent thiol protective agent and the formed GSH-NEM conjugate can directly be analysed by HPLC the relatively high limit of detection made the method unsuitable for the determination of GSH from cell organelles. The low GSH level of organelles could easily be measured by the monochlorobimane-HPLC-fluorescent method. The addition of monochlorobimane into the homogenisation buffer could prevent the oxidation of GSH during the isolation. The formation of monochlorobimane-GSH adduct in the biological samples was accelerated by their own GST activity, however the omission of GST from the standard GSH solutions could cause the overestimation of GSH concentrations of biological samples. Although the GSH recycling DTNB assay is quite simple and rapid the stabilization of GSH and the efficiency of detection lag behind the monochlorobimane-HPLC-fluorescent method. According to our results the monochlorobimane-HPLC-fluorescent method can be advised for the determination of GSH from cell organelles and tissue.

The effects of different preanalytical sample handling on the glutathione content of plant cells (*Arabidopsis thaliana*) was also investigated. The abundant tripeptide, glutathione plays a vital role in the life of plant cells too, furthermore its level and redox state acts as a biomarker of several biotic and abiotic stresses. Thus, the proper determination of GSH and its redox state is more than important in various stress responses and cell death mechanisms. *Arabidopsis thaliana* suspension cells were treated with two glutathione depleting agents: BSO, and the widespread soil pollutant, cadmium. The effect on the glutathione pool of whole cell homogenates, mitochondria, cytoplasm and ER due to the treatments was followed by two different methods: the easy-handling, fast and relatively cheap spectrophotometric DTNB assay and the more complex and expensive monochlorobimane derivatization-HPLC method. The measurable GSH concentrations by the monochlorobimane method were 30-40% higher than by the DTNB assay in the cell homogenate and in all the investigated organelles. At the same time the changes in glutathione concentrations due to BSO and Cd treatments showed similar pattern independent of the applied GSH determination method. Since monochlorobimane is a good thiol masking agent, we aimed to investigate whether it can protect (similar to the animal tissues) the GSH content of plant samples from auto-oxidation and decomposition during the long process of organelle isolation. Thus, 1 mM monochlorobimane was added to the samples at the cell homogenization. By this “glutathione protecting step”, significantly less oxidized glutathione content could be measured from all organelles, especially form cytoplasm and ER. The formation of monochlorobimane-GSH adduct in the plant samples could be accelerated by the externally added GST. The addition of GST doubled the measurable GSH content of the samples compared to those in the absence of externally added GST, indicating that the own GST activity of the samples are not enough to the wholehearted GSH-bimane conjugation. According to our results the monochlorobimane-HPLC-fluorescent method with externally added GST can be advised for the determination of GSH from plant cell organelles and tissue.

2. Quantitative data on the contribution of GSH and Complex II dependent ascorbate recycling in plant mitochondria

Before the investigation of the effect of elicitor treatment on mitochondrial ascorbate metabolism (especially on mitochondrial ascorbate recycling) we aimed at the collection of quantitative data on dehydroascorbate (DHA) reduction (ascorbate recycling) in control, elicitor non-treated plant cells. Up to date the Foyer-Halliwell-Asada cycle and the Complex II dependent DHA reduction have been characterized in plant mitochondria. However, no quantitative data on the contribution of the certain pathways to DHA reduction is known. In our study, information was gained by applying inhibitors of these two DHA reducing pathways about the contribution of these pathways to DHA reduction in plant mitochondria. To determine the extent of GSH-dependent DHA reduction, BY2 tobacco cells were treated by the gamma-glutamylcysteine synthetase inhibitor BSO. The treatment caused a dramatic decrease in the cellular GSH content. However, this dramatic decrease did not influence the viability of cells. The huge difference (90 % decrease) in GSH content of the treated cells was much smoother at mitochondrial level (38 % decrease). These observations suggest a well-regulated mitochondrial GSH transport mechanism since GSH biosynthesis is localized in the cytosol and in the plastids. The difference in DHA reduction capacity of mitochondria between the BSO treated and control cells was even smoother. This phenomenon affirms the existence of alternative, non-GSH dependent DHA reducing mechanism(s) in plant mitochondria. The contribution of mitochondrial GSH to DHA reduction was assessed by the determination of mitochondrial GSH content before and after (20 min) the addition of DHA to the mitochondrial fraction. The ascorbate production (upon DHA addition) was determined parallel. In the absence of any additional fuel (of metabolism) the pure GSH-dependence of DHA reduction in plant mitochondria could be assessed. The decrease in mitochondrial content was approximately one-fifth of ascorbate production. Hence, GSH (consumption) is responsible for the ~20 % of ascorbate production (DHA reduction). The remaining DHA reduction can be occurred by other non-GSH dependent manner. According to our previous results only the Complex II of mitochondrial electron transfer chain (mETC) has any contribution to DHA reduction in plant mitochondria. Hence, the mETC dependent part of ascorbate production can be assessed by the supplementation of incubation medium with the Complex II substrate succinate and the Complex II inhibitors TTFA and malonate. The addition of succinate caused 90 % enhancement of ascorbate production of which could be almost totally prevented by the concomitant addition of malonate or TTFA. Although succinate showed moderate inhibition on mitochondrial DHA uptake, the addition of this complex II substrate resulted in marked elevation of ascorbate generation upon DHA addition. The observed boost in ascorbate generation due to succinate addition is definitively the result of higher electron donation which could compensate its possible inhibitory effect on mitochondrial DHA uptake.

On the base of these results the importance of mETC compared to GSH-dependent mechanisms in mitochondrial ascorbate recycling has been underestimated so far.

3. The effect of bacterial elicitor treatment on ascorbate metabolism

Mitochondria are considered as one of the major source of ROS during (both biotic and abiotic) stress in plant cells. The energy-dissipating machineries of plant mitochondria and the abundant water soluble antioxidants such as ascorbate and glutathione are important parts of the ROS regulatory mechanisms of plant cells. Hence we aimed at the investigation of these ROS preventing, antioxidant systems in biotic stress induced by recombinant harpin proteins from *Pseudomonas syringae* and *Erwinia amylovora*. The harpin proteins for the treatment of Arabidopsis thaliana suspension cell cultures were prepared by our team.

As the very first step of the investigation of harpin protein induced alterations of ascorbic acid metabolism we aimed at the determination of the ideal concentration of different harpins. It has been found that the ideal concentration for the treatment of Arabidopsis suspension culture is 150nM in the case of both applied harpin proteins from *Pseudomonas syringae* pv. tomato DC3000 (HrpZpto, HrpWpto).

In the next turn of our experiments *Arabidopsis* cells were treated by these harpin proteins (HrpZpto, HrpWpto) then the viability of the cells and the level of ROS generated due to the treatment were followed. Neither of the harpins had any influence on the viability of the cells in the applied concentration even at longer incubation times (6 or 24 h). At the same time a well-defined oxidative burst could be observed. The generation rate of superoxide anion increased to 0.44 nmol/g FW/min (320 % increase) and to 0.54 nmol/g FW/min (412% increase) after 30 min of HrpZpto and HrpWpto treatment respectively. The level of H_2O_2 increased to 6.5 µM (189 % increase) and to 8.5 µM (225% increase) after 60 min of HrpZpto and HrpWpto treatment respectively. Following these peak values, the level of ROS descended to the control 120 min after the addition of harpins. The expression of the alternative oxidase (AOX1a) and vtc5, one of the paralog genes that encode the rate limiting enzyme of ascorbate biosynthesis followed the elevation of ROS. Similarly, the activity of ascorbate-peroxidase and galactono-1,4-lactone dehydrogenase (EC 1.3.2.3) (GLDH), the enzyme catalysing the ultimate, mitochondrion coupled step of ascorbate biosynthesis and the level of ascorbate and glutathione also followed the elevation of ROS due to harpin treatment. The recycling of the oxidized forms of ascorbate is as important in the regulation of its level as its biosynthesis, thus the effect of harpin treatments on the activities of the enzymes of ascorbate–glutathione cycle was also studied. The activity of ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDHAR) was enhanced by HrpZpto treatment. Both of them reached their maximal value after 90 min and then descended. However, HrpZpto did not have any effect on dehydroascorbate reductase (DHAR) and glutathione reductase (GR) activities. Similarly, HrpWpto had no notable effect on the activity of any enzyme of the cycle. The enhanced expression of AOX1a, the elevated activity of GLDH and the increased level of ascorbate and glutathione all can contribute to the mitigation or absence of programmed cell death. Furthermore on the base of these observations a new function, the fine tuning of redox balance during plant-pathogen interaction can also be proposed to vtc5.

4. Differences in the effects of bacterial and bacterial elicitor protein treatments

In the next turn of our research work we aimed at the investigation of the possible differences between the bacterial and the earlier applied bacterial elicitor protein treatments. The possible differences were investigated in the frame of a collaboration with Miklós Pogány and his research group (Plant Protection Institute, Centre for Agricultural Research of the Hungarian Academy of Sciences). *Nicotiana tabacum* was chosen as a non-host plant. Thereafter the interveinal regions of tobacco leaves were injected by *Pseudomonas syringae* DC3000 suspension with a needleless syringe. Hypersensitive response and oxidative stress was induced by the bacterial treatment of the leaves. Leaves became flaccid, and later they were totally collapsed.

The inoculation of leaves by 10^8 CFU/ml of *P. syringae* DC3000 suspension caused the collapse of them after more than seven hours. The cellular ROS production was monitored by xylenol-orange and by the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA). The level of ROS has increased by 50-100% compared to the control plants. It reached the maximum value 4.5-6 hours after the infiltration, then it has descended to the ROS level of control plants 7 hours posttreatment.

The level of total ascorbate (ascorbate + DHA) increased by 70-100% in the *P. syringae* DC3000 treated plants compared to the controls. Interestingly the redox state of ascorbate did not change. The observed increased activity of MDHAR can be in the background of this phenomenon. The activity of the other enzymes of ascorbate-glutathione cycle was also investigated. On the contrary of MDHAR significantly decreased activity of APX could be observed at 4.5 hours of bacterial treatment and it remained at the same level at 7 hours of incubation. As we already mentioned the activity of MDHAR was significantly higher at all the investigated time points (4.5-7 hours) in the treated plants compared to the controls. No significant differences could be observed in the activity of DHAR between the bacterial treated and non-treated control plants. Similarly no significant differences could be observed in the activity of GR between the bacterial treated and non-treated control plants. Summarily the activity of APX decreased by ~55%, the activity of MDHAR elevated to 4-12 fold and the activities of DHAR and GR did not change due to *P. syringae* DC3000 treatment of tobacco leaves.

5. The effect of bacterial elicitor treatment on AOX and UCP activity

Plant mitochondria contain an additional terminal oxidase (beside of Complex IV) the alternative oxidase (AOX) that catalyzes the oxidation of ubiquinone and reduction of O2 to H2O. AOX also reduces the energy yield of respiration because it is non-proton pumping and bypasses proton-pumping Complexes III and IV. Hence AOX can be called as a “safety electron valve” since one of its key roles is to prevent over-reduction of the ETC and allow the continued operation of glycolysis and the tricarboxylic cycle. Accordingly AOX maintains the

redox balance of the ubiquinone pool, thus minimizing the formation of ROS from reduced ubiquinone. Plant mitochondria contain another “safety valves”, in the form of uncoupling proteins (UCPs). The possible physiological role of UCPs is fine tuning of mitochondrial membrane potential ($\Delta\Psi m$) to a level optimal for oxidative phosphorylation and minimal ROS production.

Arabidopsis cells were treated by HrpZpto harpin proteins and the activity of both AOX and UCP were followed. The development of oxidative burst was verified by the measurement of superoxide anion and hydrogene-peroxide. The activity of AOX and UCP in the harpin treated cell suspension exceeded that of the control suspensions. The activity of AOX reached its maximum with the double value of the control 24 h post treatment then it declined. Similarly the activity of UCP reached its maximum 6-24 h post treatment. These observation suggest that UCP (beside the AOX) plays important role in the regulation of ROS generation during bacterial harpin induced oxidative burst.

6. The investigation of mitochondrial sorbitol formation in plant cells

It seems that alkaline neutral invertase and mitochondrial carbohydrate metabolism is intimately linked to (mitochondrial) ascorbate metabolism. The mitochondrial invertase activity and the putative sorbitol formation due to a mitochondrial aldose reductase activity may contribute to mitochondrial ROS homeostasis, and perhaps, also to total cellular ROS homeostasis. Thus we aimed at the investigation of mitochondrial sorbitol formation during control and stressed conditions.

Preparation of intact mitochondria requires the use of an osmoticum in high concentration that preserves the mitochondrial structure and prevents physical swelling and rupture of membranes. For this purpose, mannitol is widely accepted as a component of the homogenization medium. The large difference in the concentration of possibly formed sorbitol and mannitol of the homogenisation medium makes the determination of sorbitol difficult. Appropriate separation of mannitol and sorbitol standards in our experiments could be reached at millimolar concentrations by Gas-chromatography with flame ionization detection (GC-FID) using a nonpolar fused silica capillary column. Retention times for mannitol and sorbitol were 25.50 and 25.63 min respectively. Unfortunately a significant peak widening could be observed analyzing the mitochondrial samples due to the high mannitol concentration therefore sorbitol could not be determined reliably. Gas-chromatography coupled with mass spectrometry provided excellent qualitative analysis due to the capability of structural identification of the compounds. Despite of the same MS fragmentation pattern observed in the case of mannitol and sorbitol, the capillary column provided the sufficient separation of these sugar alcohols at millimolar concentration range. Retention times for mannitol and sorbitol were 24.14 and 24.31 min respectively. Furthermore, sorbitol could be measured at millimolar concentration, in the presence of high amount of mannitol (0.4M) after appropriate dilution of the mitochondrial samples. However the reproducibility of the derivatized mitochondrial samples was not sufficient in some cases. Mitochondrial samples derivatized parallel had

different colours and morphology after the evaporation. The application of ^{13}C labelled substrates ($^{13}C6$ glucose and fructose) and consequently the formation of ^{13}C labelled products ($^{13}C6$ sorbitol) by mitochondria offers the advantage that these compounds can be selectively measured by mass spectrometry, due to the mass difference of the labelled compounds. HPLC-MS-MS technique combined with hydrophilic interaction liquid chromatography also offers an appropriate solution to eliminate the time, sample consuming and uncertain derivatization step. The ZIC-pHILIC polymeric column gave the possibility to apply basic (pH=9) mobile phase for the separation. Retention times for sorbitol and mannitol were 20.97 and 22.40 min respectively. The use of ^{13}C labelled substrates ($^{13}C6$ glucose, fructose) and $^{12}C6$ mannitol as osmoticum allowed us to measure sorbitol production selectively. Low amount of $^{13}C6$ sorbitol could be determined in the presence of high amount of mannitol in mitochondrial samples.

7. In silico aided thoughts on mitochondrial vitamin C transport

By the spring of 2014 our picture on mitochondrial vitamin C transport became quite obscure. The initially described quasi solid role of GLUTs may have been queried and the role of initially excluded SVCTs may have been strengthened in the mitochondrial vitamin C transport. These recent observations and the supposed discrepancies on the field of the localization of mitochondrial vitamin C transporters drove us to do a computational analysis implied all the potential transport proteins which can be involved in vitamin C transport through the mitochondrial inner membrane. *In silico* prediction tools were applied in aid of the support of *in vitro* and *in vivo* results. The role of GLUT1 as a mitochondrial dehydroascorbate transporter could be reinforced by our *in silico* predictions however the mitochondrial presence of GLUT10 is not likely since this transport protein got far the lowest mitochondrial localization scores. Furthermore, the possible roles of GLUT9 and 11 in mitochondrial vitamin C transport can be proposed leastwise on the base of their computational localization analysis. In good concordance with the newest experimental observations on SVCT2 the mitochondrial presence of this transporter could also be supported by the computational prediction tools.

8. APAP induced GSH depletion leads to ferroptosis

The recently described form of programmed cell death, ferroptosis can be induced by agents causing GSH depletion or the inhibition of GPX4. Ferroptosis clearly shows distinct morphologic, biochemical and genetic features from apoptosis, necrosis and autophagy. Since NAPQI the highly reactive metabolite of the widely applied analgesic and antipyretic, acetaminophen (APAP) induces a cell death which can be characterized by GSH depletion, GPX inhibition and caspase independency, the involvement of ferroptosis in acetaminophen induced cell death was investigated. The specific ferroptosis inhibitor ferrostatin-1 failed to

elevate the viability of acetaminophen treated HepG2 cells. It should be noticed that these cells do not form NAPQI due to the lack of phase I enzyme expression therefore GSH depletion cannot be observed. However in the case of acetaminophen treated primary mouse hepatocytes the significant elevation of cell viability could be observed upon ferrostatin-1 treatment. Similar to ferrostatin-1 treatment, the addition of the RIP1 kinase inhibitor necrostatin-1 could also elevate the viability of acetaminophen treated primary hepatocytes. Ferrostatin-1 has no influence on the expression of CYP2E1 or on the cellular GSH level which suggest that the protective effect of ferrostatin-1 in APAP induced cell death is not based on the reduced metabolism of APAP to NAPQI or on altered NAPQI conjugation by cellular GSH. Since the erastin induced (ferroptotic) cell death was suppressed by the lipophilic antioxidant α-tocopherol the possible protective effect of α-tocopherol, the water soluble antioxidant vitamin C (DHA) and the combination of DHA and α-tocopherol was investigated. Interestingly DHA and the combination of DHA and α-tocopherol exceeded the protective effect of the pure α-tocopherol. The synergetic effect of α-tocopherol, ascorbate (and glutathione) is reasonable since the three key antioxidants play an interdependent role on the electron transfer stage of the cell due to their recycling. The marked beneficial effect of these antioxidants can be explained by this synergetic effect, furthermore by scavenging the lipid and non-lipid ROS. Since the formation of excess ROS can trigger at least two programmed cell death, necroptosis and ferroptosis, thus we aimed at the finding of the optimal ratio of DHA andα-tocopherol in this antioxidant cocktail. The best protective effect could be reached by the combination of 1 nM α-tocopherol and 1 mM DHA. Our results suggest that beyond necroptosis and apoptosis a third programmed cell death, ferroptosis is also involved in acetaminophen induced cell death in primary hepatocytes.

9. The interrelationship of programmed cell deaths, focused on ferroptosis

Ferroptosis was induced in HepG2 cells with the specific ferroptosis activator erastin for 12 hours. In order to investigate the effect of erastin on the cells various measurements were made every 2 hours. Cell viability was measured with the Merck Muse cell cytometer utilising a cell viability kit and LDH release was measured with a spectrophotometer. Reactive oxygen species generation was measured in cells with the Merck Muse cell cytometer utilising the fluorescent probe dihydroethidium. Lipid peroxidation was measured by the TBARS assay from the growth medium. Intracellular GSH level was determined by conjugation of GSH with monochlorobimane and HPLC-fluorescent quantification. The activation of specific cell death pathways was investigated from isolated protein samples which were subjected to western blot analysis. We found that erastin induced significant cell death in HepG2 cells after 8h treatment. Based on the results of cell viability measurements cell viability dropped to 75% after 8h, 50% after 10h and 25% after 12h of treatment. LDH release was not significantly altered possibly because during the cell death process the cellular membrane remains more or less intact. Reactive oxygen species measurements did not show the specific signs of ferroptosis, but re-evaluation of assay conditions needs to be considered. Measured intracellular reduced GSH levels showed a significant drop to 50% just after 2h, 12% after 4h, 5% after 6h of treatment

and continued to drop below quantification limit through the treatment. We investigated the activation of various cell death marker proteins which could be considered in the cell death process. PARP-1 and Caspase 3 activation were measured to investigate apoptosis. LC3 activation and ULK1 phosphorylation were measured to investigate autophagy. RIP3 activation was measured to investigate necroptosis. Western blot analysis of protein samples of erastin treated HepG2 cells showed an increase in autophagy related protein activity in the first 4h of treatment while necroptosis related protein activity was seen at 6-8 hours. These findings indicate that ferroptosis could be a mixed form of cell death in which various cell death processes are initiated.

10. The level of ALR is regulated by the quantity of mitochondrial DNA

The protein, Augmenter of liver regeneration (ALR) contributes to mitochondrial biogenesis, maintenance and to the physiological operation of mitochondria. The depletion of ALR was widely studied and had serious consequences on the mitochondrial functions. However the inverse direction, the effect of the depletion of mitochondrial electron transfer chain and mtDNA on ALR expression has not been investigated yet. Thus an mtDNA depleted, ρ0 cell line was prepared to investigate the role of mitochondrial electron transfer chain and mtDNA on ALR expression. The depletion of mtDNA did not cause any difference at mRNA level, but at protein level the expression of ALR markedly increaseded. The regulatory role of ATP and ROS levels could be ruled out as the treatment of the parental cell line with different respiratory inhibitors and an uncoupling agent did not provoke any changes in the protein level of ALR. The effect of mtDNA depletion on the protein level of ALR was proved to be not liver specific, since the phenomenon was observed in the case of two other, non-hepatic cell lines. It seems the level of mtDNA and/or its products may have a regulatory role on the protein level of ALR. The up-regulation of ALR can be a part of the adaptive response in ρ0 cells that preserves the structural integrity and the transmembrane potential despite the absence of protein components encoded by the mtDNA.

11. Reviews in the frame of the supported project

A comprehensive review was written on the role of ascorbate in protein folding. Both the role of ascorbate in protein hydroxylation reactions and the possible role of ascorbate in the formation of protein disulfide bridges are discussed. Special attention was paid to the recent observation that low tissue ascorbate levels and a noncanonical scurvy were observed in endoplasmic reticulum thiol oxidase- and peroxiredoxin 4-compromised mice. This novel observation implies that ascorbate may be involved in oxidative protein folding and creates a link between the disulphide bond formation (oxidative protein folding) and hydroxylation.

Another comprehensive review was written on ascorbate subcellular compartmentation. Beyond its general role as antioxidant, specific functions of ascorbate are compartmentalized within the eukaryotic cell. The list of organelle-specific functions of ascorbate has been

recently expanded with the epigenetic role exerted as a cofactor for DNA and histone demethylases in the nucleus. Compartmentation necessitates the transport through intracellular membranes; members of the GLUT family and sodium-vitamin C cotransporters mediate the permeation of dehydroascorbic acid and ascorbate, respectively. Recent observations show that increased consumption and/or hindered entrance of ascorbate in/to a compartment results in pathological alterations. The review focuses on the reactions and transporters that can modulate ascorbate concentration and redox state in three compartments: endoplasmic reticulum, mitochondria and nucleus.

Finally, a third review was written on the beneficial and potentially harmful effects of ascorbate in humans and plants. Growing number of evidence show that excessive ascorbate accumulation may have negative effects on cellular functions both in humans and plants, inter alia it may negatively affect signaling mechanisms, cellular redox status and contribute to the production of reactive oxygen species via the Fenton reaction. Not accidentally both plants and humans tightly control cellular ascorbate levels. The control mechanism includes its biosynthesis, transport and degradation, in order to maintain it in an optimum concentration range, which, among other factors, is essential to minimize the potentially harmful effects of ascorbate. The regulatory mechanisms controlling cellular ascorbate levels are also to be considered e.g. when aiming at generating crops with elevated ascorbate levels.

Summary

Two small molecular weight antioxidants could be recognized in the heart of our project: the ascorbate and the glutathione. Thus methods were developed for the determination of ascorbate and glutathione from biological samples. Before the investigation of the effect of elicitor treatment on mitochondrial ascorbate metabolism quantitative data was gained on the contribution of GSH and Complex II dependent ascorbate recycling in plant mitochondria. On the base of our results the importance of Complex II compared to GSH-dependent mechanisms in mitochondrial ascorbate recycling has been underestimated so far. The effect of bacterial elicitor treatment on ascorbate metabolism in plant suspension cells and green tissues was investigated and a new role, the fine tuning of redox balance during plant-pathogen interaction was proposed for to vtc5. As most important regulators of mitochondrial ROS production the activity of AOX and UCP was investigated due to bacterial elicitor treatment. The activity of both proteins was enhanced by the elicitor 6-24 h post-treatment. The formation of sorbitol in plant mitochondria was determined in control and abiotic stressed plants. The experimental results on mitochondrial ascorbate transport was reinvestigated and checked by *in silico* tools. It was first described by our group that APAP induced GSH depletion leads to ferroptosis. We showed that the level of ALR is regulated by the quantity of mitochondrial DNA.