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DEPARTMENT OF PLANT PHYSIOLOGY AND MOLECULAR PLANT BIOLOGY
BIOLOGY PH.D PROGRAMME

ROLE OF POLYAMINE TRANSPORTERS IN THE PARAQUAT RESISTANCE OF HORSEWEED (*CONYZA CANADENSIS* /L./ CRONQ.)

PH.D. THESIS

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2010

Introduction

Paraquat (1,1'-dimethyl-4,4'-bipiridyl) is a non-selective, post-emergence herbicide, widely used in the form of haloed salts as the active agent in contact herbicides for total weed control in vineyards, orchards and gardens and for the defoliation and desiccation of field crops. It exerts its toxic effect by diverting electrons from the physiological pathway on the reducing side of PSI at the FeSx level, while itself taking up an electron to form a cation radical. When this reverts to the Pq cation form by reacting with molecular oxygen, it generates a superoxide anion radical, which reacts with Fe^{3+} or H^{+} ions in the Fenton reaction, while the paraquat cation radical generates hydroxyl radicals in the presence of hydrogen peroxide in the Winterbourn reaction. The reactive oxygen species formed during these reactions, particularly the superoxide and hydroxyl radicals, damage membranes through lipid peroxidation and the destruction of the double bonds in the side chains of fatty acids, thus leading to plant mortality.

The frequent, repeated use of paraquat resulted in the development of resistant species, which at present number more than twenty. Among these plants the resistant biotype of horseweed (*Conyza canadensis* /L./ Cronq.) has got an exceptionally high value of resistance. There are several popular theories describing this extraordinary high resistance but none of them proved to be irrefutable. Deceleration of processes generating reactive species like O_2^{*-} and OH^* can provide a temporary solution. If paraquat generated superoxide species are unable to react free Fe^{3+} then less quantities of Fe^{2+}will be produced during Fenton-reaction, and it will produce less quantities of OH^* species from H_2O_2, which is important because of lipid membrane destructive property of OH^* species. However intracellular control and lower concentration of free iron radicals can result only a slight and temporary tolerance to paraquat.

There is no difference in the site of action between susceptible and resistant biotype of *Conyza*, and it has been proven too that there is no metabolism of paraquat in the horseweed plants. Higher activity of the antioxidant enzyme system is excluded since there is no difference between both biotypes in the response against other superoxide generating agents. In addition radiographic measurements showed signs of inactivated paraquat in vacuoles. Since the phenomenon of resistance has not ceased by using ABC transporter inhibitors, polyamine transporters are most likely to participate in the sequestration mechanism. Many have described that penetration of paraquat into the cells can occur partly via polyamine-uptake systems.

In yeast paraquat as substrate is recognized by the protein TPO1, which is similar to *E. coli* putrescin transporter PotE. This yeast protein is able to transport cationic substrates that share similar charge distribution with putrescin. Overexpression of TPO1 led to sequestration and higher tolerance of paraquat in yeast. Members of the cationic amino acid transporter superfamily play a key role in the polyamine transport of plants however, these have not been studied in detail yet.

Objectives

The aim of my work was the definition of the molecular mechanism of resistance of horseweed against paraquat. I wanted to describe the transporters involved in the sequestration mechanism, and the involved proteins during the initial, temporary protective phase.

1. My aim was to identify the polyamine transporter genes, which are involved in the sequestration mechanism of paraquat.

2. My aim was to determine the genes that ensure the inhibition and the protection during the initial, temporary protective phase by their activation.

3. In addition to identifying the key resistance genes my aim was to examine the changes in expression of these genes.

4. I wanted to support these molecular research results by providing *in silico* structure analysis, homology research and phylogenic tree.

Material and Methods

Plant material, treatments and measurement of the fluorescence induction

Rape (*Brassica napus*) and horseweed (*Conyza canadensis* /L./ Cronq.) plants were grown hydroponically in warmhouse under 130 μEm2 s-1 illumination regime.). Plants at the rosette stage were used in the experiments. Functional activity of leaves was characterized by variable fluorescence (Fv/Fm) and expressed as percentages of the initial control values. Fluorescence parameters were determined by PAM fluorometer (Walz, Effeltrich, Germany).

Molecular biology studies

DNA was isolated with PlantDNAzol (Invitrogen, USA), mRNA content of three-week-old plants was isolated with a GenoPrep TMDirectmRNA Kit (GenoVision) according to the instructions of the manufacturer. cDNA synthesis was performed using a RevertAid First Strand PCR Synthesis Kit (Fermentas) and oligo dT primers. DDRT-PCR reaction was performed by using protocol of GenHunter Corp and HAP arbitary primers. Following PCR amplification samples were loaded onto acrylamide gel, which was then recovered and stained with silver nitrate. The fragments of interest were excised with a scalpel and eluted by rinsing, then the eluted DNA was re-amplified directly under conditions identical to those used for the initial PCR. The amplified PCR products were cloned in pGEM or pBluescript vectors and were sequenced directly from plasmid by M13 primers. Primers used in the semiquantitative RT-PCR were designed based on the EST sequences. Semiquantitative PCR was performed by using PCR reagents of Fermentas and touch-down PCR protocol. 300bp sequence of actin was used as housekeeping.

In silico studies

During bioinformatical studies we used standard FASTA nucleotide and protein sequences from GenBank, EMBL, PDB and SwissProt (UniProt). In homology search we used BLAST 2.2.8 algorhythm. For visualization we used BioEdit and Genedoc, for sequence alignment we used CLUSTALW, for phylogenic tree we used PAUP 4.0. Conservative regions were searched in the NCBI Conserved Domain Search database, for prediction of TM domains we used TMNMM.

Results

1. My aim was to substantiate that hypothesis that the high level of paraquat resistance of horseweed is caused by the sequestration towards the metabolically inactive vacuole. Previous research has shown that the recovery phase after treatment can be prevented by using protein synthesis inhibitors and paraquat is still detectable even after several months in the case of resistant biotype. Selective vacuolar ATP proton pump inhibitor potassium nitrate was used to prove sequestration. The inhibitor has proven to be effective; in case of resistant biotype the lack of recovery phase was experienced. If the nitrate was given within 1 h after paraquat treatment, it inhibited about 50% the recovery process. Repeated spraying of nitrate and paraquat treated leaves left in nitrate-containing solution prevented the recovery process. This confirms that the sequestration is burden to proton gradient energy and paraquat is sequestered into inactive particles.

My aim was to identify the genes involved in the sequestration. In order to decide whether the up-regulation of the genes specifically responsible for resistance can be attributed to paraquat itself or to the reactive oxygen species induced by the paraquat effect, and to distinguish which genes are activated by paraquat and which by superoxide or the general stress response, changes in gene expression were studied using the DDRT-PCR technique in susceptible and resistant plants treated with paraquat and menadione. The expression profile reveals the appearance of a number of new *expressed sequence tags* (EST) in both the susceptible and the resistant biotype in response to both paraquat and menadione. After purifying and sequencing, ESTs were compared with known genes in databases, leading to the detection of four identified partial sequences induced only by paraquat: an Myb transcription factor, a H+ATPase which is involved in the establishment of proton gradient, an iron binding ferritin, and an *Arabidopsis* CAT4 cationic amino acid transporter that belongs to the APC (amino acid polyamine choline) transporter family. Homologues of APC protein family are able to transport cationic polyamines and other substrate positively charged homologues.

2. My aim was to describe the role of key proteins involved in the temporary protective phase. The multimer iron storage ferritin gene detected with DDRT-PCR is responsible for intracellular regulation of free Fe^{3+} ions that are able to produce lipid membranes destructing radicals by binding the free iron ions. Our results show that the ferritins are induced by the paraquat treatment in both susceptible and resistant biotypes therefore ferritin may play a protective key role in the temporary destructive phase.

3. My aim was the study of expression of key genes. I wanted to support my results of DDRT-PCR; therefore I used semi-quantitative RT-PCR method for examining the expression level of the cationic amino acid transporter and ferritin among actin control by treating susceptible and resistant horseweed plants with paraquat. Among standard actin expression cationic amino acid transporter showed induction and higher expression level in the resistant biotype. This supported the key role of CAT4 cationic amino acid transporter homologue in the paraquat sequestration mechanism of the resistant biotype. The ferritin gene expression increased in both biotypes during the paraquat treatment. This proves that the plant reacts with improved expression of ferritins in the temporary destructing phase to the treatment by subtraction of free Fe^{3+} ions that are able to generate destructive hydroxyl radicals in the Fenton reaction. The overexpression of ferritins in tobacco plants supports our theory since these transgenic plants showed higher oxidative stress capability.

4. My aim was to support these molecular biology results with *in silico* analysis and homologue search. The partial sequence of ferritin was completed by direct sequencing, so possessing the whole coding sequence we were able to create phylogenetic tree and further *in silico* structure analysis, like definition of characteristic structures, such as transit and extension peptides. There was no significant difference of ferritins between the susceptible and resistant biotype. The completed ferritin sequence was uploaded to GenBank nucleotide database under accession number AJ786262 GI:50787936. In the sequence analysis we identified the TP transit peptide that is responsible for the transport of precursor into the chloroplast and the EP extension peptide that is responsible for the stability of the mature protein. Phylogenic tree of plant ferritins showed that ferritin of horseweed is mostly similar to ferritin3 of *Arabidopsis* and soya bean. These phylogenic results will facilitate design of the detection of further genes.

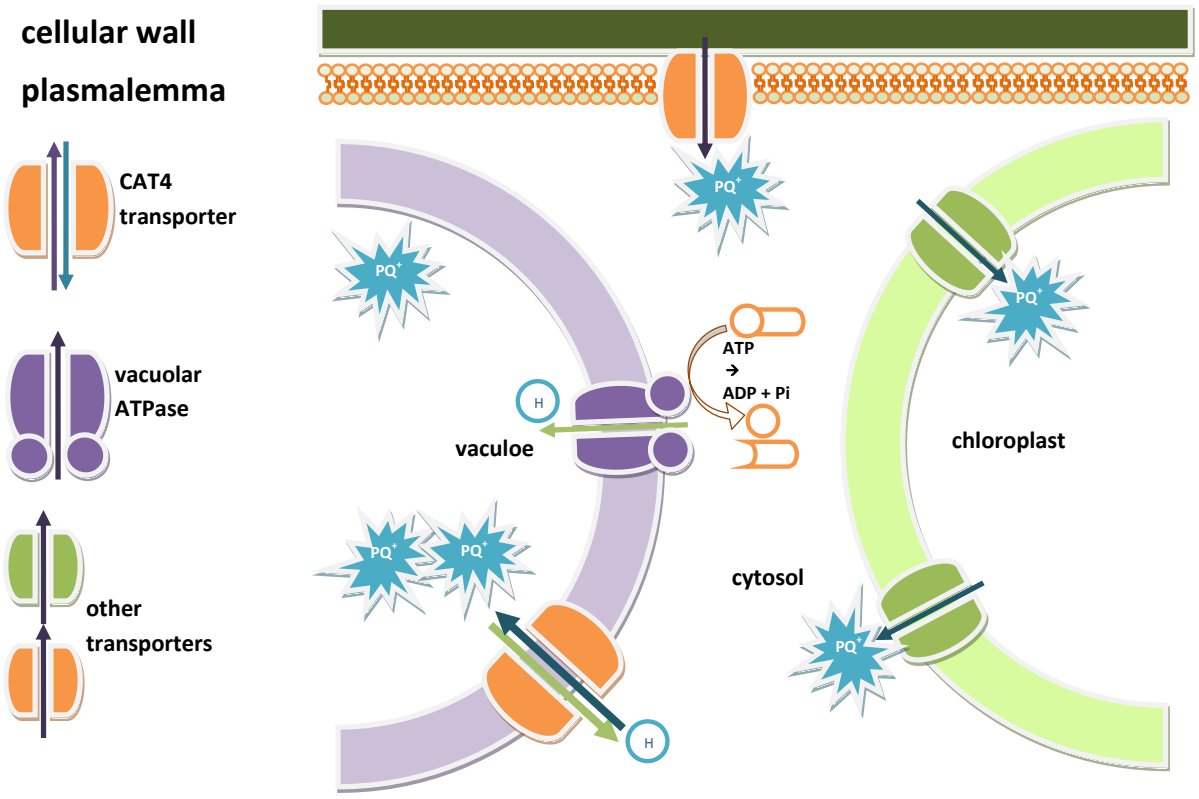
At the *in silico* analysis of CAT4 homologue cationic amino acid transporter of horseweed I looked to the structural properties of the protein for the ability of transport of substrate homologue transport such as paraquat and proved the key role of this protein in the sequestration mechanism. During the analysis we examined other polyamine and cationic amino acid transporters among CAT4, therefore we used the sequence of *Ochrobactrum* paraquat resistance protein PqrA archaic *E. coli* protein PotE, and its homologue TPO1 isolated from *Saccharomyces*, that's overexpression led to paraquat resistance in yeast. With the definition of transmembrane domains in the whole *Arabidopsis* CAT-family, *E. coli* PotE, *Ochrobactrum* PqrA and yeast TPO1 I mapped the polyamine binding sites since paraquat shares the same charge distribution with polyamines. This analysis showed that glutamic acid

in the Glu^{77}, Glu^{277}, and Glu^{433} position are in the hydrophilic site on the transmembrane part following the II. , VI and XII domain. Based on the analysis CAT4 is the only cationic amino acid transporter that fulfils all the criteria for binding paraquat analogue substrate putrescine.

The *in silico* analysis of PqrA protein responsible for paraquat resistance of *Ochrobactrum anthropi* provided meaningful lessons too. In this protein we found glutamic acid only in three regions localized on the citoplasmic site, however in one region more were found. At the same time in the CAT4 transporter more glutamic acids are located in the regions after the VI and XII. transmembrane domains. presumably these positions are important to establish the negatively charged environment that facilitates the connection of cations.

Based on structure analysis result we can say that antiporter CAT4 is the mostly similar plant protein to the putrescine and paraquat transporter TPO1 of yeast and its prokaryotic ancestor PotE, and has regions rich in glutamic acid likely to PqrA that result increased paraquat transport in *Ochrobactrum.* Detection of CAT4 from *Arabidopsis*-related rape is also an important result. Partial sequence from genomic DNA showed 93% similarity, while EST were identical from rape and *Arabidopsis*. The rape sequences contribute to further, more precise molecular biological analyzes. The results confirm our hypothesis that a single change or increased transporter expression may be responsible for a significant resistance development.

The presumed mechanism



Overall, based on the kinetics of uptake and intracellular compartmentalization of paraquat it can be concluded that paraquat is sequestered in the resistant *Conyza canadensis* (L.) Cronq. Paraquat penetrates through the plasmalemma by an unknown transporter in the cell. From the intracellular space it gets into the stroma of the chloroplast by further transporters then it translocates again to the cytosol. As a general response during the initial temporary destructive phase expression of ferritin is induced that plays a key role in the acute protection of lipid membranes against free oxidative radicals followed by the induced expression of CAT4 homologue key cationic amino acid transporter protein during the sequestration that delivers paraquat to the vacuole. The energy demand of the antiporter is covered by a vacuolar H+ ATPase, which could blocked by the NO_3^--treatment. It is likely that the CAT4 homologue transporter of the resistant biotype of horseweed has a higher substrate affinity to the cationic substrates, than its susceptible biotype or by its increased expression more transporters are located in the vacuolar membrane, therefore sequestration of paraquat into the metabolic inactive vacuoles is more effective in the resistant biotype of *Conyza canadensis* (L.) Cronq.

List of publications related to the thesis

Articles in referred scientific papers:

Soós V., Páldi E., Jóri B., Szigeti Z., Rácz I., Lásztity D. (2006): Ferritin2 gene in paraquat susceptible and resistant biotypes of horseweed *Conyza canadenesis* (L.) Cronq. Journal of Plant Physiology, 163(9):979-982.

Jóri B., Soós V., Szegő D., Páldi E., Szigeti Z., Rácz I., Lásztity D. (2007): Role of transporters in the paraquat resistance of horseweed (*Conyza canadenesis* /L./ Cronq.). Pesticide Biochemistry and Physiology, 88(1):57-65

Further articles in scientific papers:

Visnovitz T., Soós V., Jóri B., Rácz I., Szigeti Z. (2008): Staying alive: Insight into the resistance mechanism of *Conyza canadensis* to xenobiotic paraquat. Acta Biologica Iugoslavia Seria Acta Herbologica, 17:173-178

Conference abstracts in referred scientific papers:

Soós V., Jóri B., Szegő D., Páldi E., Szigeti Z., Rácz I., Lásztity D. (2005): Paraquat-induced genes in horseweed (*Conyza canadensis* /L./ Cronq.). FEBS Journal, Vol 272. Suppl., p. 429.

Soós V., Jóri B., Szegő D., Bratek Z., Rácz I., Lásztity D. Szigeti Z. (2005): Role of transporters in the mechanism of paraquat resistance of horseweed (*Conyza canadensis* /L./ Cronq.). Acta Biologica Szegediensis, 49: 191-193.

Szigeti Z., Soós V., Jóri B., Rácz I., Lásztity D. (2004): Resistance of *Conyza canadensis* (L.) Cronq. Acta Physiologiae Plantarum, Vol. 26. Suppl., p. 227

Halász K., Soós V., Jóri B., Rácz I., Lásztity D., Szigeti Z. (2002): Effect of transporter inhibitors on paraquat resistance of horseweed (*Conyza canadensis* /L./ Cronq.). Acta Biologica Szegediensis, 46.(3-4): 23-24.

Summaries in conference abstract books:

Szigeti Z., Visnovitz T., Jóri B., Soós V., Lásztity D., Rácz I. (2009): Insight into the Resistance Mechanism of *Conyza canadensis* to Xenobiotic Paraquat. International Conference of Plant Abiotic Stress Tolerance, Vienna, Austria; p. 142

Szigeti Z., Soós V., Jóri B., Páldi E., Rácz I., Lásztity D. (2006): Transporters in the paraquat resistance of horseweed (*Conyza canadensis* /L./ Cronq.). Proceedings of the 15th FESPB Congress Lyon, France; p. 157.

Szigeti Z., Jóri B., Soós V., Páldi E., Rácz I., Lásztity D. (2006): Role of transporters in the paraquat resistance of horseweed (*Conyza canadensis* /L./ Cronq.). Proceedings of the 3th EPSO Conference Visegrád, Hungary; p. 164.

Soós V., Szigeti Z., Jóri B., Rácz I., Bratek Z., Lásztity D. (2004): Novel aspects of the paraquat resistance of *Conyza canadensis* (L.) Cronq. Proceedings of the 4th International Weed Science Congress, Durban, Republic of South Africa; p. 53.

Jóri B., Lásztity D., Soós V., Rácz I., Szigeti Z. (2004): Paraquat resistance and polyamine transporters. Proceedings of the 4th International Weed Science Congress, Durban, Republic of South Africa; p. 50.

Szigeti Z., Soós V., Jóri B., Rácz I., Lásztity D., Lehoczki E. (2004): Paraquat resistance of *Conyza canadensis* (L.) Cronq. Proceedings of the 4th International Weed Science Congress, Durban, Republic of South Africa; p. 40.

Halász K., Soós V., Jóri B., Rácz I., Lásztity D., Szigeti Z. (2002): Influence of transporter inhibitors on paraquat resistance in horseweed (*Conyza canadensis* /L./ Cronq.). Proceedings of the European Workshop of ESSA, Varna, Bulgaria; p. 27.

Other publications

Jóri B (2004): Növényi genomika, Botanikai közlemények 91:39-55