Final Technical Report

Project Title: Towards a map of the Populus biomass protein-protein interaction network

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Project overview

Wood (secondary xylem) comprises the vast majority of stable biomass produced by forest trees. The development of secondary xylem in models such as Arabidopsis and poplar has been the subject of intensive investigation in recent years. While there is a large body of published work devoted to documenting the transcriptomes of differentiating xylem cells, relatively little is known about the protein-protein interactions necessary for wood formation. The main objectives of this project were to 1) identify new protein-protein interactions relevant to wood formation, and 2) perform in-depth characterizations of selected protein-protein interactions.

For the first main objective, to identify relevant protein-protein interactions, we cloned a set of approximately 400 genes that were highly expressed in the wood-forming tissue of poplar (*Populus trichocarpa*). We tested whether the proteins encoded by these biomass genes interacted with each other in a binary matrix design using the yeast two-hybrid (Y2H) method for protein-protein interaction discovery. We also tested a subset of the 400 biomass proteins for interactions with all proteins present in wood-forming tissue of poplar in a biomass cDNA library screen design using Y2H. Together, these two Y2H screens yielded over 270 interactions involving over 75 biomass proteins.

For the second main objective, we selected several interacting pairs or complexes of interacting proteins for in-depth characterizations. Characterizations involved both in vivo and in vitro independent methods such as pull-downs, co-immunoprecipitations, and bimolecular fluorescence complementation (BiFC) assays to confirm protein-protein interactions. We also evaluated phenotypes of transgenic poplar and Arabidopsis plants engineered for increased or decreased expression of the selected genes coding for interacting proteins. Transgenic poplar trees were studied in growth chamber, greenhouse, and two separate replicated field trials involving over 25 distinct wood-associated proteins. In-depth characterizations yielding positive novel results include the following. First, a NAC domain transcription factor (NAC154) that is a promoter of stress response and dormancy in trees was discovered. Increasing expression of NAC154 caused stunted growth and premature senescence, while decreasing expression led to both delayed bud and leaf expansion in spring and delayed leaf drop (i.e., prolonged leaf retention) in fall. Second, we discovered and characterized a new connection between a negative regulator of wood formation, the NAC domain transcription factor XND1, and an important regulator of cell division and cell differentiation, RETINOBLASTOMA-RELATED (RBR). Third, we identified a new network of interacting wood-associated transcription factors belonging to the MYB and HD families. One of the HD family proteins, WOX13c, was used to prepare transgenic poplar for high-level expression, resulting in significantly increased lateral branch growth. Finally, we modeled and performed in vitro analyses of the insect protein rubber resilin and we prepared transgenic Arabidopsis plants for expression of resilin to test the feasibility of using resilin to modify lignin cross-linking in wood and reduce recalcitrance and improve yield of fermentable sugars for biofuels production. Analysis of these and additional transgenics created with this support is continuing.

Published products and manuscripts in revision or in preparation that were made possible by and acknowledge funding from FG02-07ER64449

1. Zhao C, T Lasses, L Bako, D Kong, B Zhao, B Chanda, A Cruz- Ramírez, B Scheres, AM Brunner, EP Beers (2016) XYLEM NAC DOMAIN1: A potential innovation regulating xylem differentiation in angiosperms through multiple conserved linear motifs interacting with RETINOBLASTOMA-RELATED. In preparation

Abstract

In Arabidopsis, NAC domain transcription factors VASCULAR NAC DOMAIN6 (VND6) and VND7 promote xylem vessel differentiation. *XYLEM NAC DOMAIN1* (*XND1*) is a direct target of VND7. We previously showed that overexpression of *XND1* repressed the final stages of xylem vessel differentiation (secondary cell wall deposition and programmed cell death). Here we report that *XND1* and its orthologs are evident only in angiosperms and that the highly conserved 32-amino-acid C-terminal region of XND1 is comprised of multiple linear motifs for interaction with the cell cycle and differentiation regulator RETINOBLASTOMA RELATED (RBR). Deletion of the overlapping LXCXE/E2F transcriptional activation domain-like or LXCXE-mimic motifs eliminated or reduced, respectively, the XND1-RBR interaction, and deletion of all three RBR-interaction motifs abolished XND1 function. Overexpression of an XND1-RBR fusion where full-length RBR was substituted for the C-terminal RBR-binding domain of XND1 phenocopied a weak XND1 overexpression phenotype. Moreover, the dominant-negative activity of N-terminal NAC domains of VND6 and VND7 was greatly enhanced by fusion with the C-terminal domain of XND1. Together these findings indicate that *XND1* is an angiosperm innovation for expanding the VND7-dependent gene regulatory network to include RBR-mediated negative regulation of cell wall biosynthesis and programmed cell death during xylem vessel differentiation.

Relevance to FG02-07ER64449

We previously published work showing that XND1 is a negative regulator of wood formation. This manuscript builds on this earlier work by characterizing the role of the RBR-XND1 interaction in regulation of wood formation.

2. Petzold HE, B Chanda, C Zhao, AW Dickerman, AM Brunner, EP Beers (2016) DIVARICATA AND RADIALIS INTERACTING FACTOR (DRIF) also interacts with WOX, and KNOX proteins associated with wood formation in *Populus trichocarpa*. In preparation

Abstract

DIVARICATA AND RADIALIS INTERACTING FACTOR (DRIF) from snapdragon (*Antirrhinum majus*) is a MYB/SANT protein that interacts with related MYB/SANT proteins, RADIALIS and DIVARICATA, through its N-terminal MYB/SANT domain. In addition to the MYB/SANT domain, DRIF contains a C-terminal Domain of Unknown Function (DUF3755). Here we describe novel protein-protein interactions involving a poplar (*Populus trichocarpa*) homolog of DRIF, PotriDRIF1. In addition to interacting with poplar homologs of RADIALIS (PotriRAD1) and DIVARICATA (PotriDIV4), PotriDRIF1 interacted with members of other families within the homeodomain-like superfamily, including PotriWOX13c, a WUSCHEL-RELATED HOMEOBOX protein, and PotriKNAT7, a KNOTTED1-LIKE HOMEOBOX protein. PotriRAD1 and PotriDIV4 interacted with the MYB/SANT-containing N-terminal portion PotriDRIF1, while DUF3755 was both necessary and sufficient for interactions with PotriWOX13c and PotriKNAT7. Of the two MYB/SANT domains present in PotriDIV4, only the N-terminal MYB/SANT domain interacted with PotriDRIF1. GFP-PotriDRIF1 expressed alone or with PotriRAD1 localized to the cytoplasm, while co-expression of GFP-PotriDRIF1 with PotriDIV4, PotriWOX13c, or PotriKNAT7 resulted in nuclear localization of GFP-PotriDRIF1. Modified yeast two-hybrid and BiFC experiments using PotriDRIF1 as a bridge protein revealed that PotriDRIF1 simultaneously interacted with PotriRAD1 and PotriWOX13c but could not form a heterotrimeric complex when PotriKNAT7 was substituted for PotriWOX13c or when PotriDIV4 was substituted for PotriRAD1. The discovery of an additional protein-protein interaction domain in DRIF proteins, DUF3755, and its ability to form heterodimers and heterotrimers involving MYB/SANT and wood-associated homeodomain proteins implicates DRIF proteins as mediators of a broader array of processes than previously reported.

Relevance to FG02-07ER64449

All of the interactions described in this manuscript were discovered from our initial Y2H assays of wood-associated proteins. This is the first report showing that DRIF proteins can interact with an expanded range of members from homeodomain-like superfamily, including MYB and HD proteins. One of these HD proteins, PotriWOX13c, causes increased growth of lateral branches when overexpressed. This latter finding is currently being intensively characterized.

3. Khandaker MSK, DM Dudek, EP Beers, DA Dillard, DR Bevan (2016) Molecular Modeling of Repeating Motifs of Disordered Elastomeric Proteins. In preparation, awaiting submission by corresponding author Bevan.

Abstract

Elastomeric proteins can be found in a wide range of living organisms, and structural disorder is one of the critical features of these proteins. About 40% of highly structured proteins contain disordered domains. Despite their ubiquity, there is little understanding about the mechanisms responsible for the mechanical properties of these disordered elastomeric proteins. To better understand the structure-property relationship between elastomeric behavior and their amino acid sequence, we investigate the repetitive motifs from different species using molecular dynamics (MD) simulations. Repeating motifs are considered from elastin: VPGVG, resilin: exon-3: GYSGGRPG, dragline spider silk: GGYGPGS, flagelliform silk: GPGGY and mussel byssus: GPGGG. The results after clustering analyses show that all the motifs adopt a bent structure, presumably through a combined effect of intramolecular hydrogen bonding and lack of steric hindrance. These hydrogen bonds within the motifs apparently play a role in maintaining the bent conformation. During SMD pulling of these motifs, the hydrogen bonds break and they reform again when the peptides are released to move freely, returning to similar bent conformations. Adhikary et al. and Takano et al. also observed intramolecular hydrogen bonds which stabilizing the protein conformations. Moreover, our experimental work on recombinant resilin and other work on natural resilin of dragonflies and cockroaches suggested that the measured transitions could potentially be within the rubbery plateau and due to intramolecular hydrogen bonds, though the transitions might also due to true glass transitions or altered structural organization.

Relevance to FG02-07ER64449

This manuscript expands our modeling efforts beyond resilin to include other elastomeric proteins. As high expression of glycine-rich disordered proteins is a common feature of xylem, such studies can be used to develop hypotheses about the role of these proteins and how to exploit their properties for wood modification.

4. Khandaker MSK, DM Dudek, EP Beers, DA Dillard (2016) Expression, crosslinking and developing modulus master curves of recombinant resilin. In revision for Journal of Biomechanical Behavior of Biomedical Materials

Abstract

Resilin is a disordered elastomeric protein found in specialized regions of insect cuticles, where low stiffness and high resilience are required. It has a wide range of functions and varies with the insect species – operating across a wide frequency range from 5 Hz for locomotion to 13 kHz for sound production. We synthesize a recombinant resilin from clone-1 (exon-1 + exon-2) of the gene, and determine the water content and dynamic mechanical properties, along with estimating surface energies relevant for adhesion. The recombinant resilin-like hydrogel has 80wt% water and does not exhibit tack even though it satisfies the Dahlquist criterion. Finally, dynamic moduli master curves have been developed by applying the time-temperature superposition principle (TTSP) and time-temperature concentration superposition principle (TTCSP), and compared with the reported master curves for natural resilin from locusts, dragonflies and cockroaches. The resulting master curves show that the synthetic resilin undergoes a pronounced transition with increasing ethanol concentrations, with the storage modulus increasing by approximately three orders of magnitude. Although possibly a glass transition, alternate explanations include the formation of intramolecular hydrogen bonds or that the chitin binding domain (ChBD) in exon 2 might change the secondary structure of the normally disordered exon 1 into more ordered conformations that limit deformation. All these possibilities have been reported in the literature for polymeric materials.

Relevance to FG02-07ER64449

This manuscript continues our analysis of resilin properties by expanding our efforts to include examination of dynamic mechanical properties of recombinant resilin.

5. Khandaker MSK, DM Dudek, EP Beers, DA Dillard, DR Bevan (2016) Molecular modeling of the elastomeric properties of repeating units and building blocks of resilin, a disordered elastic protein. Journal of Biomechanical Behavior of Biomedical Materials 61: 110-121. Doi:10.1016/j.jmbbm.2016.01.017

Abstract

The mechanisms responsible for the properties of disordered elastomeric proteins are not well known. To better understand the relationship between elastomeric behavior and amino acid sequence, we investigated resilin, a disordered rubber-like protein, found in specialized regions of the cuticle of insects. Resilin of *Drosophila melanogaster* contains Gly-rich repetitive motifs comprised of the amino acids, PSSSYGAPGGGNGGR, which confer elastic properties to resilin. The repetitive motifs of insect resilin can be divided into smaller partially conserved building blocks: PSS, SYGAP, GGGN and GGR. Using molecular dynamics (MD) simulations, we studied the relative roles of SYGAP, and its less common variants SYSAP and TYGAP, on the elastomeric properties of resilin. Results showed that SYGAP adopts a bent structure that is one-half to one-third the end-to-end length of the other motifs having an equal number of amino acids but containing SYSAP or TYGAP substituted for SYGAP. The bent structure of SYGAP forms due to conformational freedom of glycine, and hydrogen bonding within the motif apparently plays a role in maintaining this conformation. These structural features of SYGAP result in higher extensibility compared to other motifs, which may contribute to elastic properties at the macroscopic level. Overall, the results are consistent with a role for the SYGAP building block in the elastomeric properties of these disordered proteins. What we learned from simulating the repetitive motifs of resilin may be applicable to the biology and mechanics of other elastomeric biomaterials, and may provide us the deeper understanding of their unique properties.

Relevance to FG02-07ER64449

In this publication we are modeling the mechanism responsible for elastomeric proteins as a prelude to using such proteins to modify wood quality in transgenic plants.

6. Jia X, B Chanda, M Zhao, AM Brunner, EP Beers (2015) Instability of the Arabidopsis mutant csn5a-2 caused by epigenetic modification of intronic T-DNA. Plant Science 238:53-63. Doi: 10.1016/j.plantsci.2015.05.015

Abstract

T-DNA insertion mutants play a crucial role in elucidating Arabidopsis gene function. In some cases, two or more T-DNA mutants are combined to study genetic interactions between homologous genes or genes hypothesized to act in the same pathway. We studied the significance of protein–protein interactions between CSN5A and ROP11 by crossing three independent rop11 T-DNA insertion mutants with csn5a-2, a partial loss-of-function intronic T-DNA insertion mutant. The csn5a-2 single mutant is severely stunted, but double rop11 csn5a-2mutants were rescued and exhibited increased CSN5A transcript and protein levels. The rescued phenotype was maintained in non-Mendelian fashion when the csn5a-2 single mutant was re-isolated from the rop11-1 csn5a-2 double mutant, and was sensitive to two inhibitors of DNA methylation. Loss of kanamycin resistance was also observed in re-isolated csn5a-2. These findings indicate that the rescue of csn5a-2 resulted from a trans T-DNA-mediated epigenetic effect on the csn5a-2 intronic T-DNA, similar to recent reports involving the intronic T-DNA mutants ag-TD, ben1-1, and cob-6. Thus the work reported here provides further support for the recommendation that mutants created through novel combinations of T-DNA alleles should be carefully evaluated for evidence of epigenetic modification of T-DNA before final conclusions are drawn.

Relevance to FG02-07ER64449

We discovered an interaction between poplar CSN5A and poplar ROP proteins. To expediently characterize this interaction, we used loss-of-function alleles in Arabidopsis. An epigenetic silencing effect was observed and is described here mainly as a cautionary report to benefit other members of the plant science community.

7. Jervis J, SB Hildreth, X Sheng, EP Beers, AM Brunner, RF Helm (2015) A metabolomic assessment of *NAC154* transcription factor overexpression in field grown poplar stem wood. Phytochemistry 15:112-120. Doi: 10.1016/j.phytochem.2015.02.013

Abstract

Several xylem-associated regulatory genes have been identified that control processes associated with wood formation in poplar. Prominent among these are the NAC domain transcription factors (NACs). Here, the putative involvement of *Populus NAC154*, a co-ortholog of the Arabidopsis gene *SND2*, was evaluated as a regulator of "secondary" biosynthetic processes in stem internode tissues by interrogating aqueous methanolic extracts from control and transgenic trees. Comprehensive untargeted metabolite profiling was accomplished with a liquid chromatography–mass spectrometry platform that utilized two different chromatographic supports (HILIC and reversed phase) and both positive and negative ionization modes. Evaluation of current and previous year tissues provided datasets for assessing the effects of *NAC154* overexpression in wood maturation processes. Phenolic glycoside levels as well as those of oligolignols, sucrose and arginine were modulated with phenotypic and chemotypic traits exhibiting similar trends. Specifically, increased levels of arginine in the *NAC154* overexpressing tissues supports a role for the transcription factor in senescence/dormancy-associated processes.

Relevance to FG02-07ER64449

This publication describes the impact on transgenic tree growth due to overexpression of one of the wood-associated NAC transcription factors, NAC154, first described by Grant et al., 2010, an earlier publication supported by this project and listed below in this report.

8. Liu LJ, M Zinkgraf, HE Petzold, EP Beers, V Filkov, A Groover (2015) The Populus ARBORKNOX1 homeodomain transcription factor regulates woody growth through binding to evolutionarily conserved target genes of diverse function. New Phytologist 205: 682-694. doi: 10.1111/nph.13151

Abstract

The class I KNOX homeodomain transcription factor ARBORKNOX1 (ARK1) is a key regulator of vascular cambium maintenance and cell differentiation in Populus. Currently, basic information is lacking concerning the distribution, functional characteristics, and evolution of ARK1 binding in the Populus genome. Here, we used chromatin immunoprecipitation sequencing (ChIP-seq) technology to identify ARK1 binding loci genome-wide in Populus. Computational analyses evaluated the distribution of ARK1 binding loci, the function of genes associated with bound loci, the effect of ARK1 binding on transcript levels, and evolutionary conservation of ARK1 binding loci. ARK1 binds to thousands of loci, which are highly enriched proximal to the transcriptional start sites of genes of diverse functions. ARK1 target genes are significantly enriched in paralogs derived from the whole-genome salicoid duplication event. Both ARK1 and a maize (Zea mays) homolog, KNOTTED1, preferentially target evolutionarily conserved genes. However, only a small portion of ARK1 target genes are significantly differentially expressed in an ARK1 over-expression mutant. This study describes the functional characteristics and evolution of DNA binding by a transcription factor in an undomesticated tree, revealing complexities similar to those shown for transcription factors in model animal species.

Relevance to FG02-07ER64449

Seven biomass transcription factors cloned for this project were used in a yeast one-hybrid assay to identify transcription factors that bind to PopREVOLUTA promoter. The results are shown in Figure 5b of this publication.

9. Petzold HE, M Zhao, EP Beers (2012) Expression and functions of proteases in vascular tissues. Physiologia Plantarum 145: 121-129. doi:10.1111/j.1399-3054.2011.01538.x

Abstract

With the emergence of new models for wood formation and the increasing emphasis on improving the efficiency of cellulosic biofuel production, research on vascular tissue biology has intensified in recent years. Some of the most active areas of research focus on manipulating activity of enzymes in the cellulose, hemicellulose, pectin and lignin pathways. In addition, great strides have been made in the characterization of transcriptional networks controlling genes that affect differentiation, secondary cell wall synthesis and programmed cell death in xylem. Less attention has been devoted to the characterization of proteases that may be important regulators of post-translational events that affect vascular cell differentiation and function and cell wall composition. Several genes for proteases and components of the ubiquitin/26S proteasome pathway are upregulated in xylem and phloem and in cell culture systems for studying the differentiation of xylem tracheary elements (TEs). Although small molecule protease inhibitors have been used to explore the roles of proteases during the differentiation of cultured TEs, only a small number of vascular tissue-associated protease genes have been directly tested to determine whether they play roles in vascular tissue biology. In this report, we review roles for proteases in vascular cell differentiation and function as determined through the use of protease inhibitors and genetic analyses and conclude by identifying opportunities for future research in this area.

Relevance to FG02-07ER64449

A review that focuses on the state of knowledge of proteases as they impact wood formation and the opportunity for future research in this area.

10. Zhao C, A Hanada, S Yamaguchi, Y Kamiya, EP Beers (2011) The Arabidopsis Myb genes MYR1 and MYR2 are redundant negative regulators of flowering time under decreased light intensity. Plant Journal 66: 502-515. doi: 10.1111/j.1365-313X.2011.04508.x

Abstract

Changes in the duration, quality and intensity of light affect flowering time. Compared with the effects of light duration and quality, less is known about the effects of light intensity on flowering. Here we describe two paralogous single Myb domain genes, MYB-RELATED PROTEIN 1 (MYR1) and MYB-RELATED PROTEIN 2 (MYR2), and their roles as repressors of responses to decreased light intensity in Arabidopsis. Homozygous myr1 myr2 double mutants flowered early under low light intensities. Additionally, myr1 myr2 mutants exhibited increases in petiole length, leaf angle and apical dominance. Genetic analyses involving mutants in the long-day, gibberellin (GA) and phyB flowering pathways indicated that all aspects of the myr1 myr2 phenotype required GA biosynthesis. The early-flowering phenotype of myr1 myr2 also required FLOWERING LOCUS T, and myr1 myr2 mutants showed an epistatic interaction with the phyB-9 mutant. Over-expression of MYR1 or MYR2 produced GA-deficiency symptoms that were rescued by application of gibberellic acid (GA_3). Loss of MYR1 and MYR2 function was associated with a twofold increase in GA20ox2 expression and a 30% increase in GA_4 levels, while over-expression of MYR2 led to a threefold decrease in GA20ox2 expression and a 50% decrease in GA_4 levels. Considered together, these results suggest that the ability of MYR1 and MYR2 to repress flowering and organ elongation is at least partly due to their negative effect on levels of bioactive GA.

Relevance to FG02-07ER64449

The MYB genes characterized in this report also interact with one of the wood-associated MYB genes, PotriRAD1, the subject of another manuscript listed above in this report.

11. Grant EH, T Fujino EP Beers AM Brunner (2010) Characterization of NAC domain transcription factors implicated in control of vascular cell differentiation in Arabidopsis and Populus. Planta 232: 337-352. Doi: 10.1007/s00425-010-1181-2

Abstract

Wood has a wide variety of uses and is arguably the most important renewable raw material. The composition of xylem cell types in wood determines the utility of different types of wood for distinct commercial applications. Using expression profiling and phylogenetic analysis, we identified many xylem-associated regulatory genes that may control the differentiation of cells involved in wood formation in Arabidopsis and poplar. Prominent among these are NAC domain transcription factors (NACs). We studied NACs with putative involvement as negative (XND1 from Arabidopsis and its poplar orthologs PopNAC118, PopNAC122, PopNAC128, PopNAC129), or positive (SND2 and SND3 from Arabidopsis and their poplar orthologs PopNAC105, PopNAC154, PopNAC156, PopNAC157) regulators of secondary cell wall synthesis. Using quantitative PCR and in situ hybridization, we evaluated expression of these Populus NACs in a developmental gradient and in association with reaction wood and found that representatives from both groups were associated with wood-forming tissue and phloem fibers. Additionally, XND1 orthologs were expressed in mesophyll cells of developing leaves. We prepared transgenic Arabidopsis and poplar plants for overexpression of selected NACs. XND1 overexpression in poplar resulted in severe stunting. Additionally, poplar XND1 overexpressors lacked phloem fibers and showed reductions in cell size and number, vessel number, and frequency of rays in the xylem. Overexpression of PopNAC122, an XND1 ortholog, yielded an analogous phenotype in Arabidopsis. Overexpression of PopNAC154 in poplar reduced height growth and increased the relative proportion of bark versus xylem.

Relevance to FG02-07ER64449

This is the first published characterization of poplar XND1 and NAC154, both of which are the subjects of additional publications and manuscripts listed in this report.