PHENOMICS ANALYSIS

Authors | ORGANIZATION

Helmut Kirchhoff — Washington State University

Ricarda Hoehner — Washington State University

Magnus Wood — Washington State University

Ahmad Zia — Washington State University

Norman Lewis — Washington State University

Barri Herman — Washington State University

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LIST OF ACRONYMS

ADT	arogenate dehydratase
Fv/Fm	maximal photochemical quantum efficiency of PSII,
Phi 2	photochemical quantum efficiency of PSII in the light
PSII	photosystem II
qE	photoprotective high energy quenching
qL	fraction of open PSII centers

EXECUTIVE SUMMARY

The goal of this project was to employ recently developed high-throughput optical screening based on non-invasive image technology (Phenomics) for characterization of plant varieties and mutants used in the NARA consortium. Furthermore, the Phenomics technology was used for identification of plants that either responded stronger or less sensitive to drought stress. Plants with these traits of interest that have been identified by the Phenomics approach can then be used for time- and resource-intensive follow-up omics and biochemical studies. In this way, the Phenomics approach helps efficient development of biofuel crops. Over the entire funding period, measurements were performed with (i) Arabidopsis mutants with reduced lignin contents, (ii) hybrid and transgenic poplars, and (iii) Douglas-fir families (from Oregon State University). In addition, for selected plants, more in-depth biochemical and spectroscopic characterizations were done in the Kirchhoff laboratory. A main part of the project dealt with optimization of experimental conditions for using Phenomics for different trees (poplars and Douglas-fir) since application of this technology was new for these plants. This optimization included finding the right spacing between individual plants in the Phenomics chamber (required for automated image analysis), improving growth conditions for the different species in the chamber (including reduction of infestations by parasites), developing measuring protocols for probing photosynthetic performance (used as screening parameters), identification of the best conditions to induce drought stress, and optimization of computer based image analysis, data extraction, and interpretation. The Phenomics measurements were integrated in other characterizations by the Lewis lab (biochemical measurements of secondary metabolites) and Jayawickrama (developing SNP chips for Douglas-fir). Key results of our studies included: (i) unraveling the impact of reduced lignin content on photosynthetic performance, primary metabolism, and overall plant performance (manuscript re-submitted after revision for Plant Physiology, 5-year impact factor 8.03), (ii) identification of hybrid poplar plants that perform better under drought stress, and (iii) preliminary recommendation of Douglas-fir families for SNP chip development that show differential drought response. During the project, four manuscripts were published and one is under revision (acknowledging NARA support). Furthermore, four congress presentations acknowledged NARA support. Finally, during this project, one postdoctoral scholar was trained in Phenomics technology.

INTRODUCTION

Over the last decade, the Phenomics approach became an essential emerging field in plant biology and agricultural research (Tester and Langridge, 2010; Araus and Cairns, 2014; Fahlgren et al., 2015). This approach allows non-invasive assessment of the plant status and response to different biotic and abiotic stresses over different time periods including developmental studies. In that way, it closes a critical bottleneck of screening a huge number of plants (mutants or germplasm collections) for traits of interest (Tester and Langridge, 2010; Araus and Cairns, 2014). WSU acknowledges the importance of Phenomics for plant biology and agricultural research that is manifested by establishing a full-automated Phenomics facility in 2010 that was one of the first facilities in the US. Over the years, we constantly improved the facility (WSU purchased a Phenomics prototype). The functional plant status is derived from photosynthesis parameters measured by chlorophyll fluorescence (Kramer et al., 2004, Baker, 2008, Brooks and Niyogi, 2011) recorded with an automated image acquisition system combined with a computer controlled camera positioning and lighting system. The WSU Phenomics facility was mainly applied for characterization of Arabidopsis mutants (Puthiyaveetil et al., 2014, Tietz et al., 2015). For the NARA project, the Phenomics approach offered a unique opportunity for efficient and reliable characterization and screening of plant types that were used by the consortium for feedstock production. The first project was studying how reduced lignin levels affected primary plant metabolism and plant performance. For this purpose, arogenate dehydratase Arabidopsis mutants (from Dr. Lewis lab, WSU) were employed that show drastically reduced lignin contents (Corea et al., 2012). In the context of feedstock engineering, it is a central question whether plants can be generated with reduced lignin content since lignin cannot be converted into biofuels and interferes with technical conversion processes (ligno-cellulosic recalcitrant). Furthermore, it binds a significant amount of fixed carbon that is lost for biofuel production. For the second task, hybrid and transgenic poplar lines were analyzed that were designed by the Lewis lab for higher biofuel production (woody biomass) and improved wood quality for technical biofuel generation. The aim was to correlate biomass characteristics of the different poplar lines with plant performance (studied by photosynthetic parameters) and resistance against drought stress. In the third task, the Phenomics approach was used for identification of Douglas-fir families that are either more susceptible of more resistant against drought stress. The aim was to establish a SNP chip (with Dr. Jayawick-rama, Oregon State University) to identify critical genes for drought resistance in Douglas-fir by compare drought robust and sensitive families that were selected by Phenomics.

TASK 1: CHARACTERIZATION OF ARABIDOPSIS AROGENATE DEHYDRATASE (ADT) MUTANTS FOR GROWTH AND PHOTOSYNTHETIC PERFORMANCE PHENOTYPES AND IN-DEPTH FUNCTIONAL CHARACTERIZATION

Task Objective

The aim of Task 1 was to identify the relationship between changes in lignin levels caused by knocking down ADT genes and the efficiency of photosynthetic energy conversion. Since plant metabolism is highly integrated, the question is whether changes in energy demand in an engineered secondary metabolism causes feed-back inhibition in primary (photosynthesis) metabolism. This study will inform about possibilities and limitation of genetic engineering biofuel crops to reduce lignin content.

Methodology

The strongest reduction in lignin content is apparent for the *3456adt* knock-out qua-druple mutant plant. Although other ADT mutants were studied in this project, the following will concentrate on the comparison of the quadruple mutant with wild-type plants. In total, about 160 *3456adt* knock-out mutant and 160 wildtype plants were analyzed in four sets of measurements. Plants grew in the Phenomcis facility (9 hours light per day at 200 micro mol quanta m^{-2} s^{-1}) and were measured once per day in the light and dark respectively for 35-50 days. The following photosynthetic parameters were measured that were used to access plant performance: maximal photochemical quantum efficiency of photosystem II (PSII, Fv/Fm), photochemical quantum efficiency of PSII in the light (Phi 2), photoprotective high energy quench-ing (qE), and the fraction of open PSII centers (qL). These combined parameters gave detailed insight in plant energy metabolism. Growth curves were derived from total leaf projection area determined from chlorophyll fluorescence images (allows easy discrimination between plant and non-plant areas). In addition to Phenomcis studies, selected plants were analyzed in more detailed follow-up measurements in the Kirchhoff lab. Statistical significance was evaluated by student's t-test.

Results

Overall, plant growth, under these unstressed conditions, was similar for *3456adt* knock-out and wildtype plants with a slight but significant retardation in leaf area growth in *3456adt* mutant in the growth phase with highest biomass production. Photosynthetic electron transport was slightly lower (about 5%) in the mutant correlated with a higher energization of the thylakoid membrane (higher proton motive force), higher photoprotective qE, higher ATP/ADP ratio, and higher starch content.

Conclusions/Discussion

The data indicates that reduction of lignin content in Arabidopsis plants has only mild impact on plant growth and photosynthetic performance. The increase in membrane energization, ATP levels, and starch content give conclusive evidence that the consumption of ATP is impaired in *3456adt* knock-out plants, i.e. the altered secondary metabolism in the mutant leads to lower consumption of ATP. This is partially compensated with a higher accumulation of transitory starch in the mutant. Thus, fine-tuning of photosynthetic primary reactions to lowered lignin content is required to optimize growth in lignin-reduced plants. It must be high-lighted that these measurements were performed under green-house conditions for non-stressed plants. It is possible that the small difference in plant performance found in this project could be exaggerated under non-favorable conditions. It is therefore advisable to repeat these studies for stressed plant and include field trials. This work is re-submitted after revision to *Plant Physiology*.

TASK 2: CHARACTERIZATION AND SCREENING HYBRID AND TRANSGENIC POPLAR VARIETIES FOR IMPROVED DROUGHT TOLERANCE

Task Objective
Poplar lines (hybrid and transgenic) were raised and selected by Dr. Barri Herman (WSU Puyallup) for improved biomass and physiochemical wood characteristics. In this task, the growth characteristics and photosynthetic parameters (as indicator of primary metabolism and stress) of these different poplar lines were analyzed to study whether improvements of biomass/wood quality impact the overall plant performance. Furthermore, the impact of drought stress on these lines was studied.

Methodology
Hybrid and transgenic poplar plant lines (from the Lewis lab) were provided by Dr. Barri Herman (WSU Puyallup Research and Extension center). Plants in Pullman were grown in greenhouses of the Lewis lab until they were about 1 m high and then transferred to the Phenomics facility. Photosynthetic parameters as detailed in Task 1 (Methodology) were measured daily over several weeks. One set of experiments were done with hybrid poplar and two sets with hybrid poplar. Six biological replicates per line were measured.

Results
To our knowledge, these types of phenotyping experiments performed in Task 2 with poplar trees have not been reported before. To establish these measurements, the first goal was to optimize Phenomics measurements, tree growth, and drought protocols. A key optimization point was the right spacing between individual trees required for automated tree detection by image analysis software. A main problem with vulnerability of the poplar plants for biotic attacks could be solved. The Phenomics analysis revealed that the photosynthetic performance of four hybrids (A19, A44, 1732-48, P3) were lower than for control trees (OSU alba poplar). Four other hybrids showed higher performance (A16, 2R-35, 1732-85, 1732-84) relative to the control. In the two independent sets for hybrid polar lines, 2R-35, 1732-85, and 1732-84 performed better than average under drought stress. Two of them (1732-85, and 1732-84) also showed higher photosynthetic performance. After spider mite infestation, only 2R-35 performed better.

Conclusions/Discussion
Based on phenotyping characterization, three poplar lines (2R-35, 1732-85, and 1732-84) showed photosynthetic performance that was higher than the average. However, since only two of them also showed increase photosynthetic rates, it is expected that mainly these two (1732-85, and 1732-84) will have increased biomass production. It is now interesting whether these lines will also show higher biomass and/or altered wood properties under field conditions. If yes, the two lines are promising candidates for feedstock production. Interestingly, 2R-35 shows better resistance against biotic stress. This indicates that higher biomass production and resistance against pests are independent traits.

TASK 3: SCREENING DOUGLAS-FIR FAMILIES FOR SNP CHIP DEVELOPMENT

Task Objective

The overarching goal of Task 3 was the generation of a new SNP chip to identify gene patterns that correlate with drought response in Douglas-fir trees. The chip production was organized by other members of the feedstock team. The purpose of the Phenomics measurements was selection of Douglas-fir lines with differential response to drought that can then be used for SNP chip for development.

Methodology

The Douglas-fir trees were organized by Dr. Jayawickrama (Oregon State University). The project had two sub-projects. The first part was a pilot project with a relative small number of plants for coastal and inland trees (44 trees for habitat, respectively). The motivation of the pilot project was identification of conditions in the Phenomics facility to study drought response of Douglas-fir trees. The second, main experiment was a drought response study, done with 98 families and nine individual trees per family, i.e. with 882 trees. The trees were 0.5 to 1 m high and grew in special pots optimized in the pilot run. Before the Phenomics measurements, the trees (shipped from Oregon State by WSU personnel) grew until used in a greenhouse. After transfer to the Phenomics facility, the trees were acclimated for one week under non-stressed conditions. After that, drought was induced by stopping watering for one to two weeks. Due to the high number of trees, the experiment was split into two sets. Photosynthetic parameters as described in Task 1 were measured twice a day (middle of day and end of night period).

Results

The pilot experiment reveals that the spacing of the trees must be optimized for computer-based detection of trees. Furthermore, it turned out that trees must be grown in small pots to accelerate drought response. For the main screening experiment of drought response in Douglas-fir, a new analysis routine was developed based on the photosynthetic Fv/Fm parameter that immediately identified plants that were more susceptible on drought stress or more robust. Based on these screening results, we identified families #11636, # 49614, 31782, and #49012 as more drought resistant than the average and families #8419, #9258, #7950, and #31847 are more sensitive to drought.

Conclusions/Discussion

After optimization of phenotyping screening for differential drought response of Douglas-fir trees, the main experiment identified six families that responded either earlier or later on water shortage. These families can serve as candidates for SNP chip development. However, this should be regarded as preliminary since our experience with plant screening reveals that experiments should be repeated to ensure correct assignments of selected lines.

NARA OUTPUTS

Poster presentations

- 2014: Hoehner, R. 16th International Photosynthesis congress (St. Louis, MO) in the Phenomics symposium, NARA support acknowledged

- 2014: Hoehner, R. Gordon conference on Photosynthesis (West Dover, VT), NARA support acknowledged

- 2013: Hoehner R, Annual NARA meeting: A non-invasive, large-scale screening of Arabidopsis lignin mutants reveals significant changes in Photosynthesis, NARA support acknowledged

Publications

R. Höhner, J.V. Marques, T. Ito, Y. Amakura, A.D. Budgeon, Jr., L.B. Davin, H. Kirchhoff, N.G. Lewis, Modulating arogenate dehydratase gene expression in *Arabidopsis thaliana*: Photosynthetic energy conversion and metabolism ramifications. *Plant Physiology under revision*. (NARA support acknowledged)

H. Kirchhoff and R. Yarbrough, Evaluation of lipids for the study of photosynthetic membranes. In: Photosynthesis: Methods and Protocols (Ed. S. Covshoff), Springer Press, accepted (NARA support acknowledged)

H. Kirchhoff, M. Li, S. Puthiyaveetil (2017) Sublocalization of cytochrome b6f complexes in photosynthetic membranes. *Trends Plants Science*, in press (NARA support acknowledged)

H. Kirchhoff (2014) Diffusion of molecules and macromolecules in thylakoid membranes. Biochimica et Biophysica Acta 1837: 495–502 (NARA support acknowledged)

H. Kirchhoff (2013) Structural changes of the thylakoid membrane network induced by high light stress in plant chloroplasts. Phil. Trans. R. Soc. B 369: 20130225 (NARA support acknowledged)

NARA OUTCOMES

Change in knowledge

The project laid the foundation for applying non-invasive optical Phenomics to screen young trees. During the project, measurement protocols were refined in particular to screen for drought response. For both tree types that were studied in this project (poplar and Douglas-fir), lines and families were identified that show differences in overall (photosynthetic) performance and responses to drought stress. This knowledge can be used for improvements of biofuel prospects and for development of SNP chips that identifies differential gene expression associated with drought stress (Douglas-fir). Furthermore, results on ADT mutants indicate that engineering of secondary metabolic pathways (lignin) requires co-engineering of primary metabolism (photosynthesis).

Change in action

To further improve lignin-reduced mutants, co-engineering of photosynthetic processes is advisable. The data indicate that in ADT mutants, fixed carbon is transitorily stored as starch. Redirecting this stored carbon into biomass would significantly improve the value of lignin-reduced biofuel prospects. The identified poplar and Douglas-fir lines that reveal differential response to drought stress should be further used in follow-up performance studies under field conditions. This could be combined with photosynthetic measurements. Also, follow-up studies in the Phenomics facility are recommended for further validation (Douglas-fir) and identification of new lines.

NARA
Northwest Advanced Renewables Alliance

FUTURE DEVELOPMENT

Future Development

The outcomes of this NARA sub-project demonstrated that high-throughput Phenomics is a valuable tool for optimizing feedstock development for biofuel production. A prerequisite is careful optimization of growth and screening conditions that should be adjusted individually for different plant species (e.g. drought response is different in different species). Used properly, Phenomics can give clear advice about what plants should be used for more detailed, time-consuming, and expensive follow-up studies. Therefore, tight integration into other approaches is necessary to make biofuel optimization by using Phenomics successful. In that sense, the Douglas-fir data should be regarded as preliminary and require further experimental validation by Phenomics measurements.

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