Novel plants optimised for lignin, growth, and biofuel production via re-mutagenesis and co-expression analysis

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Abstract

The objectives of this project were to find novel genes that might be useful for optimising plant biomass to facilitate biofuel production. Since the phenolic polymer, lignin, surrounds and cross-links to other cell wall components, reducing the amount of lignin or altering its structure to make it easier to extract have become major targets for improving sugar release from plant biomass (saccharification). Previous published work from our lab and others has shown that mutation in lignin biosynthesis genes can improve saccharification but there are sometimes costs to plant growth and mutant plants can be stunted. This project was designed to (1) determine whether greater improvements in saccharification could be achieved by stacking new mutations on top of lignin biosynthesis gene mutations; (2) explore whether the growth defect in some lignin mutants could be repaired by secondary mutations; and (3) discover new genes involved in lignin biosynthesis that could be manipulated to improve saccharification. Several approaches were used towards these goals. Firstly, mutagenic screens were performed to find mutants that improved the saccharification potential of existing monolignol biosynthesis mutants. One screen involved EMS mutagenesis of a weak *Arabidopsis ref3* mutant in the lignin biosynthesis gene *C4H* that already had a small reduction in lignin with no major growth defect [1] and some increase in saccharification. In order to find re-mutagenised plants with further increases in saccharification potential due to a second mutation, a high-throughput saccharification assay at the University of York was used to screen progeny families of the mutagenized *ref3* population [2]. Using this screen, 26 potential high saccharification and 30 potential low saccharification mutants were identified. These potential mutants were then re-screened in replicate using the same saccharification assay. Out of the 26 potential high saccharification mutants, seven had significantly increased release of sugars compared to the *ref3* mutant upon re-screening. Many of these mutants had reduced lignin levels and most were mutations in different positions within a single gene that has been mapped and identified using next generation sequencing (NGS). The mapped mutants provide evidence that it is possible to stack two genes, one in the lignin biosynthesis gene *C4H* and another on an interconnected pathway, to optimise sugar release. We know from other work that we have been involved in that this is quite a novel discovery as it is difficult to find combinations of genes that act additively or synergistically to enhance sugar release without

adversely impacting on plant growth. This work is currently being written up for publication. One of the low saccharification mutants from this screen has also been mapped and will be the subject of an additional paper.

In a second, distinct, screen, 43 mutants were identified that rescued the reduced size phenotype of the Arabidopsis *irx4* mutant in the lignin biosynthesis gene *CCR*. This mutant has reduced lignin and releases more sugar upon saccharification of stem tissue, but has a severe growth defect [3]. This founder mutant was treated with EMS and 80,000 plants from the resulting M_2 generation were screened for increased plant size. Of 71 mutants originally identified, 43 reproducibly rescued the plant size defect of the founder mutant on repeated screening. A large portion of these restored mutants still retain significant reductions in lignin and a corresponding increase in sugar release upon saccharification. Many are mutants in the same second gene. One of these mutants was selected for mutation mapping to identify the causal mutation and we hope that this work will soon be the subject of a publication.

A third approach that we used to identify novel genes involved in lignin synthesis and regulation was co-expression analysis. Over 65 mutants in genes identified as being co-expressed with lignin biosynthesis genes were characterized in a variety of assays. Two of these mutants were shown to contain both reduced lignin and elevated sugar release in our saccharification assay. One of these was extensively studied (in collaboration with Wout Boerjan's group (VIB, Gent) who we discovered were working on a similar mutant). The gene mutated in this mutant encodes an enzyme that had no previously known connection to lignin biosynthesis. The mutant deposits 36% less lignin than wild-type plants, and the composition of the polymer is also altered with H units increased over 30-fold (analysis performed by John Ralph, University of Madison). The high levels of H units in the mutant suggested that the normal gene is active in the lignin biosynthetic pathway at some point after the branch leading to H units but before G and S units are produced. Using phenolic metabolite profiling, our collaborators determined that the lignin pathway intermediate caffeoyl shikimate accumulated in the mutant, suggesting it may be the substrate for the enzyme encoded by the (mutant) gene. The recombinant wild-type enzyme was, indeed, able to hydrolyze caffeoyl shikimate into caffeate and shikimate and so we called the enzyme caffeoyl shikimate esterase, the product of the *CSE* gene. The cellulose-to-glucose conversion of stems from the *cse* mutants without pretreatment released 75% more glucose per plant than wild-type plants, illustrating the potential value of the discovery for improving biomass processing. By manipulating the *CSE* gene, it may be possible to release sugars more efficiently for fuel production from crops such as switchgrass, poplar and eucalyptus that all appear to have similar *CSE* genes. A patent [4] and a scientific paper [5] have been published on this work, and it generated a lot of interest and additional commentary articles in the media [6] on publication in *Science* magazine.

Finally, we have also used protein complex purification to identify interacting partners of lignin biosynthesis proteins in the hope of identifying novel genes involved in lignification. A known lignin biosynthesis enzyme, C4H, and the CSE enzyme were used as baits for tagged purification strategies and several potentially interacting proteins were identified that require further study.

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Publications and Patents

1. Vanholme, R., Cesarino, I., Rataj, K., Xiao, Y., Sundin, L., Goeminne, G., Kim, H., Cross, J., Morreel, K., Araujo, P., Welsh, L., Haustraete, J., McClellan, C., Vanholme, B., Ralph, J., Simpson, G.G., Halpin, C*., and Boerjan, W*. Caffeoyl Shikimate Esterase (CSE) is an Enzyme in the Lignin Biosynthetic Pathway in Arabidopsis. **Science** 341:1103-6, 2013. *joint corresponding/senior authors.
2. Bao, Z., Benson, S.M., Cui, Y., Dionne, J.A., Maher, K., Boerjan, W., Halpin, C., Nelson, R., Nichols, D., Ralph, J., and Ramakrishnan, T.S. In Search of Clean, Affordable Energy. Oilfield Review 26(1), 4-15, 2014.
3. Published patent: Halpin, C., Simpson, G.G. and Boerjan, W. (2013) Modified Plants PCT/GB2013/051206.

Presentations (a selection only)

1. Talk: Claire Halpin 'Spinning straw into gold' at the International Bioenergy Conference, Manchester, March 2014.
2. Talk: Claire Halpin 'Tailoring biomass feedstocks for biorefineries' at the Lignin Meeting, University of Warwick, March 2014.
3. Talk: Claire Halpin 'Barley, biofuels and biosequestration' at the University of Edinburgh, June 2013.
4. Talk: Claire Halpin 'Tailoring biomass feedstocks for biorefineries' at BBSRC-organised workshop on the ABC of Biofuels, Buenos Aires, Argentina, Dec 2013.
5. Talk: Claire Halpin 'Spinning straw into gold: Barley, biofuels and biosequestration' at retreat of the ARC Centre of Excellence in Plant Cell Walls, Maroochydore, Australia May 2013.
6. Talk: Claire Halpin at UKPlantSci 2013, Dundee 'Spinning straw into gold: Barley, biofuels and biosequestration' April 2013
7. Talk: Claire Halpin at Monogram Network Meeting 'Improving barley straw for bioenergy' March 2012
8. Talk: Chris McClellan at UK PlantSci 2012, Norwich, UK: 'Improving plants for the production of 2nd generation biofuels,' 18 April 2012
9. Poster: Kasia Rataj at 23rd International Conference on Arabidopsis Research, Vienna,
10. Austria: 'Optimization of lignin content in Arabidopsis to facilitate biofuel production' 3-7 July 2012
11. Talk: Claire Halpin 'Tailoring Plant Biomass for Bioenergy Applications by Manipulating Lignin' at the GCEP symposium, Palo Alto, USA, Sept 2012.
12. Poster: Chris McClellan at UK PlantSci 2012, Norwich, UK: 'Genetic screening for improved saccharification properties in *Arabidopsis thaliana*' 18-19 April 2012
13. Talk: Claire Halpin at The RCUK (Research Councils United Kingdom) Review of Energy (October 2010). This review was organised by the EPSRC on behalf of all UK Research Councils and in conjunction with learned societies to provide an independent assessment of the quality and impact of UK energy research.
14. Talk: Claire Halpin at the BBSRC/South East Asia Workshop on Biofuels (February 2011, Hanoi, Vietnam)
15. Talk: Claire Halpin at the College of Life Sciences, University of Dundee, Annual Symposium, Crieff Hydro Hotel, Scotland, March 2011)
16. Talk: Claire Halpin at the GCEP symposium, Palo Alto, USA, Sept 2010.
17. Talk: Claire Halpin "Lignin biosynthesis and manipulation" at the Gordon Conference on "Cellulosomes, Cellulases & Other Carbohydrate Modifying Enzymes" July 26-31, 2009, Proctor Academy, Andover, NH.
18. Talk: Claire Halpin "Barley Straw – A fuel for the Future?" at the BCPC09 (British Crop Production Council Congress 2009), November 2009, Glasgow, UK.

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2. Gomez, L.D., C. Whitehead, A. Barakate, C. Halpin, and S.J. McQueen-Mason, Automated saccharification assay for determination of digestibility in plant materials, Biotechnology for Biofuels, 3, 23, 2010.
3. Mir Derikvand, M., J.B. Sierra, K. Ruel, B. Pollet, C.T. Do, J. Thévenin, D. Buffard, L. Jouanin, and C. Lapierre, Redirection of the phenylpropanoid pathway to feruloyl malate in *Arabidopsis* mutants deficient for cinnamoyl-CoA reductase 1, Planta, 227, 943-956, 2008.
4. Published patent: Halpin, C., Simpson, G.G. and Boerjan, W. (2013) Modified Plants PCT/GB2013/051206.
5. Vanholme, R., Cesarino, I., Rataj, K., Xiao, Y., Sundin, L., Goeminne, G., Kim, H., Cross, J., Morreel, K., Araujo, P., Welsh, L., Haustraete, J., McClellan, C., Vanholme, B., Ralph, J., Simpson, G.G., Halpin, C*., and Boerjan, W*. Caffeoyl Shikimate Esterase (CSE) is an Enzyme in the Lignin Biosynthetic Pathway in Arabidopsis. **Science** 341:1103-6, 2013. *joint corresponding/senior authors.
6. August 2013 on publication of Science paper subsequent publicity included: 4 radio interviews by Claire Halpin plus 1 local TV news plus many short articles on the web and in scientific or popular press including:
    a. Stecker, T. and ClimateWire. New Enzyme May Lead to Cheaper Biofuels. In Scientific American. http://www.scientificamerican.com/article/new-enzyme-may-lead-to-cheaper-biofuels/
    b. Everts, S. New Way To Reduce Lignin Output. Chemical and Engineering News 91(33) p7. http://cen.acs.org/articles/91/i33/New-Way-Reduce-Lignin-Output.html
    c. Service, R.F. Want better biofuels, get wood out! Science Now. http://news.sciencemag.org/biology/2013/08/want-better-biofuels-get-wood-out
    d. Bullis, K. A Novel Way to Cut the Cost of Advanced Biofuels. MIT Technology Review. http://www.technologyreview.com/news/518061/a-novel-way-to-cut-the-cost-of-advanced-biofuels/
    e. New Possibilities for Efficient Biofuel Production. Science Daily. http://www.sciencedaily.com/releases/2013/08/130815145034.ht
    f. Byrne, M. The Key To Making More Efficient Biofuels Is Taking the Wood Out of Wood. http://motherboard.vice.com/blog/the-key-to-making-more-efficient-biofuel-is-taking-the-wood-out-of-wood
    g. Stecker, T. New enzyme discovery could lead to cheaper cellulosic fuel. http://www.eenews.net/stories/1059986088/print
    h. Dundee Researchers In Biofuel Production Move. TayAM
    i. Christie, L. Plant find could boost biofuel push. Herald Sun (Australia). http://www.heraldsun.com.au/news/breaking-news/plant-find-could-boost-biofuel-push/story-fni0xqll-1226698983590
    j. Pultarova, T. Genetically modified plants for better biofuel. Engineering and Technology Magazine
    k. Watson, J. Scientists hail new lignin biofuel breakthrough. Energy Voice
    l. New Component of Lignin Biosynthesis Pathway Suggests Possible Biofuel Strategy. Genome Web Daily News. http://www.genomeweb.com/new-component-lignin-biosynthesis-pathway-suggests-possible-biofuel-strategy
    m. Discovery of new lignin biosynthetic pathway could aid biofuel production. Green Car Congress. http://www.greencarcongress.com/2013/08/20130816-cse.html
    n. Christie, L. Finding may boost biofuels. IrishExaminer.com

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