Botanica Pacifica. A journal of plant science and conservation. 2015. 4(2): 95–101
DOI: 10.17581/bp.2015.04207

RESEARCH PAPER

Stomatal Movement and Stomatal Formation Mechanisms Utilize the Same Regulatory Genes

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Manuscript received: 07.08.2015
Review completed: 16.09.2015
Accepted for publication: 18.09.2015
Published online: 26.09.2015

ABSTRACT

The climate changes across geological time are mirrored in paleontological fossils of stomata morphology. Professor Krassilov significantly contributed to a study of stomata development in modern plants. As a continuation of his work we approached the stomata regulation problem in modern plants by mining of the publically available transcriptome data on regulation of the stomata movement and formation by three of key regulators: SOC1, SPEECHLESS, and YODA. A goal of the study was to integrate heterogeneous data collections on stomata regulation and disclose the underlying basic regulation pathways that belong to stomata as the specific cell type independently on perturbations of its regulatory pathways. By a fresh algorithmic approach, we managed to extract the underlying stomata regulation genes from divergently designed stomata projects. In particular, we defined groups of genes associated with the stomata patterning, formation, and movement. Additionally, groups of genes were associated with specific pairs of these processes.

Keywords: stomata, transcription factors, kinases, gene expression, RNA-Seq

РЕЗЮМЕ

Муха Д., Острецов Б., Муха Дм., Бродский Л. Механизмы образования и функционирования устьичного аппарата используют одни и те же регуляторные гены. Изменения климата в геологическом времени находят отражение в морфологии устьиц палеонтологических окаменелостей. Профессор В.А. Красилов внес значительный вклад в изучение формирования устьиц современных растений. В развитие его идей мы подошли к проблеме регуляции работы устьичного аппарата современных растений, проанализировав массив имеющихся данных по транскрибированию функционирования и образования устьичного аппарата тремя ключевыми регуляторами: SOC1, SPEECHLESS и YODA. Цель исследования – интегрировать разнородные данные по регулированию работы устьиц и раскрыть основные пути контроля устьиц, не зависящие от специфических особенностей регуляторных механизмов. С использованием нового алгоритмического подхода нам удалось определить основные регуляторные гены, ассоциированные с устьичным аппаратом, используя разнородные экспериментальные данные. В частности, мы определили группы генов, ассоциированных с расположением, формированием и функционированием устьиц. Кроме того, нами определены группы генов, вовлеченные в несколько регуляторных процессов одновременно.

Ключевые слова: устьица, транскрипционные факторы, киназы, экспрессия генов, RNA-Seq

Переведено редколлегией

INTRODUCTION

The current climate change is becoming a main issue in agrobiology, health science, industry, and most profoundly in politics and mass media. When we spoke about climate changes with Valentin Abramovich Krassilov, he very opposed the popular hypothesis about the major impact of human activity on the global warming caused by "greenhouse effect". Indeed, as he showed in his published works, the adaptability of living organisms reflected dramatic climate changes occurred in the geological history of the Earth with many periods of extreme volcano activity. Surprisingly, because of the plasticity of the bio-world, land plants flourished even upon geological crises. He warned that the observed growth of CO_2 level in the atmosphere leads to another scientific question. Why the present day Earth cannot absorb this relatively small addition of the greenhouse gases. An answer to this question may be found in studying the storage of CO_2 in soil capillaries and in consumption of CO_2 by land plants and marine phytoplankton. An issue of high interest is the gene regulations of the utilization mechanisms in modern organisms as well as manifestations of these mechanisms in paleontological plant fossils. Certainly, the central elements of the CO_2 utilization in plants are stomata.

Stomatal patterning/formation and stomatal movements are the major responses of plants to drought, CO_2 content in atmosphere, and climate changes. Prof. Krassilov studied mechanisms of stomata development in the modern plants mostly as a key to understanding evolution of these mechanisms across geological periods. He published several seminal articles and book chapters on stomata development, connecting, in particular, stomata response reconstructed from paleontological data with the estimated climate of the corresponding geological periods (Krassilov 1968, 1978a, b, 1995, Krassilov & Karasev 2009).

Plants started to conquest land more than 475 million years ago (Wellman et al. 2003). As organisms with an aquatic past, the first land plants faced with severe challenges in the new environment. They evolved impermeable outer waxy cuticle to prevent desiccation, but also inhibiting gas exchange (Chater et al. 2013). Very early in the evolutionary timescale, land plants developed microscopic pores for control of evaporation and O_2/CO_2 exchange. It is believed that stomatal pores existed more than 410 million years ago, appearing in sporophyte generation of basal groups of land plants, known as bryophytes (Chater et al. 2011). Surprisingly, in old vascular Rhynie flora, stomata presents on both generations. The origin of stomata as gametangial conceptacles evolutionary appearing together with cutinization is widely accepted concept (Krassilov et al. 2013).

Major factors controlling stomata in plants are light, CO_2 concentration and drought hormone ABA. Stomata on the sporophytes of hornworts are believed to functioning as gas exchange and also exhibit closure in response to ABA (Hartung et al. 1987). There are several genes involved in orchestration of stomata development and functioning. SPCH (SPEECHLESS) is a transcription factor and along with FMA and MUTE, it regulates the stomata formation. As UniProt describes it, SPCH is required for the initiation and the formation of stomata, by promoting the first asymmetric cell divisions. There are no stomata in the SPCH-mutant of *Arabidopsis thaliana*.

SOC1 transcription activator is active in flowering time control. SOC1 may integrate signals from the photoperiod, vernalization and autonomous floral induction pathways. Can modulate class B and C homeotic genes expression. When associated with AGL24, mediates effect of gibberellins on flowering under short-day conditions, and regulates the expression of LEAFY (LFY), which links floral induction and floral development.

YODA functions in a MAP kinase cascade that acts as a molecular switch to regulate the first cell fate decisions in the zygote and the early embryo. YODA promotes elongation of the zygote and development of its basal daughter cell into the extra-embryonic suspensor. In stomatal development, it acts downstream of the LRR receptor TMM, but upstream of the MKK4/MKK5-MPK3/MPK6 module to regulate stomatal cell fate before the guard mother cell (GMC) is specified. YODA plays a central role in both guard cell identity and pattern formation. This MAPK cascade also functions downstream of the ER receptor in regulating coordinated local cell proliferation, which shapes the morphology of plant organs. Upon brassinosteroid signaling, YODA is inhibited by phosphorylation of its auto-inhibitory N-terminal domain by the GSK3-like kinase ASK7. The YODA-mutant exhibits an excess of stomata.

Production of stomata is influenced by a network containing SPCH, EPF (downregulates stomata), TMM (too many mouths, downregulates stomata), stomagen (upregulates stomata, inhibits SPCH), as well as ERL and YODA that downregulate stomata development.

In order to understand the functioning of stomata as a whole and separately for three functional processes, we took the NGS and microarray transcriptome projects on stomata regulation by three of key regulators: SOC1 (Kimura et al. 2015), SPEECHLESS (Lau et al. 2015), and YODA (Bergmann et al. 2004). These projects were designed to study gene regulations that are specifically associated with each regulator separately. The goal of our integrative analysis was to detect the basic underlying gene regulations in stomata of *A. thaliana*. The integration was done by a generalization of the GSEA algorithmic approach (Mootha et al. 2003, Subramanian et al. 2005) this allowed for the extraction of underlying stomata regulation genes from divergently designed stomata projects. In particular, we associated groups of genes either with the stomata patterning, formation, and movement together, or associated with specific pairs of these processes.

Our analysis of public domain data on gene regulation mechanisms of the stomatal opening and stomatal distribution, some results of which are presented here, was initiated by Valentin Abramovich.

MATERIALS AND METHODS

We took three *A. thaliana* transcriptome analysis projects from the GEO NCBI database with accession IDs GSE60183 (SOC1), GSE57953 (SPEECHLESS), and GSE991 (YODA). The first project was focused on regulation of opening/closing of the stomata guard cells, as it is influenced by the SOC1 transcription factor. The remaining, two projects were focused on formation and distribution of stomata holes on the leaf surface that are regulated by the SPEECHLESS and YODA. Our goal was to process the NGS/array data in these three divergent projects of completely different experimental design, and find genes that are associated with basic underlying mechanisms for both types of stomata responses to drought, light, and CO_2 atmospheric content.

NGS and microarray transcriptome data used in the present study

The SOC1 project, GEO database GSE60183 (Kimura et al. 2015) consists of 6 samples. The RNA was taken from six *A. thaliana* plants, which were grown under 16 h light / 8 h dark, constant 22°C conditions for 4 to 5 weeks. Three of those plants, replicates of the mutant phot1-5 phot2-1, were used as a control samples. Plants, replicates of the double mutant pGC1:SOC1-GFP/ phot1 phot2, were used as a treatment. Epidermis, including guard cells, was isolated from leaves of these six plants with followed isolation and sequencing of RNA.

The SPCH project, GEO GSE57954 (Lau et al. 2015) consists of two parts: RNA-seq (GSE57953) and ChIP-

seq (GSE57497). RNA-seq contained 11 samples extracted from 4-day-old light grown seedlings. Five of this samples have a Col ecotype, those was used as controls. Six of samples have a ESTpro:SPCH1-4A-YFP ecotype and were used as treatments. Both groups contain three types of samples: (1) not incubated in 10 uM beta-estradiol, (2) incubated for 6 hours, and (3) incubated for 8 hours. Anti-Myc (Cell Signaling Technology, catalog# 71D10, lot# 5) antibodies was used for get SPCHp:SPCH2-4A-MYC in spch3 ecotype and Col ecotype ChIP-seq probe. In all samples 4-day-old light grown seedlings were used.

The YODA project, GEO GSE991 (Bergmann et al. 2004) contains four biological replicates of 7dpg yda seedlings, four biological replicates of 7dpg N-term deletion (NB89) YDA seedlings of no stomata Ler ecotype, three controls 7dpg seedlings Ler ecotype, and three controls 7dpg seedlings of line CS9159 C24 ecotype. The array images were analyzed with the Affymetrix Microarray Suite 5.0 with the target intensity set to 500. The expression levels of the genes were obtained using GeneSpring 4.2 software (Silicon Genetics).

Processing the raw NGS data: obtaining gene expressions across samples

The data processing was performed by our T-bioinfo platform which was developed at the Tauber Bioinformatics Research Center, University of Haifa. The NGS data processing pipeline from the RNA-seq section consists of stages as follows: (i) cleaning of reads from primers and PCR artifacts; (ii) correction of errors in raw reads; (iii) mapping of reads on the whole *A. thaliana* genome; (iv) estimation of gene expression levels; (v) differential expression of genes (Fig. 1).

Two pipelines from the T-bioinfo RNA-seq section were used for data processing:

- PCR duplicate cleaning → eMER(error correction) → Bowtie2 (mapping) → RSEM (gene expression estimation)
- PCR duplicate cleaning → eMER → TopHat (mapping) → CuffLinks(splicevariant detection) → CuffMerge → CuffDiff (expression and differential expression of genes/isoforms)

The resulting gene expression tables of the SOC1 and SPEECHLESS projects, as well as the microarray gene expression table of the YODA project were used for the following up integration of three projects.

Gene ontology analysis

We analyzed gene ontology by two approaches: (i) by means of NCBI DAVID service (Huang et al. 2009a, b); (ii) using hypergeometric test for gene ontology categories extracted from UniProt (UniProt Consortium 2015) and Panther (Mi et al. 2013) databases. Enrichment was asserted in all pairs of projects, for genes associated by three projects together, as well as for union of the lists of genes mentioned. All P-values were corrected with respect of multiple testing (Benjamini & Hochberg 1995).

RESULTS

Integration of data on stomata development and functioning from three divergent projects

An objective of the integration was to find a set of genes that are shared regulators of both stomatal opening/closing and stomatal patterning/formation. The SOC1, SPEECHLESS, and YODA projects were designed to study a gene regulation that is specifically associated with each transcription factor separately. Thus, the obtained gene expression differentiations are project oriented: the differentiations depend on what treatments and controls were used in each individual project. Nevertheless, despite different designs and specific narrow objectives of each of the three projects, they are united by the basic underlying fact that all three projects are studying gene regulations in stomata of *A. thaliana*.

Any disturbance of the gene expression pathways in the same organ/tissue/group of cells of an organism manifests itself as a multi-dimensional distribution of points (gene expression profiles) in a multidimensional space. Some statistical characteristics of these distributions are defined by the functioning of major signal transduction and enzymatic pathways that are kept in this specific organ under any project specific disturbances by the treatments.

As these basic statistical characteristics across the SOC1, SPEECHLESS, and YODA projects, we took directions in the multidimensional distributions (axes through the distribution center) of gene profiles in three projects. With a focus to those axes that are mutually associated, and therefore are independent on particular treatments applied in each individual project: coordinated variability of genes along these axes define common denominators of all three multidimensional distributions (projects) together. We defined the inter-axis association as similarity of the axis values for the same gene after projecting its gene-profiles on each axis separately in project's own multidimensional distributions.

A level of coordination of projections of gene-profiles of the same set of the *Arabidopsis* genes on two centralized axes in two different project-specific multidimensional distributions is evaluated as follows. A list of differences of projection values on two axises for the same genes is prepared as a first step. Next, a segmentation of this profile is performed where the segments enriched by small absolute values are selected (Brodsky et al. 2010). The total statistical significance of these enriched fragments was taken as a measure of association between two axes in two different multidimensional distributions.

Detection of associated centralized axes was performed based on PCA analysis of the project-specific multi-dimensional distributions of gene-profiles for each project separately. Namely, since the principal components (lines) define mutually independent directions of major variations in a multi-dimensional distribution, any axis with a significant variability of gene projections on it is either a PC component, or a linear combination of several PCs with high enough variability of data point projections across the PC line (eigenvalues). Thus, a search for major associated

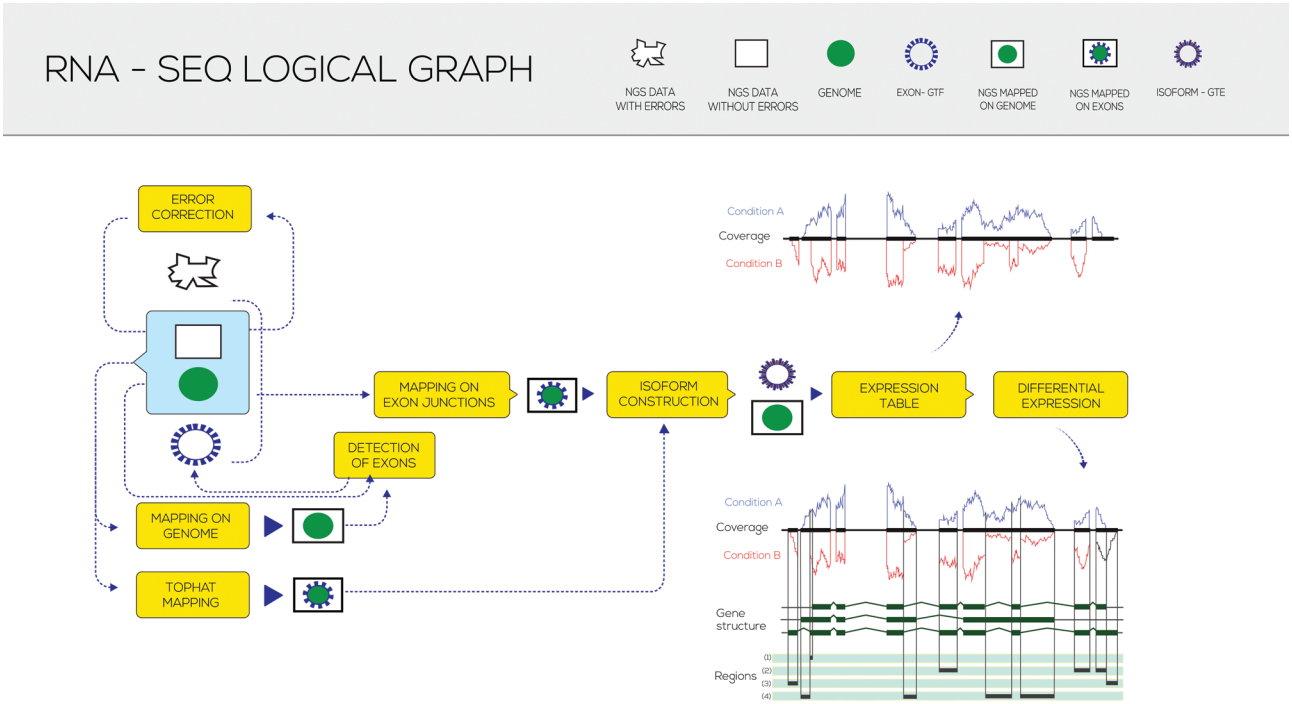

Figure 1 A conceptual scheme of the RNA-seq analysis stages in the T-bioinfo platform

axes can be performed in a combinatorial way, as an optimal pair of two linear combinations of PCs taken from two groups of PCs in two projects that are already one-to-one associated. In this project only individual PCs from different projects were checked on one-to-one associations.

Orchestration of stomata formation and movements by SPEECHLESS – YODA – SOC1 triumvirate

An objective of the integration was to find a set of genes that are shared regulators of both stomatal opening/closing and stomatal formation. We integrated RNA-seq data from all three projects, as well as made all three pairwise integrations. On a level of major regulators (Fig. 2), formation of stomata is controlled by interactions between SPCH, MUTE, FAMA that promote development (Peterson et al. 2010), and TMM, COP1, YODA, which inhibit stomata formation (Serna 2009). A crosstalk between stomata patterning, formation, and movement is highlighted by associations of genes across all three experiments. Participants of particular parts of the process were identified in pairwise associations.

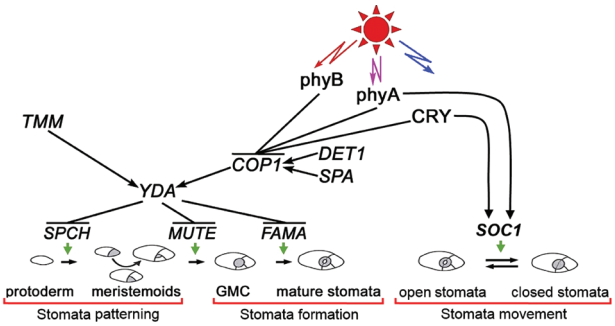

Figure 2 Major regulators of stomata patterning, formation, and movements

Enrichment by stomata-related genes

In the integrated results, identified enrichment of genes that were assigned as having relation to stomata in previous studies (Fig. 3). Enrichment was found for association of the three experiments, as well as for all pairwise integrations. These genes include EPIDERMAL PATTERNING FACTOR 1 (EPF1); GDSL esterase/lipase LTL1; glycine-rich protein gene AT4G29030; F14K14.11; chloroplast-related sulfur transferase STR9 and a few genes belonging to biosynthesis of waxes. As expected, the list of genes associated with stomata contains YODA and other MAPK kinases.

Associations in all three experiments

After the screening, the best on-to-one association of PCs from the SOC1, SPEECHLESS, and YODA projects gave pair associations as follows: (i) SOC1 PC1 – SPEECHLESS PC1; (ii) SOC1 PC1 – YODA PC2; (iii) SPEECHLESS PC1 – YODA PC2. The DAVID NCBI analysis of the matching genes gave the following enriched clusters including a group of WRKY transcription factors, which are regulators of ABA-mediated drought responses (Rushton et al. 2012). Also, a group of MYB (SANT) transcription factors that may be involved in control of stomatal aperture was overrepresented in results (Cominelli et al. 2005).

The list of stomata regulators can be extended by inclusion of genes that share the same GO categories as common regulators. In the integrated data, we identified three groups of genes that are involved in stomata development and functioning: response to red light, suberin biosynthesis, and lipid catabolism and signaling (Fig. 4).

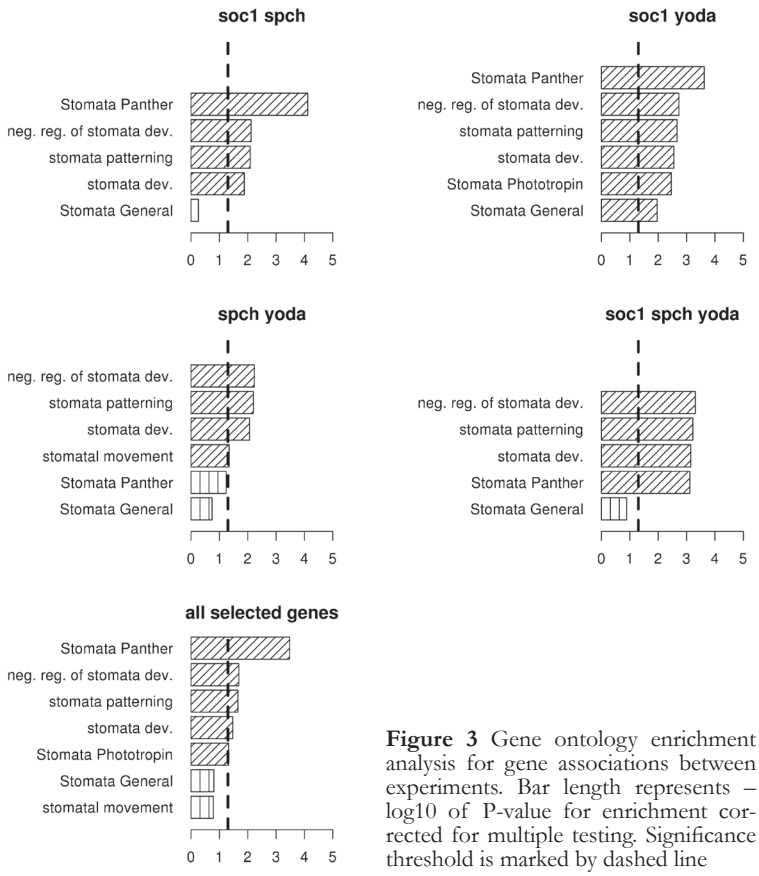

Figure 3 Gene ontology enrichment analysis for gene associations between experiments. Bar length represents – log10 of P-value for enrichment corrected for multiple testing. Significance threshold is marked by dashed line

Ion channels and transporters involved in stomata regulation

We have identified several ion channels and ion transporters that are promising candidates for regulation of stomata formation and, especially, movement. AT4G18290 encodes KAT2, a member of the Shaker family potassium ion (K^+) channel. It is critical to stomatal opening induced by blue light. KAT2 controls circadian rhythm of stomatal opening. It is involved in plant development in response to high light intensity. AT5G46240 (ATKAT1, KAT1) encodes a potassium channel protein. ABA triggers KAT1 endocytosis in epidermal cells, as well as in guard cells. Upon removal of ABA, KAT1 is recycled back to into the plasma membrane.

AT4G19690 (IRON-REGULATED TRANSPORTER 1, IRT1) encodes Fe^{2+} transporter protein. It is a member of the Zrt/Irt-like protein (ZIP) family of transporters. AtIRT1 has broad specificity for divalent heavy metals, mediating the transport of zinc, manganese, cobalt and cadmium under Fe-deficient conditions. IRT1 is monoubiquitinated to promote endocytic trafficking. AT5G50740 belongs to heavy metal transport/detoxification superfamily. This superfamily functions in metal ion binding and metal ion transport.

Genes participating in signal transduction and abiotic stress response

Genes related to stress response appear to be important for stomatal control as well. AT4G18480 is the Mg-protoporphyrin IX methyltransferase that specifically affects ABA signaling in the control of stomatal aperture and have no effect on ABA-induced gene expression (Du et al. 2012). AT4G14480 is a BLUE LIGHT SIGNALING1 (BLUS1). Phosphorylation of BLUS1 kinase by phototropins is a primary step in stomatal opening (Takemiya et al. 2013). AT5G20630 is ARABIDOPSIS THALIANA GERMIN 3, which is involved in the abiotic stress response. AT2G46070 encodes a MAP kinase protein 12; it is highly expressed in guard cells and function as positive regulators of ROS-mediated ABA signaling in guard cells (Jammes et al. 2011).

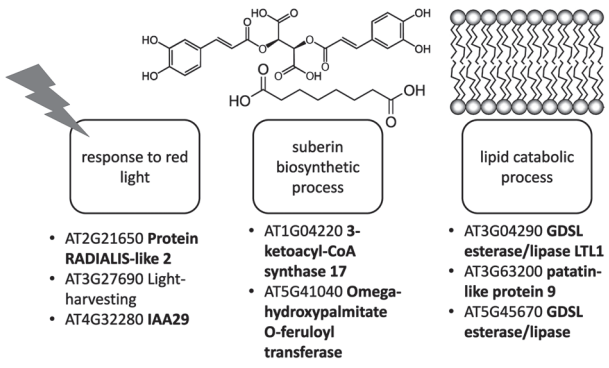

Figure 4 Genes correlated in all three projects

CONCLUSIONS

Integration of RNA-seq data from several projects shed light on the key mechanism underlying processes of stomata patterning, formation, and motion are control by plants. The new algorithm of integration allows for the use of results from very divergent experimental studies, and joining them in order to find a robust core of genes involved in the processes of interest. Our study supports a hypothesis that several types of signals regulate stomata as a whole. On the cell level, the signal is represented by ion current through membrane activating kinase cascade. From the other hand, to pass the information to neighboring cellular environment, cells utilize lipid signaling. We clearly showed that stomata regulation is tightly connected to ABA-associated stress response and light sensing. Both blue and red light sensing systems are important for stomata regulation. Biosynthesis of waxes are associated with stomata development and movements. This process can be co-regulated and involved in production of chemical signals.

Changes in the global environment including rise of CO_2 concentration and temperature will be challenging for land plants. It is expected, that abiotic factors will influence the efficiency of photosynthesis since gas exchange will be limited, as well as the amount of water evaporated from the surface of leaves (Fig. 5). These changes in plant physiology need to be accounted for in models that are used for prediction of global climatic factors.

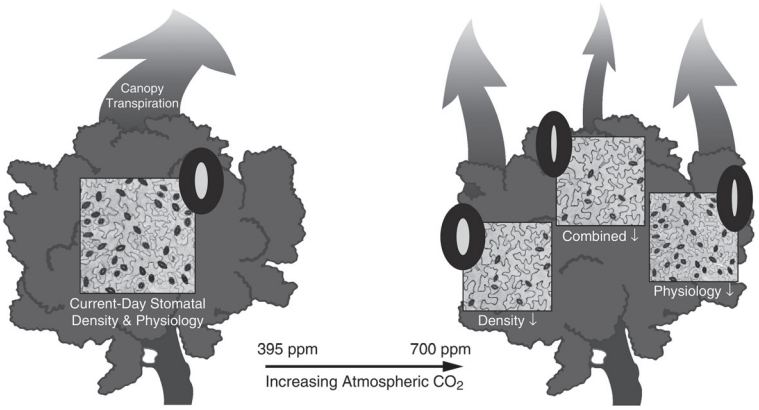

Figure 5 Ongoing changes of CO_2 concentration will influence on both physiology and morphology of plants. It is anticipated that land plants will change rate of photosynthesis and slow down transpiration. Adapted from (Dow & Bergmann 2014)

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Sophia Barinova for initiation the manuscript, many valuable discussions, and ongoing support of the study. We are thankful to members of the Tauber Bioinformatics Research Center Avi Titievsky and Eran Beit Halachmi, and Jaclyn Williams from Pine Biotech for their help with calculations and manuscript preparation.

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Leonid Brodsky:

I met Professor Krassilov for the first time in 2006, at the Institute of Evolution, University of Haifa: I was highly impressed by his lecture on evolution of life from a perspective of the paleontological history of plants at the International Conference on Evolution at the Institute. Indeed, the lecture was about paleobotany, but its most impressive part was his deep thinking on philosophical basis of Evolution. We spoke a bit with Valentin Abramovich during the Conference, and after that I became a frequent visitor of his office at the Institute of Evolution.

Our discussions varied from technical issues of the bioinformatics projects I worked with to the most general ideas about life and human society. Valentin Abramovich was a polymath -- his expertise span almost whole human knowledge: from his professional area of science, evolution, biology, botany and paleontology to philosophy, where he worked intensively and made significant input. The most spectacular was to see how non-orthodox and deep was his thinking about any issue that rose in our conversations. After these discussions I always felt that I am speaking with really great Thinker, one of the Great Minds we only dream to listen to.

Together with Valentin Abramovich we planned several projects, where bioinformatics analysis of data in molecular biology can help in several issues of his interest and of high importance. One of these projects was a project on stomata regulations in relation to climate changes that is presented at this volume. One of my deep sorrows is that I will never listening the thoughtful comments of Valentin Abramovich about the analysis results.