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Application of PlantSize phenotyping software for functional characterization of *SPQ* genes which confer abiotic stress tolerance

Ph.D. Thesis

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INTRODUCTION

On molecular level the adaptation of plants to extreme environmental conditions is coordinated by several factors. The main target of plant research is to reveal the regulatory hubs and metabolic pathways which control stress responses. Research on model organisms, like *Arabidopsis thaliana* has identified a number of genes, which play important role in stress perception and defense mechanisms. However, study of this model organism in extreme stress conditions is more challenging. Extremophile plants, such as xerophytes and halophytes are able to grow in arid regions or on saline soils, which are otherwise lethal to nonadopted species. Extremophile plants are valuable sources of genes conferring tolerance traits, which can be useful to improve stress tolerance of crops. (Ahuja et al., 2010; Mishra and Tanna, 2017).

Plant phenotype is determined by the genetic background and environmental conditions. Interaction of the genotype and environmental factors influences plant growth and development, physiological and molecular traits of plants. Therefore, phenotypic characterization of plants requires precise description and monitoring of multiple structural and physiological traits. While classical methods are generally precise and reliable, they usually destroy the plants and provide information just in the endpoint of the experiments. Besides, standard physiological techniques often require numerous analytical steps and measurements, making large-scale analysis difficult or impossible. of large number of plants is a time-consuming and error-prone procedure. To circumvent such limitations, non-destructive methods have been developed to analyze different morphological and physiological parameters. Such methods are usually based on imaging technologies, which allow serial measurements, and simultaneous detection of morphological and physiological parameters in time (Furbank and Tester, 2011; Dhondt et al., 2013; Rungrat et al., 2016). However, most of the image analysis software and automatic phenotyping systems rely on sophisticated and expensive equipment, and personnel experienced in computer science, not available for most research laboratories.

We have developed a novel *in vitro* imaging system based on phenotyping software called PlantSize. We have demonstrated the utility of the software, which include in the characterization of wild type, mutant and transgenic *Arabidopsis* plants in a fast and cost-effective way. With the application of PlantSize we have successfully characterized the *Lepidium crassifolium SPQ* gene (*LcSPQ*-Small Paraquat resistance) and its *Arabidopsis thaliana* homologue, which confer stress tolerance to negative environmental factors. We have proved that both SPQ protein are novel players in the regulation of hormone signaling and stress responses in higher plants.

AIMS

At the start of my Ph.D. work I was taking part in an applied research project which was related to the identification of novel stress tolerance genes in *Lepidium crassifolium*. I studied some previously identified and selected *Lepidium* genes, which were expressed in transgenic *Arabidopsis* plants. Nowadays high-resolution and expensive imaging system are often required to use during research studies, which are designated to improve the stress tolerance of plants. Applying these systems during phenotyping gives a great assistance to perform the gene identification related studies on more effective and precise way. In future studies the identification and characterization of genes can improve the genetic background of crop plants. According to this our aims were:

1. To develop a cost-effective and user friendly phenomics tool;

2. To apply the image analysis method in practice, under the characterization of transgenic and mutant lines in stress tolerance studies;

3. To test the genes what were isolated from the halophyte *Lepidium crassifolium* and were showed tolerance against environmental stresses, and the characterization of one selected gene;

4. To describe and compare Small Paraquat resistance (*SPQ*) genes which confer paraquat resistance in *Lepidium crassifolium* and *Arabidopsis thaliana*;

5. To reveal the role of SPQ protein in the regulation of hormone signaling and other stress mechanism;

6. To observe the adaptation of SPQ overexpressed *Arabidopsis thaliana* transgenic plants to dehydration.

APPLIED METHODS

Development of PlantSize phenotyping software:

- The PlantSize software was developed for multiple image analysis using MATLABs (version 2016b) with the Image Processing Toolbox™
- Conventional digital camera was used for image capturing
- To calibrate the imaging system *Arabidopsis thaliana* Columbia ecotype (Col-0) were used as control and test plant. To verify the utility of our system *phyB-9* mutant were used as test plants
- To calibrate the imaging system for plant size, shape and color determination the following methods were applied:
    - Fresh and dry weight measurements;
    - Chlorophyll and anthocyanin content determination by spectrophotometer.
- The following morphological and physiological parameters were measured by the PlantSize software:
    - Projected rosette area (Pixel Area): the area that is occupied by the green rosette in a top view image in pixel unit;
    - The convex hull of the rosette (Convex Area): the convex hull is the area defined by the smallest convex set containing the rosette in pixel unit;
    - Ratio of plant area within the convex hull (Convex %): ratio of the detected leaf area divided by the convex hull area in pixel unit;
    - Chlorophyll content: the calculated chlorophyll content from measured Hue parameter of seedling in µg/pixel unit;
    - Anthocyanin content: the estimated anthocyanin from Hue frequency of detected leaves in ng/pixel unit based on pixel and Hue color appearance parameters.

Characterization of *Lepidium crassifolium* and *Arabidopsis thaliana SPQ*:

- SPQ (LcSPQ, AtSPQ) protein were identified with *in silico* bioinformatic tools
- Full length of *SPQ* cDNAs were expressed in the presence of constitutive (pCaMV 35S) promoter and transformed into wild-type (Col-0) plants
- To examine the intracellular localization of SPQ proteins *LcSPQ-GFP* and *AtSPQ-GFP* gene construct were created and transformed into *Arabidopsis* root cell suspension and wild-type (Col-0) plants
- For the functional characterization of *AtSPQ* gene two individual T-DNA insertion mutant were genotyped
- To study the transgenic and mutant line PlantSize phenotyping software were used for *in vitro* characterization
- To determine the differences under stress treatment FIJI image processing and analysis tool were used to evaluate the differences in germination, root growth and hypocotyl length
- Analyzation of pulse amplitude modulation by Imaging-PAM
- Lipid-peroxidation assay
- Determination of polyamine content
- Measurement of stomatal apertures
- Regeneration affinity under dehydration: determination of relative water content (RWC), survival tests, measurement of PSII linear electron transport rate (ETR) by Imaging-PAM

Other technics and methods:

Differential centrifugation, Cell fraction, Western blot, PCR and qRT-PCR, Confocal Spinning Disk Microscopy.

RESULTS OF THE THESIS

1. Identification of Small Paraquat resistance (SPQ) protein:

- We have previously identified the Small Paraquat resistance (SPQ) protein in the halophytic plant *Lepidium crassifolium* as upon overexpression it could confer paraquat resistance to transgenic *Arabidopsis* plants (Rigó et al., 2016).

- With bioinformatic tools we have determined that SPQ-like genes exist in all plants in one or two copies, including *Arabidopsis*, in which the *AT3G52105* gene encoded a small, 8 kD protein composed of 70 amino acids, which has 95% similarity to LcSPQ. All SPQ-type proteins had a conserved N-terminal signal sequence, but no other known protein domain could be identified in them.

- Expression of the *Arabidopsis* and *Lepidium SPQ* genes was not influenced by salt, osmotic or oxidative stresses, suggesting that these genes are not regulated by abiotic stress stimuli. While transcriptional regulation did not suggest that *SPQ* genes could be implicated in stress responses, overexpression of *AtSPQ* or *LcSPQ* cDNA in transgenic *Arabidopsis* considerably enhanced paraquat resistance, suggesting that the elevated expression and not a particular sequence feature of *LcSPQ* was important to define the paraquat resistance (Rigó et al., 2016).

- Sequence analysis revealed no similarity to previously published proteins which are implicated in paraquat resistance, suggesting, that SPQ proteins are novel players in herbicide resistance.

2. SPQ proteins play novel role in the regulation of hormone signaling and oxidative stress responses

- SPQ overexpression could reduce paraquat-triggered damage of photosynthesis and prevent decline of PSII functions as confirmed by sustained PSII quantum yield (Y(II)) or maximum efficiency of PSII (Fv/Fm), which otherwise declined in paraquat-treated wild type plants. However, SPQ overexpression promoted resistance to paraquat not only in illuminated green plants but also in dark-germinated, etiolated seedlings and in non-photosynthetic roots, indicating that protective function of this protein is not confined to photosynthetic electron transport. Cytoplasmic localization of SPQ-GFP fused protein in *Arabidopsis* cells also suggests, that primary action of SPQ is not connected directly to photosynthesis.

- It is unlikely that SPQ protein is involved in cellular uptake of paraquat as the GFP-tagged protein was not located in the plasmalemma.

- Polyamine content of SPQ overexpressing plants was however similar to wild type in the presence of putrescine, spermine and spermidine, suggesting that SPQ is not implicated in polyamine metabolism or uptake.

- ABA sensitivity was enhanced by SPQ overexpression, while the knockout *spq1* mutant was insensitive to this hormone as demonstrated in germination, growth and stomata closure data.

- ABA hypersensitivity of SPQ overexpressing plants might influence paraquat resistance through modulating ROS signaling.

3. SPQ overexpression plants confer drought tolerance:

- ABA hypersensitivity of SPQ overexpressing plants can be responsible for the observed drought tolerance, through promoting stomata closure.

- Besides stomata closure, SPQ overexpression might contribute to drought tolerance by reduction of oxidative damage. Photosynthetic electron transport was less reduced by drought in SPQ overexpressing plants than in wild type plants, suggesting that some photosynthetic activity could be sustained in such stress conditions contributing to viability.

- Better recovery rates of drought-stressed SPQ overexpressing plants can therefore be the consequence of ABA hypersensitivity, as well as the reduced oxidative damage and sustained photosynthetic capacity. Although deciphering the precise biological function of SPQ proteins requires further studies, our data suggest, that they are implicated in a regulatory circuit which connects ABA and H_2O_2 signals with paraquat resistance. Enhancement of drought tolerance and paraquat resistance through increased expression of the *SPQ* genes can have biotechnological perspectives in crop plants.

4. Developing of image analysis tool, PlantSize:

- In order to make our stress tolerance related research work more effective during the characterization of transgenic lines which overexpressed *Lepidium* genes, we have developed a phenotypic system based on our image analysis software to facilitate the easy and fast evaluation of basic characters of plants, which can be precisely measured.

- Our technology is rather simple and does not need heavy investment, as it relies on standard laboratory equipment, a digital camera and a standard desktop computer.

- The PlantSize application is able to perform simultaneous analysis of a number of plants and has the capability to simultaneously analyze size, shape and color of the plants. The PlantSize based system therefore offers simultaneous analysis of the most commonly studied morphological parameters describing size and shape and provides information on chlorophyll and anthocyanin contents of the same plant.

- The technology is available for all research and biotechnology laboratories, which needs high throughput image analysis, but cannot afford an expensive phenotyping platform.

- The technology has been optimized for *Arabidopsis. In vitro* grown seedlings and small plants of other species can also be analyzed, requires optimization of the experimental conditions and calibration of PlantSize.

- With the application of PlantSize we have determine growth rate, chlorophyll and anthocyanin content of studied transgenic and mutant line under different stress condition (salt, osmotic and oxidative stresses) in a precise and cost-effective way.

- We have executed high-resolution image analysis measurement, provide us great assistance during the characterization of transgenic lines.

- Our method is suitable to reveal small but significant differences in plant sizes, shapes and color, which can contribute to the functional characterization of important regulatory genes such as Small Paraquat resistance (SPQ)

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SUMMARY

The Small Paraquat resistance protein (SPQ) of *Lepidium crassifolium* has previously been identified due to its capacity to confer paraquat resistance to overexpressing transgenic *Arabidopsis* plants. *LcSPQ* overexpression of the closely related but previously unknown Arabidopsis SPQ can also enhance resistance to paraquat, while the knockout *Arabidopsis* mutant is slightly hypersensitive to this herbicide. The AtSPQ and LcSPQ proteins are composed of 70 and 69 amino acids, respectively, and both of them are localized in the cytosol. Besides being implicated in paraquat response, overexpression of SPQs enhance sensitivity to abscisic acid (ABA), while the T-DNA insertion of *AtSPQ* is insensitive to ABA. As consequence of ABA hypersensitivity, SPQs can considerably improve drought tolerance by reducing water loss, stabilizing photosynthetic electron transport and enhancing plant survival in water-limited environment. Although deciphering the precise biological function of SPQ proteins requires further studies, our data suggest, that SPQ proteins has pleiotrop function connecting multiple regulatory pathways in stress responses. Enhancement of drought tolerance and paraquat resistance through increased expression of the SPQ genes can have agrobiotechnological perspectives in crop plants.

Image analysis of plants through color imaging is an increasingly popular method to define growth parameters, characterize plant development in time. We have developed a non-invasive method, which simultaneously measures basic morphological and physiological parameters of *in vitro* cultured plants such as *Arabidopsis thaliana*. Changes of plant size, shape and color is monitored by repeated photography with a commercial digital camera. Images are analyzed with the Matlab-based computer application PlantSize, which simultaneously calculates several parameters including projected rosette area (pixel area, fresh weight, convex area and ratio), and color (chlorophyll and anthocyanin contents). Numerical data are exported in MS Excel format. Subsequent data processing provides information on growth rates, chlorophyll and anthocyanin contents. The developed technology offers a simple, affordable and fast way to measure several morphological and physiological parameters of *Arabidopsis* plants. The methods are based on non-destructive imaging allowing repeated measurements and monitoring changes of various growth parameters in time. Using the PlantSize technology we were able to study precisely the effects of different stress conditions on *Arabidopsis* plants and characterize growth and basic physiological parameters of transgenic *Arabidopsis* plants, expressing *Lepidium* cDNA clones, which modulate their stress tolerance.

PUBLICATIONS

Article on which the thesis was based:

Faragó D. és Sass L., Valkai I., Andrási N., Szabados L (2018) plantsize offers an affordable, non-destructive method to measure plant size and color *in vitro. Front. Plant Sci.* **9**, 219. doi: 10.3389/fpls.2018.00219
MTMT: 3343909
Impact factor: 4,298

Article which is closely related to the thesis, but was used in the thesis:

Rigó G. és Valkai I., **Faragó D.**, Kiss E., Van Houdt, S., Van de Steene, N., Hannah, M., Szabados L. (2016). Gene mining in halophytes: functional identification of stress tolerance genes in *Lepidium crassifolium. Plant Cell Environ.* 39, 2074–2084.
doi: 10.1111/pce.12768
MTMT: 3080577
Impact factor: 6,960

Faragó D., Zsigmond L., BenyóD., Ayaydin F., Alcazar R, Rigó G., Szabados L. (2019) Small Paraquat resistance proteins modulate paraquat and ABA responses and confers drought tolerance to overexpressing *Arabidopsis* plants. *Plant Cell and Enviroment.* **Manuscript in preparation.**

Patent:

SPQ proteins confer drought tolerance (Under revision in Hungary and Europe).

Conferences:

1. Conference of Scientific Student Association 2012 (Rewarded).

2. Szabados L., Papdi Cs., Pérez-Salamó I., Rigó G., Joseph M. P., Valkai I., Andrási N., **Faragó D.**, Hannah M., Koncz Cs. (2016) Functional identification of stress regulatory genes in model and extremophile plants using the Conditional Overexpressing System (COS). International Conference on "EMERGING BIOTECHNOLOGIES", Kakatiya University, Warangal, India.

3. Szabados L., Rigó G., Valkai I., **Faragó D.**, Kiss E., Van Houdt S., Van de Steene N., Hannah M. A. (2016) Gene mining in extremophile plants: stress tolerance genes from Lepidium crassifolium. Plant Biology Europe, EPSO/FESPB 2016 Congress, Prague, Czech Republic, ID841.

4. Szabados L., Rigó G., Valkai I., **Faragó D.**, Kiss E., Koncz Cs., Van Houdt S., Van de Steene N., Hannah M. A. (2017) Gene mining in model and halophytic plants: functional identification of stress regulatory genes by random gene transfer and large-scale genetic screens. TASARD 2017 Conference, New Delhi, India.

5. Szabados L., Rigó G., Valkai I., **Faragó D.**, Kiss E., Van Houdt S., Van de Steene N., Hannah M. A. (2017) Gene mining in extremophile plants: stress tolerance genes from Lepidium crassifolium. Hungarian Molecular Life Sciences 2017 Conference, Eger, Hungary.

6. Szabados L., Rigó G., Valkai I., **Faragó D.**, Kiss E., Koncz Cs., Van Houdt S., Van de Steene N., Hannah M. A. (2017) Gene mining in model and halophytic plants: functional identification of stress regulatory genes by random gene transfer and large-scale genetic screens. Straub Napok, Szeged.

7. **Faragó D.**, Sass L., Valkai I., Andrási N., Szabados L. (2017) PlantSize: an affordable, non-destructive method to measure plant size and color *in vitro*. A MAGYAR NÖVÉNYBIOLÓGIAI TÁRSASÁG XII. KONGRESSZUSA, Szeged.

8. **Faragó D**., Sass .L, Valkai I., Andrási N., Szabados L. (2018) PlantSize: an affordable, non-destructive method to measure plant size and color *in vitro*. Plant phenotyping for future climate challenges, COSTFA1306 Meeting, 2018.03.20-21. Leuven, Belgium.

9. **Faragó D**., Sass L., Valkai I., Andrási N., Szabados L. (2018) PlantSize: an affordable, non-destructive method to measure plant size and color in vitro. (NE3) FIATAL BIOTECHNOLÓGUSOK ORSZÁGOS KONFERENCIÁJA, Budapest.

10. **Faragó D**., Rigó G., Zsigmond L., Szabados L. (2019) Small paraquat resistant protein controlling stress responses in higher plants. Hungarian Molecular Life Sciences 2019 Conference, Eger, Hungary.

11. **Faragó D.**, Sass L., Valkai I., Andrási N., Szabados L. (2019) PlantSize: an affordable, non-destructive method to measure plant size and color in vitro. Hungarian Molecular Life Sciences 2019 Conference, Eger, Hungary.

Scientific trainings:

- CNR-IGV Institute of Plant Genetics, Portici, Italy, (2015.11.30-12.14) Scientific Cooperation between CNR and Hungarian Academy of Sciences (MTA), Hungary, Grant no. AMMCNT – CNR 72935.

- Summer School on Image Analysis for Plant Phenotyping. (2016) Trainee Grant (COST-TS-ECOST-TRAINING_SCHOOL-FA1306), Wageningen, Netherland.

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