Title
Glycobiology in yeast: production of bio-ative biopolymers and small molecules:

Author
Scheller, Henrik

Publication Date
2014-04-30

a5. Qualitative Report of Grant from the Strategic Research Council

Glycobiology in yeast

Vanillin and gum Arabic are the high value glycosylated bio-molecules and reach market values of US$ 100-200 mio. However, since both are natural products, unstable supply and high cost have been a problem. Therefore, the project aimed to establish sustainable production of vanillin and gum Arabic-variant in yeast. To achieve the goal, the major challenges were i) to optimize the vanillin biosynthetic pathway by introducing vanillin glucosyltransferase and by flux control, and ii) to identify glycosyltransferases involved in the biosynthesis of gum Arabic glycan (arabinogalactan) and to construct the pathway in yeast.

Vanillin

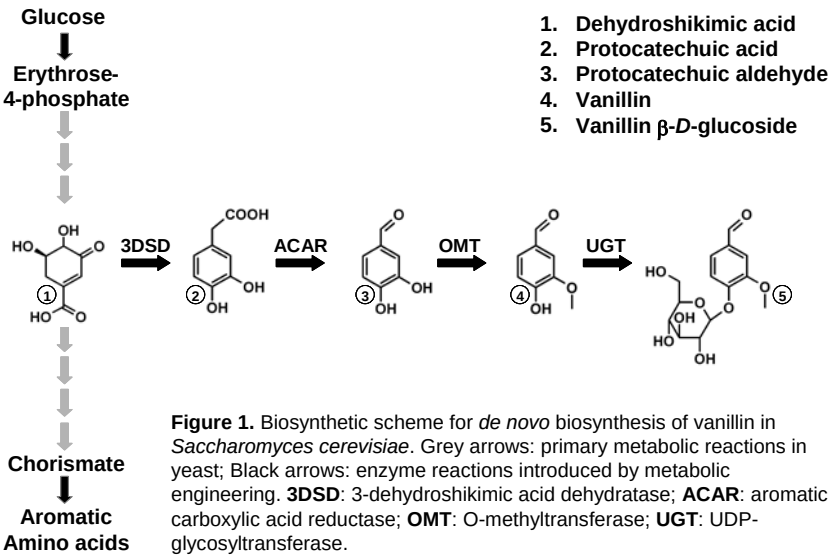
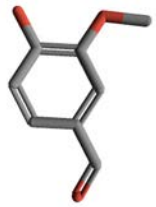


Figure 1. Biosynthetic scheme for *de novo* biosynthesis of vanillin in *Saccharomyces cerevisiae*. Grey arrows: primary metabolic reactions in yeast; Black arrows: enzyme reactions introduced by metabolic engineering. **3DSD**: 3-dehydroshikimic acid dehydratase; **ACAR**: aromatic carboxylic acid reductase; **OMT**: O-methyltransferase; **UGT**: UDP-glycosyltransferase.

The vanillin part of the project has progressed according to the plan and progresses have been made on most deliverables. Deliverable 6 and 7 concern the construction and testing of mutants of certain genes in the precursor pathways in yeast, specifically the DAHP synthase (the first committed enzyme in the aromatic amino acid biosynthesis pathway) and Aro1p, a penta-functional enzyme delivering the actual precursor for the introduced vanillin pathway (Hansen et al., 2009). Due to the fact that precursor levels earlier in the project were not limiting to vanillin and vanillin glucoside production, these strategies were postponed. A study comparing the two most commonly used yeast based cell factories for their potential to make vanillin glucoside surprisingly demonstrated that vanillin glucoside yields vary up to ten-fold between the two backgrounds. The study therefore shows that it is important to test different genetic backgrounds for their production potential early during cell factory construction (paper submitted to Metabolic Engineering). During these past year, bottlenecks in the vanillin glucoside pathway were partly alleviated, which made it possible resume the precursor boost strategies of deliverable 6 and 7. Both the mutant DAHP synthase and ARO1 strategies were tested in high producing vanillin glucoside strains and analysed using fed batch fermentations. Both strategies appeared to work, and precursor levels were increased up to 100% (Brochado et al., 2010). To exploit this new high precursor availability, a renewed focus was put on deliverable 8 and 9, which involves improving the vanillin pathway by using alternative enzymes or enzyme scaffolding.

For deliverable 8 we identified several highly active OMT enzymes that can be used in the vanillin pathway.



Vanillin structure, cited from Science News 2009

For deliverable 9 we have also tested an advanced overexpression system designed to coordinate expression in complex pathways. For example, we have constructed a strain that harbours a single copy vanillin pathway. This strain can now be combined with a mating type alpha strain containing multiple (up to 10) copies of selected genes in the vanillin pathway. Importantly, all genes are integrated in the genome at defined loci. In this way gene dosages have been titrated to optimize vanillin production. Similarly, we have constructed a diploid strain with 17 copies of the entire vanillin pathway. In both cases, we have obtained significantly higher yields.

Variation of expression levels at each step in the vanillin pathway (varying gene copy number and promoter strength) helped to increase vanillin glucoside production titers and productivities significantly, to a level where scale-up to commercial production can be initiated. We therefore consider the final deliverable 11 to be achieved, and even with a performance that exceeds the original expectations.

For deliverable 10, we have tested a number of beta-glucosidases, and we have managed to identify one enzyme that is a particular enzyme that is highly active in cleaving vanillin glucoside. The enzyme can be expressed and even secreted in yeast, which would be desirable for use in the vanillin downstream process. Upscaling and feasibility study for using this enzyme in the process are in the planning phase.

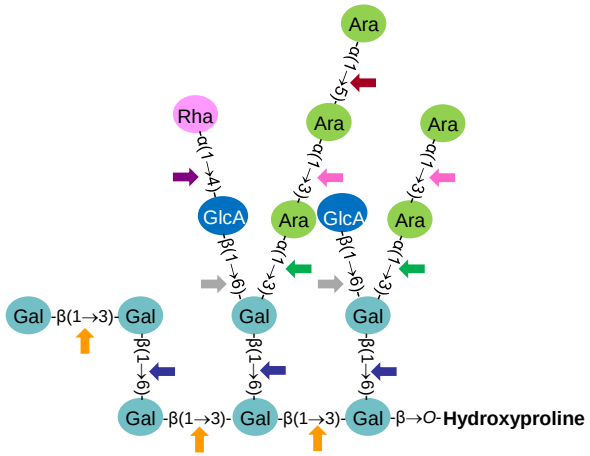
In addition, the pathway for synthesis of vanillin in the Vanilla orchid has been elucidated (Gallage et al. 2014). To identify genes and enzymes involved in vanillin glucoside biosynthesis in *V. planifolia*, a combination of transcriptomic and proteomic approaches were undertaken with an initial focus on candidates representing the five major enzyme families suggested from the literature to play a possible role in vanillin biosynthesis, namely the PAL, Cytochrome P450s (C4H and C3H), OMTs, UGTs and the carbon chain shortening enzyme *Vp*VAN. A review of the entire vanillin project by Gallage and Møller has been accepted for publication in Molecular Plant following minor revision.

For the deliverable 3, 4, they have been completed, which was stated in previous reports.

As mentioned the vanillin project has led to a yeast strain capable of producing levels of vanillin suitable for commercial production. This strain is now being scaled up, into production size fermenters and down-stream process to purify the vanillin is being developed. This will eventually mean that the vanillin will come on the market. Initially it will be marketed as natural vanillin in EU, while it with further development and optimisation may also go on market globally and compete with various other lower priced products. The research project to develop this strain is therefore looking to also become a commercial success.

Arabinogalactan proteins (AGPs)

The project has progressed according to the plan. For the gene discovery part of the gum Arabic (AGP) glycosylation, we identified four novel glycosyltransferases. We have biochemically characterized the enzymes expressed heterologously (deliverable 16), which resulted in the several international peer-reviewed articles (Geshi et al., 2013)(Knoch et al., 2013)(Dilokpimol et al., 2014)(Dilokpimol and Geshi, 2014). Due to the detection limit, we could not identify the peptides in isolated protein complexes as we originally planned (deliverable 15), but instead, we performed heterologous co-expression of candidate partner proteins and demonstrated interaction by FRET. Furthermore, we demonstrated the significance of protein interactions in the increase of the enzyme activity. The result has been published in (Dilokpimol et al., 2014). Also, we found that the arabinogalactan glycosyltransferases target to an unknown subcellular compartment partially overlapped with unconventional protein secretion pathway when expressed heterologously (Poulsen et al., 2014). Further investigation of the targeting signals etc is important for the construct of the pathway in a heterologous host such as in yeast.



Proposed primary structure of arabinogalactan attached to hydroxyproline. GTs illustrated in different colors are required to synthesize the entire structure.

Further, we identified that AtGlcAT14A (Knoch et al., 2013) adds extra glucuronic acids to gum Arabic and improves the performance of the gum (deliverable 20). We have tested the emulsifier property using the method described in (Heidebach et al., 2013) and found that the enzyme-modified gum Arabic is superior than the unmodified gum in the formation of oil-in-water emulsions (Dilokpimol and Geshi, manuscript submitted). This result implicates the synthesis of even superior gum Arabic-variant by overexpressing glucuronosyltransferase in future.

For the gene discovery effort, we also performed a RNA sequencing analysis of AGP gum secretion by the New Zealand Puka plant, *Meryta sinclairii*. Through analysis of this transcriptome we have identified candidate glycosyltransferase-like genes that are highly up-regulated during gum secretion and are predicted to possess appropriate activities for AGP glycan biosynthesis. Previously identified genes known to be involved in production of AGPs and AGP peptide encoding genes were significantly up-regulated in the transcriptome data. A specific class of proline-4-hydroxylase was upregulated as well as *Meryta* orthologs of the hydroxyproline galactosyl-transferase, AtGalT31A, AtGlcAT14, and AtGalT29A. Thus the study was successful in capturing the transcriptome of tissues over-producing AGPs. Further analysis identified previously un-known candidates for enzymes involved in the production of AGP glycans. We silenced orthologs of these new candidate enzymes in *Nicotiana benthamiana* to test their involvement in the production of AGP glycans (deliverable 16). To date we have silenced 22 candidate genes. Three candidate genes from distinct, GT-like gene families have significant effects on total cell wall composition when silenced in *N. benthamiana* (See Figure 2B). Immunological analysis showed changes in AGP glycan content in plants silenced for two of these candidate genes. Further work with stable Arabidopsis mutants and heterologous expression of these genes will determine the specific roles of these genes in AGP glycan biosynthesis.

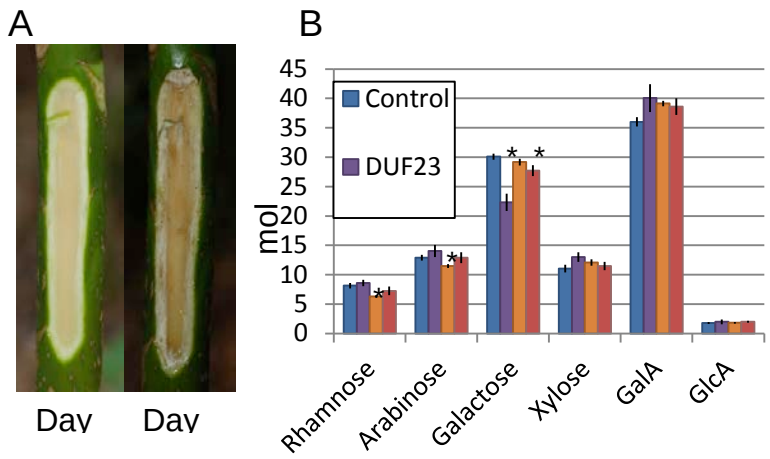


Figure 2: Wounding of *Meryta sinclairii* induces secretion of an arabinogalactan gum (A). Gum secreting (Day5) and non-secreting (Day1) tissues were used to prepare RNA sequencing libraries for the identification of candidate genes involved in the production of AGP glycans. (B) Monosaccharide composition analysis of cell walls from *N. benthamiana* plants silenced for AGP-glycan biosynthetic genes shows reductions in arabinose and galactose content. *: p<0.001.

For deliverable 12, 13 and 18, we had completed the yeast strain carrying GAGP peptide, epimerase, UDP-Gal transporter, At4PH1 in yeast *S. cerevisiae*. We have isolated the expressed GAGP peptide and analyzed proline hydroxylation using ESI-MS (deliverable 18). We have made DNA constructs for newly identified glycosyltransferases mentioned above as well as recently reported hydroxylproline galactosyltransferase (Basu et al., 2013, JBC) and transformed to the yeast strain described above (deliverable 17).

Report other than scientific outcomes

The collaboration among different partners has in this project, been very fruitful, and the project has benefitted immensely from the interdisciplinary expertise and experiences from the partners. The US partners made multiple visits to Denmark to discuss progress and to conduct joint research. Likewise, several Danish participants visited the US partner, especially during a joint Berkeley-Denmark Synthetic Biology Workshop held in August 2011. A joint meeting in Berkeley has also been scheduled in November 2014 to maintain and further strengthen the positive international collaboration. The project has also resulted in many publications in the high impact peer-reviewed journals, two patents, and two very successful PhD theses, from Tomas Strucko (Graduated April 25th 2014) and Nethaji Gallage (Graduated June 17th 2014).

Task	Year 1				Year 2				Year 3				Year 4			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Specific Goal 1: Flux analysis of vanillin biosynthesis pathway & identification of metabolic engineering target																
1.1 Identification of target genes		1														
Specific Goal 2: Flux-improved vanillin yeast strain construction																
2.1 Construct and characterize (fluxome & transcriptome) yeast mutants as predicted by flux models										2						
Specific Goal 3: Vanillin glycosyl- and methyltransferase discovery and construction																
3.1 Screen with Family 1 UGT enzyme library to identify optimal enzyme				3												
3.2 Test available methyltransfearses to identify optimal enzyme						4										
3.3 Construct and test UGT mutants and/or hybrids (optional)								5								
Specific Goal 4: Shikimate pathway deregulation																
4.1 Test feedback-resistant DAHP synthase genes								6								
4.2 Construct and test Aro1p enzyme mutants (precursor containment)								7								
Specific Goal 5: Watchmaker-based vanillin gene combination optimization																
5.1 Test analogues of vanillin biosynthesis pathway steps to find optimal combination												8				
Specific Goal 6: Synthetic enzymology for increased intermediate availability																
6.1 Employ protein-based system to "scaffold" enzymes in the vanillin pathway												9				
6.2 A protein-based glycosyltransferase-scaffold library for optimizing detoxification of metabolites (vanillin will serve as model system)												9				
Specific Goal 7: Vanillin product harvest strategies																
7.1 Identify efficient beta-glucosidase for conversion of vanillin glucside to vanillin									10							
7.2 Test a selection of C18 resin systems for vanillin adsorption											11					
OVERALL GOAL: Yeast producing vanillin glucoside or vanillin in commercially attractive amounts																11
Specific Goal 8: Construction of a yeast strain producing GAGP carrying single Gal																
8.1 Construct for yeast expression		12														
8.2 Yeast transformation and product analysis				13												
Specific Goal 9: gene discovery for GAGP glycosylation																
9.1 HTP screening pf membrane-bound GTs								14								
9.2 Co-expression database search																
9.3 GT complex isolation and peptide sequencing								15								
9.4 Heterologous expression and enzyme characterization												16				
Specific Goal 10: construct yeast strain with newly identified GTs																
10.1 Construction for yeast expression, transformation														17		
Specific Goal 11: Characterization of recombinant GAGP																
11.1 Purification, structural analysis of recombinant GAGP														18		
11.2 Product characterization with SAXS, AFM, and TEM														19		
11.3 Functional assays including emulgator test																20

References

Hansen et al., (2009) De novo biosynthesis of Vanillin in fission yeast (Schizosaccharomyces pombe) and baker's yeast (Saccharoyces cerevisiae). Applied Environmental Microbiology 75: 2765-2774

Brochado et al., (2010) Improved vanilin production in baker's yeast through in silico design. Microbial Cell Factories 9: 84-

Gallage et al., (2014). Vanillin formation from ferulic acid in Vanilla planifolia is catalysed by a single enzyme. Nature Communications 5, doi:10.1038/ncomms503

Nethaji J Gallage, PhD thesis with a title of "Elucidation of the Vanillin Biosynthetic pathway in Vanilla planifolia"

Tomas Struckos, PhD thesis with a title of "Synthetic yeast based cell factories for vanillin-glucoside production"

WO/2013/022881, COMPOSITIONS AND METHODS FOR THE BIOSYNTHESIS OF VANILLIN OR VANILLIN BETA-D-GLUCOSIDE

WO/2014/067534, VANILLIN SYNTHASE

Dilokpimol, A., and Geshi, N. (2014). Arabidopsis thaliana glucuronosyltransferase in family GT14. *Plant Signal. Behav.* 9, e28891.

Dilokpimol, A., Poulsen, C. P., Vereb, G., Kaneko, S., Schulz, A., and Geshi, N. (2014). Galactosyltransferases from Arabidopsis thaliana in the biosynthesis of type II arabinogalactan: molecular interaction enhances enzyme activity. *BMC Plant Biol.* 14, DOI: 10.1186/1471–2229–14–90.

Geshi, N., Johansen, J. N., Dilokpimol, A., Rolland, A., Belcram, K., Verger, S., Kotake, T., Tsumuraya, Y., Kaneko, S., Tryfona, T., et al. (2013). A galactosyltransferase acting on arabinogalactan protein glycans is essential for embryo development in Arabidopsis. *Plant J.* 76, 128–137.

Heidebach, T., Sass, M., and de With, A. (2013). Enzymatic treatment of gum arabic. WO2013091799 A1.

Knoch, E., Dilokpimol, A., Tryfona, T., Poulsen, C. P., Xiong, G., Harholt, J., Petersen, B. L., Ulvskov, P., Hadi, M. Z., Kotake, T., et al. (2013). A β-glucuronosyltransferase from Arabidopsis thaliana involved in biosynthesis of type II arabinogalactan has a role in cell elongation during seedling growth. *Plant J.* 76, 1016–29.

Poulsen, C. P., Dilokpimol, A., Mouille, G., Burow, M., and Geshi, N. (2014). Arabinogalactan glycosyltransferases target to a unique subcellular compartment that may function in unconventional secretion in plants. *Traffic*, accepted, doi:10.1111/tra.12203.

DISCLAIMER