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 are to find novel genes that will be useful for optimising plant biomass to facilitate biofuel production. These include genes influencing saccharification (the release of sugars from biomass), genes involved in the synthesis or control of lignin, and genes that restore normal growth to lignin mutants. Several approaches are being used towards this goal. Firstly, mutagenic screens to find mutants which improve the saccharification potential of existing monolignol biosynthesis mutants have been performed. After re-screening mutants initially identified as having increased saccharification, seven mutants have been isolated which significantly increase the release of sugars. Many of these mutants have reduced lignin levels, and one has already been through mutation mapping by next generation sequencing (NGS) while others are being prepared for mapping. The mapped mutant provides evidence that it is possible to stack two genes, one a gene of lignin biosynthesis and the other an identified gene on an interconnected pathway, to optimise sugar release. In another screen, 43 mutants have been identified that rescue the reduced size phenotype of a lignin biosnthesis mutant. A large portion of these restored expression mutants still contain significant reductions in lignin and a corresponding increase in sugar release upon saccharification. One of these mutants has been selected for mutation mapping to identify the causal mutation. A second approach used to identify novel genes involved in lignin synthesis and regulation is co-expression analysis. Over 65 mutants in genes identified as co-expressed with lignin biosynthesis genes have been analysed. Two of these mutants have been shown to contain reduced lignin and elevated sugar release in the saccharification assay, one of which has been rescued by over-expression of the targeted gene. Protein complex purification and identification has also been used to identify interacting partners of lignin biosynthesis proteins. A known lignin biosynthesis enzyme and a protein identified from the co-expression analysis have been used as baits for tagged purification strategies. Further work involves mapping more mutants from the large-scale screens and characterization of the gene isolated by co-expression analysis.

Introduction

The aim of this project is to identify novel genes involved in limiting the sugar release from plant cell walls, particularly genes involved in lignin deposition by screening for plants that have improved saccharification properties or improved

growth characteristics. Novel genes will be identified through mutagenesis of existing lignin-defective mutants, as well as screening plants defective in genes that are co-expressed with known lignin biosynthetic genes. Further analysis of lignin-related proteins will be undertaken by isolating interaction partners of known monolignol biosynthesis enzymes and these interaction partners will be identified through liquid chromatography/mass spectroscopy analysis. Genes identified with these strategies will be good candidates for manipulation in crops used in biofuel production.

Background

Many recent advances have shown the potential for improving biofuel production. Since the phenolic polymer lignin coats is cross-linked to other cell wall components, the reduction or alteration of this compound has long been a target for improving sugar release from cell walls. It has recently been shown that disrupting the lignin biosynthesis gene *CAD1* causes improved saccharification in *Brachypodium distachyon* [1]. In addition to disrupting known lignin genes, the engineering of plants to produce altered lignin structure has proved effective at increasing saccharification. *Arabidopsis* plants engineered to incorporate hydroxybenzaldehydes into lignin rather than the usual hydroxycinnamates have shown improvements in saccharification [2]. Additionally, an engineered enzyme which methylates lignin precursors, making it more difficult for lignin polymerization to occur, increases the digestion efficiency of cell walls in plants expressing this enzyme [3]. Enzymes outside of lignin biosynthesis are possible targets as well, since overexpression of a BAHD acyltransferase increases *p*-coumarate incorporation into cell wall polysaccharides and improves saccharification [4]. However, more research is needed to obtain the necessary range and specificity of mutants for saccharification engineering. To find novel genes involved in lignin biosynthesis, we are using mutagenic screens of known lignin mutants, an approach that so far, is unique and promises to yield genes not known to be involved in lignin biosynthesis. We are also using co-expression analysis on specific pathways and processes using publicly available, high-quality data sets from microarray expression experiments. Previously, co-expression experiments have been used successfully to explore novel genes involved in some cell wall pathways and processes [5,6]. Recently, it has been found that two monolignol biosynthesis proteins, C3H and C4H interact at the endoplasmic reticulum membrane, and may interact with two other monolignol biosynthesis proteins, HCT and 4CL [7,8]. However, protein interactions beyond the known monolignol biosynthesis proteins are not well studied. We are using protein complex purification to potentially identify novel gene products involved in lignin biosynthesis.

Results

Mutant Screening

Two main approaches have been used in the model plant *Arabidopsis thaliana* to find novel genes involved in improved biofuel production. The first approach, a forward genetics approach, uses genetic screening to identify mutants which are more desirable for bioethanol production. Two mutagenic screens have been performed. In both cases, a mutant, which has a defect in one of the monolignol biosynthesis genes, was mutagenised with the aim of finding new mutations which improve the original line's ability to be converted to bioethanol.

One mutagenic screen involves screening for saccharification improvement in a mutant which already has a small improvement in this phenotype. The mutant used for this screen is *ref3*, which is mutated in the *C4H* gene in *Arabidopsis*. This allele has small reductions in lignin and no visible plant growth phenotypes [9]. The mutant also has an increase of sugar release upon saccharification. It was hypothesized that further increases in saccharification potential could be found without affecting plant growth. To find such plants, a high-throughput saccharification assay was used to screen a mutagenized *ref3* population [10]. This population was generated by treating *ref3* with ethyl methanesulfonate (EMS), a mutagen, and stem material from each member of the M_2 screening population was collected. Stem material was then ground to powder for use in the saccharification screen. 20,000 M_2 plants were grown for the screen, but plant death and a lack of sufficient stem material for some plants led to only 10,000 plants being used in the screen.

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 were determined to be significantly different from the background upon re-screening (Figure 1).

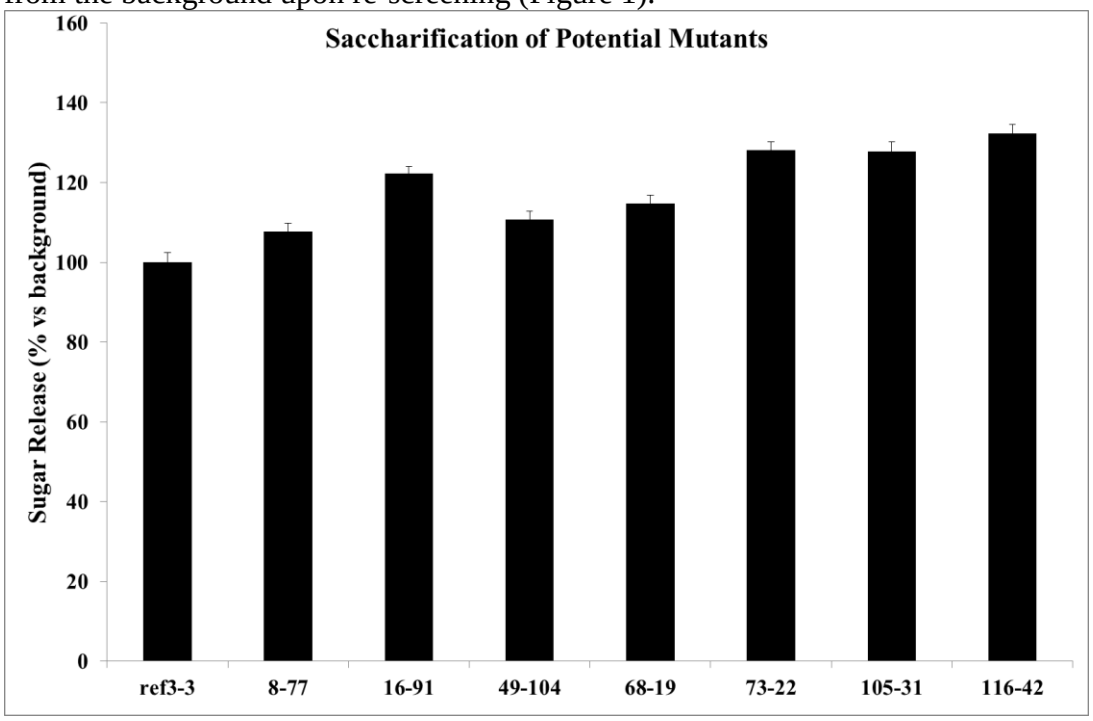


Figure 1 – *ref3* enhancer mutants show increases in sugar release upon saccharification. *Arabidopsis* plants were grown for twelve weeks, and stems were collected and ground to powder. Powder was used in the high-throughput saccharification assay developed by Gomez *et al.* [10]. Each data point represents four technical replicates of four biological replicates. Error bars represent standard error of the mean.

Since saccharification can be affected by changes in lignin levels, the acetyl bromide assay was used to assess lignin in these mutants (Figure 2). Many of the mutants also displayed reductions in lignin level.

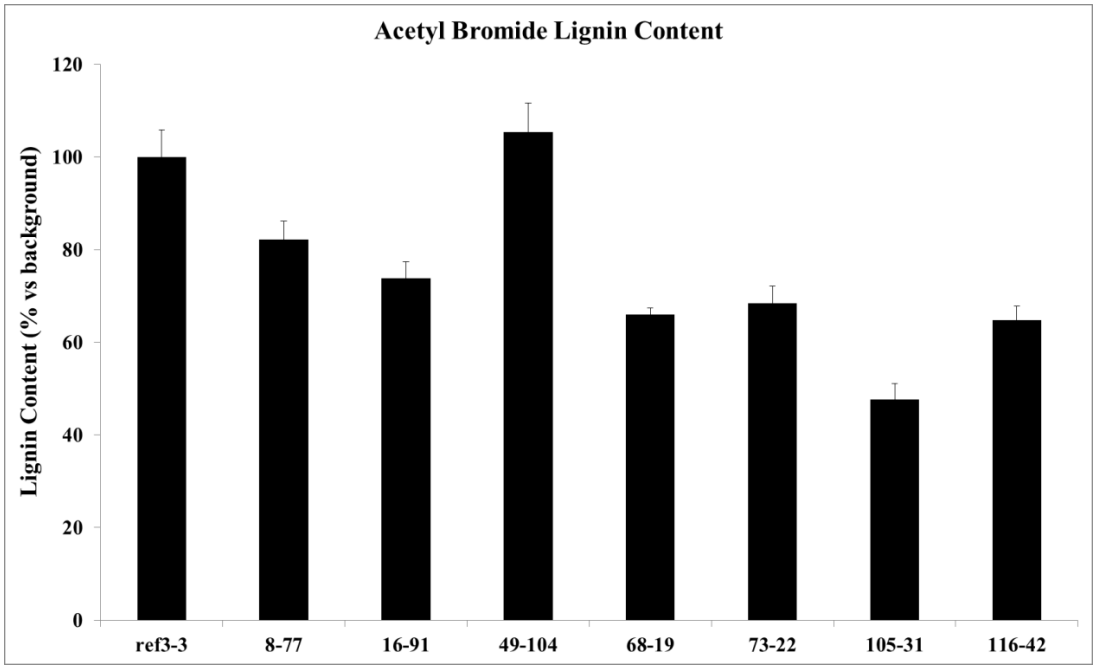


Figure 2 – *ref3-3* enhancer mutants show reductions in lignin content. *Arabidopsis* plants were grown for twelve weeks and stems were ground to powder. Powder was extracted four times with 80% ethanol and once with chloroform:methanol (2:1 v/v). Extracted powder was used in the acetyl bromide assay [11]. Data represents three technical replicates of two biological replicates. Error bars represent standard error of the mean.

Due to its increase in saccharification and reduction in lignin, the 16-91 mutant was chosen for further analysis. Stem sections from the 16-91 mutant were stained with a reagent to detect lignin (Figure 3). Interestingly, the 16-91 shows a marked decrease in phloroglucinol staining, an indication of decreased lignin content in the xylem of 16-91 mutant stems. The 16-91 enhancer mutant also displays a collapsed xylem phenotype (Figure 3), a phenotype indicative of cell wall compositional defects.

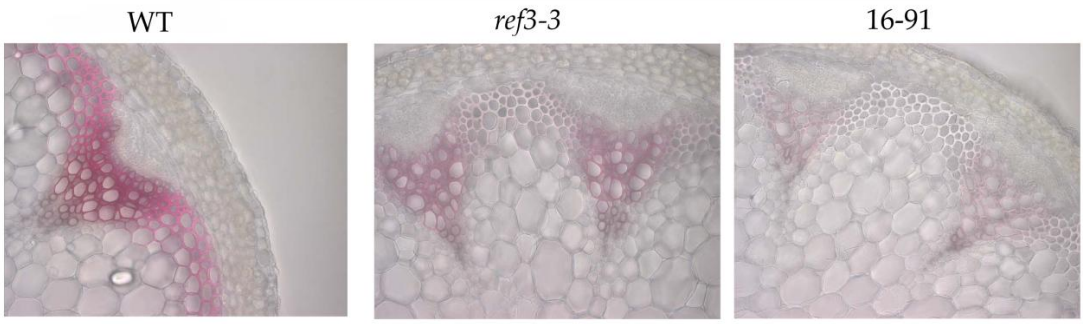


Figure 3 – Lignin staining in stem sections of the *ref3* enhancer 16-91. Stems from six-week old *Arabidopsis* plants were collected and sliced into 100 μm sections. Sections were stained with 0.67% (w/v) phloroglucinol and imaged with a light microscope.

To find the gene responsible for the high saccharification phenotype of the 16-91 mutant, a next generation sequencing (NGS) approach was used. The MutMap process, developed by Abe *et al*. [12] was used. In this approach, there is no need to cross the desired mutant to a different genetic background. Instead, the 16-91 mutant

was crossed back to the wild type and F_2 plants from this cross were used to generate a mapping population. Plants that were homozygous for the *ref3* allele were selected and used in the high-throughput saccharification assay (Figure 4).

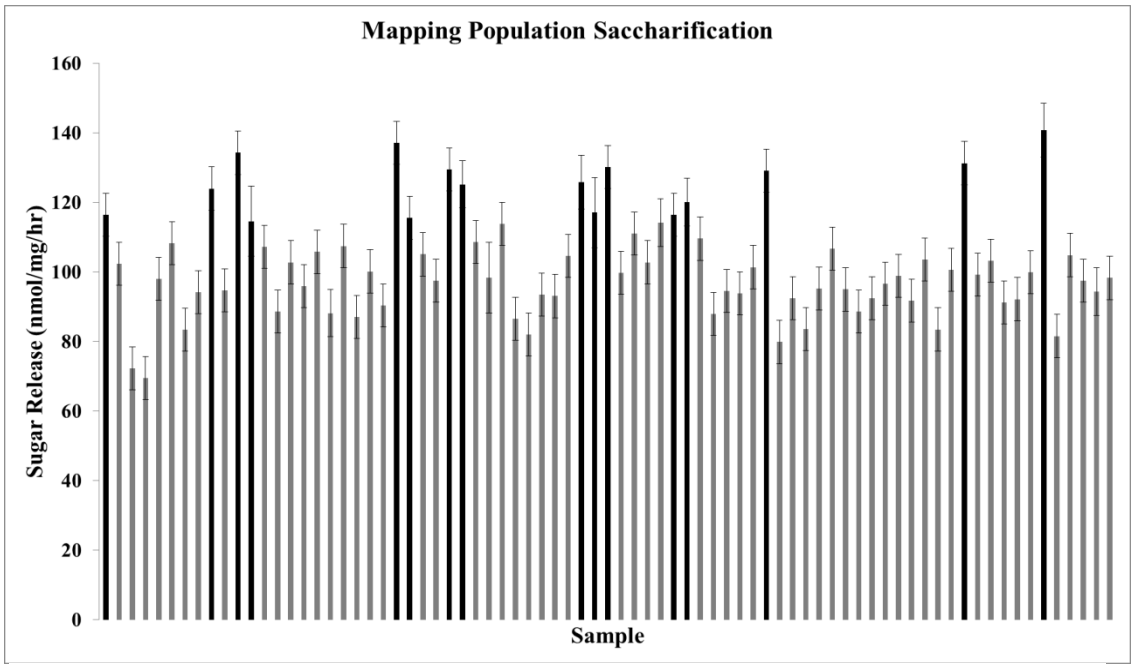


Figure 4 – A mapping population generated by a high-throughput saccharification assay. *Arabidopsis* plants were grown for twelve weeks, and stems were collected and ground to powder. Powder was used in the high-throughput saccharification assay developed by Gomez *et al.* [10]. Each data point represents four technical replicates. Error bars represent standard error of the mean.

Plants that exceeded the cut-off were then selected for the mapping population. Genomic DNA was isolated from these plants (n=16), pooled, and used for sequencing on an Illumina HiSeq2000 platform. The resulting sequences were aligned with the *Arabidopsis* reference sequence and polymorphisms were detected using VarScan. The resulting single nucleotide polymorphisms (SNPs) were plotted based on the frequency of the mutation in the sequencing pool. This index was plotted for all SNPs over the genome. Two peaks were identified, one on the bottom arm of chromosome 2, and the other on a different chromosome. The peak on chromosome 2 can be explained by the selection of the *ref3* allele, so the peak on the other chromosome was determined to be responsible for the improved saccharification phenotype.

A mapping window was defined by at least four consecutive SNPs with an index value ≥ 0.9. A total of 13 protein coding changes were found within the mapping window. Of these, there was one nonsense mutation, within the gene X. Subsequent Sanger sequencing of the 68-19 and 105-31 mutants also identified stop codon mutations within gene X, demonstrating that mutating this gene is responsible for a high saccharification phenotype when combined with *ref3*.

Another screen to find new genes which improve the saccharification properties of plants involved the mutagenesis of the founder mutant, which contains a mutation in a gene encoding a lignin biosynthesis gene in *Arabidopsis thaliana*. This mutant has

decreased lignin amounts and releases more sugar upon saccharification of stem tissue, but has a plant growth defect [13]. The founder mutant was treated with EMS and 80,000 plants from the resulting M_2 generation were screened for increased plant size. 71 mutants were identified originally, but upon re-screening, only 43 of these potential suppressors rescued the plant size defect of the founder mutant. For these 43 mutants, the stem weight of each mutant was quantified (Figure 5).

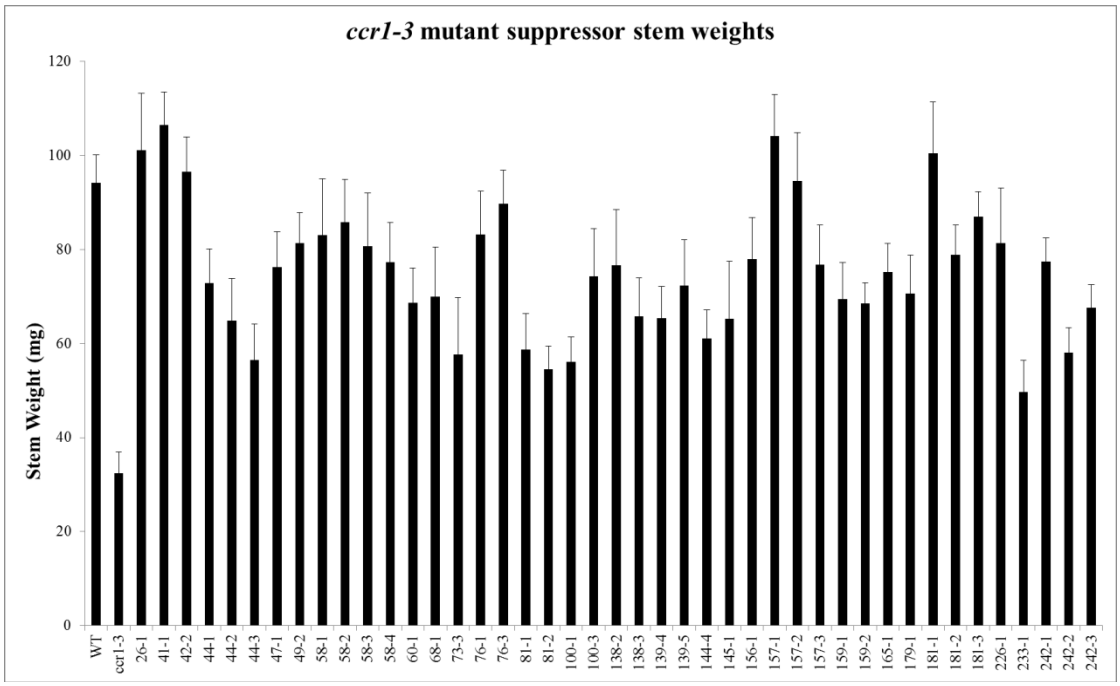


Figure 5 – Stem weights of suppressor mutants isolated from a screen for increased plant size. *Arabidopsis* plants were grown for twelve weeks and stems collected. Each data point represents the mean of eight to ten stems, with error bars representing standard error of the mean.

While not all mutants were rescued back to the size of the wild type, every mutant identified has an increase in average stem weight that is statistically different from the founder mutant, with the exception of two (mutants 73-3 and 233-1 in Figure 1). Many of the mutant lines isolated still show reductions in lignin content when assayed by the acetyl bromide method (Figure 6) [11].

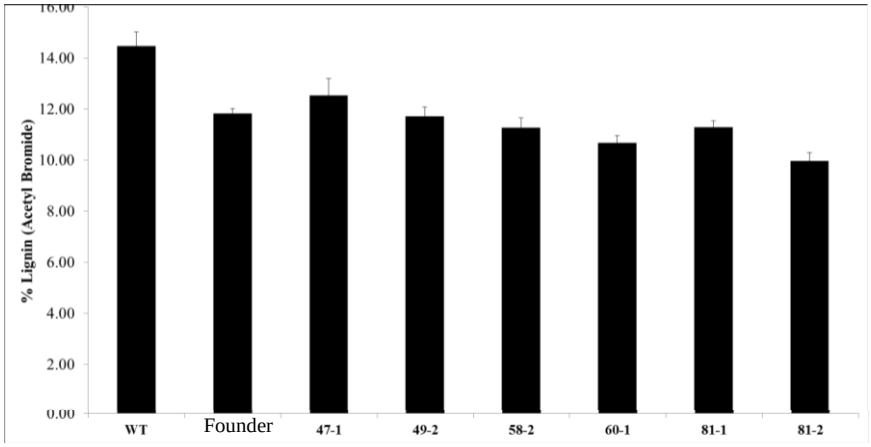


Figure 6 – Acetyl bromide lignin content of several founder mutant suppressors. *Arabidopsis* stems were collected after twelve weeks of growth and ground to powder. Powder was extracted four times with 80% ethanol and once with 2:1 chloroform: methanol. Extracted stem powder was treated with 25% acetyl bromide at 70˚C for 30 minutes, and absorbance at 280 nm measured to quantify lignin [11].

Furthermore, when tested for saccharification, five of the six lines above showed significant differences from the wild type, with the exception being line 47-1 (Figure 7).

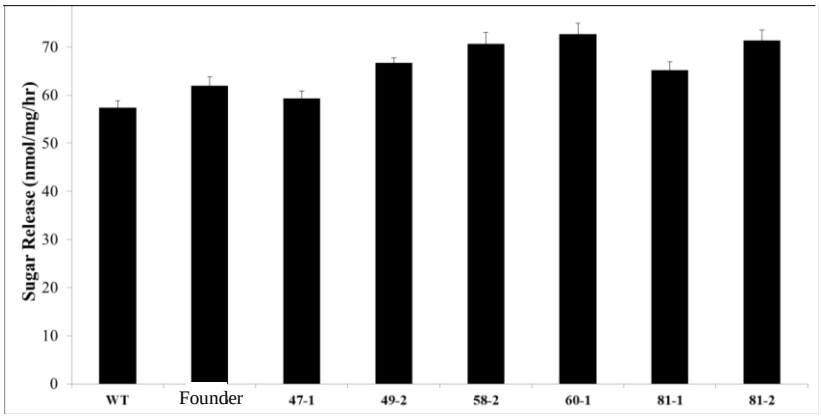


Figure 7 – Saccharification analysis of founder mutant suppressors. Stems were collected from twelve-week old *Arabidopsis* plants and ground into powder. Powder was then used in high-throughput saccharification analysis as in Gomez *et al.* [10].

One of the lines presented in figures 3 and 4 is now being used in mutation mapping. Each line has been crossed to the wild type, and plants from the resulting F_2 generation that exhibit the rescued plant size defect have been selected for the mapping population. DNA from the mapping population will be isolated and a next generation sequencing approach will be used to identify the causal mutation, as developed by Abe *et al*. [12].

Co-expression analysis and protein interactions

The second main approach used to identify novel genes involved in lignin production was a reverse genetics approach, gene co-expression analysis. In order to retrieve potential candidates involved in lignification, co-expression analysis with known monolignol biosynthetic genes was performed with three data sets: ACT [14], CressExpress [15] and Ray (Raymond Wightman and Simon Turner- personal communication). A total of 255 genes were retrieved, several of which were selected for further analysis. T-DNA insertion lines were ordered for the selected genes and these lines were tested for acetyl bromide lignin [11] and several showed reduced lignin levels. A saccharification test on these mutants identified two mutants showing increased sugar release.

The Gb mutant was selected for further analysis. To this end, plants overexpressing the Gb gene in the mutant background were prepared. The recovery of wild-type features in these plants was tested to confirm that detected developmental defects and reduced lignin levels are a consequence of the Gb mutation.

Gb overexpressing plants were compared to wild type (Col-0) plants, the original *Gb9* knock-out mutant and the weak Mx12 knock-down mutant, which shows partial reduction of Gb transcript expression. Phenotypic analysis revealed that wild-type plant growth can be restored in plants with overexpressed *Gb:GFP* or *Gb:HBH* introduced (8).

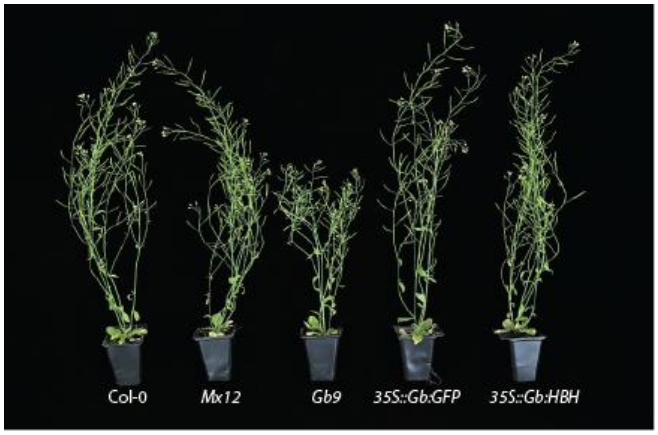
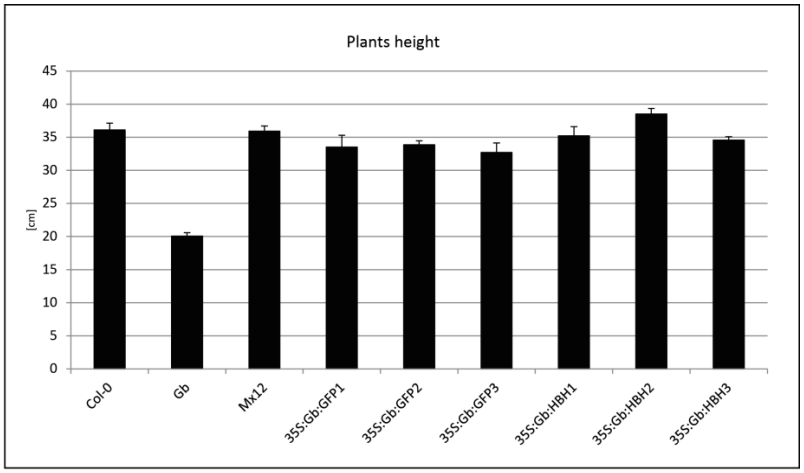


Figure 8 – Overexpression of tagged Gb proteins results in rescue of stunted growth. Plants overexpressing Gb:GFP and Gb:HBH proteins were grown alongside wild type (Col-0) and *Gb9* and *Mx12* mutant controls. A reduced growth phenotype can be observed only in *Gb9* plants.

Transgenic plants were also scored for their height (Figure 9a) and total weight (Figure 9b). Recovery of the wild type features was observed in independent transgenic lines.

a



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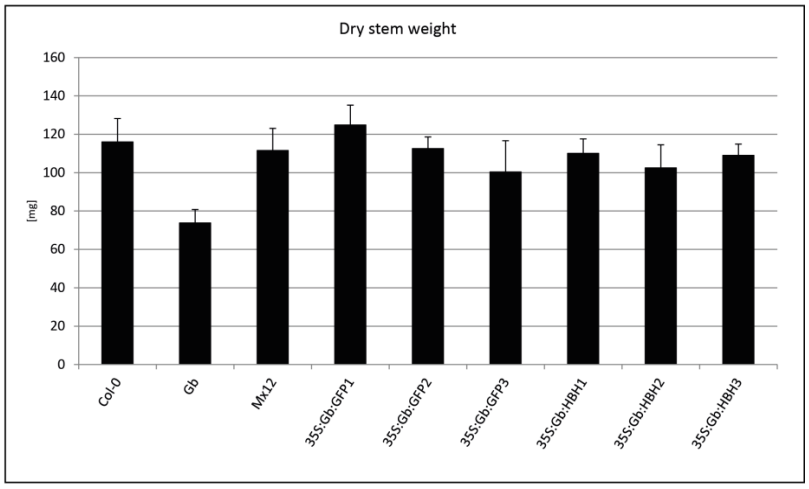

Figure 9 – Overexpression of tagged Gb proteins results in rescue of wild-type growth. Plants overexpressing Gb:GFP and Gb:HBH proteins were grown alongside wild type (Col-0) and *Gb9* and *Mx12* mutant controls. Reduced growth phenotype can

be observed only in *Gb9* plants. **(a)** Final plant height was measured. **(b)** Dry stem weight of transgenic plants.

Gb9 mutants show reduced lignin levels and increased saccharification. To confirm that these characteristics are due to specific gene disruption levels of lignin in *Gb9* plants with introduced *Gb:GFP* overexpressing transgenes were tested. Lignin was detected using phloroglucinol reagent (Figure 10).

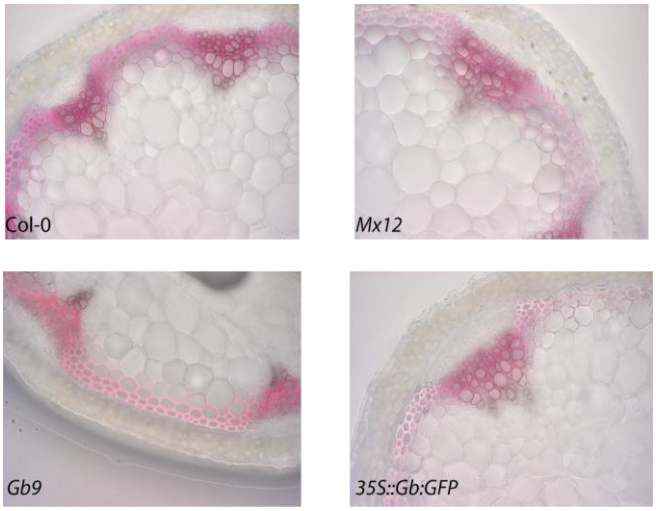

Figure 10 – Lignin staining in stem sections of plants overexpressing *Gb:GFP* transgene compared to wild type (Col-0) and *Gb9* and *Mx12* mutant controls. Stems from five-week old *Arabidopsis* plants were collected and sliced into 100 μm sections. Sections were stained with phloroglucinol reagent.

Stem staining revealed that lignin reduction was largely restored in complementation lines. Additionally, collapsed vessels that are present in *Gb9* mutant plants could not be detected in plants overexpressing the *Gb:GFP* transgene. Reduced lignin levels in *Gb9* mutants are linked with increased sugar release from these plants. To test if restored lignin levels and vessel element structure also leads to restored sugar release, a saccharification assay was performed. This revealed that increased lignin levels observed in *Gb:GFP* and *Gb:HBH* overexpressing complementation lines results in decreased sugar release, which indicates that the increased saccharification observed in *Gb9* mutants is directly linked with the decreased lignin level seen in the mutant.

Analysis of protein complexes formed by known lignin biosynthesis enzymes could reveal new proteins involved in the lignification process. In order to identify protein interactions occurring *in vivo*, potential protein complexes in the lignin biosynthesis pathway are being purified directly from plants, including complexes involving the Gb protein. Presence of the tagged proteins in transgenic lines was confirmed using western blotting with tag-specific antibodies.

The presence of GFP-tagged proteins could be detected in selected transgenic lines. These transgenic lines were used for protein purification. In order to stabilise protein complexes present in the plants, a protein cross-linking step was first performed. This cross-linked material was used in protein purification. GFP-tagged proteins can be purified using GFP trap, which consists of a small peptide that specifically binds GFP [16]. To purify complexes formed by the GFP-tagged

proteins, GFP trap fused with agarose beads was used. Presence of GFP-tagged proteins in the protein purification samples was confirmed by western blot. Additional proteins co-purifying with tagged proteins were visualized with Coomassie blue stain and were subsequently identified using using liquid chromatography/mass spectrometry (LC/MS).

In order to analyse protein interactions present in lignifying tissue, large scale protein purification of GFP-tagged protein was performed using *Arabidopsis* stems. Additional proteins co-purifying with tagged Gb:GFP and CCR1:GFP proteins were detected.

LC/MS analysis of proteins co-purifying with Gb:GFP revealed the presence of cell wall related proteins, xyloglucan hydrolase 24 being the most abundant. However, no interactions between Gb and core components of lignin pathway could be detected in *Arabidopsis* stem samples. This analysis reveals additional previously unstudied proteins that could be involved in cell wall formation in *Arabidopsis*.

Progress

Several mutants have been identified in *Arabidopis thaliana* that improve the saccharification potential of the plant. These mutants have been quantified for lignin levels, and found to have decreased lignin. One mutant has had its causal mutation mapped and the mutated gene was identified. Another screen has identified a mutant which has rescued growth but maintains reduced lignin levels and increased saccharification. Co-expression analysis has identified a gene, that when mutated, contains less lignin and releases more sugars upon saccharification. Transformation of this mutant with an overexpression rescue construct confirms that these phenotypes are the result of the studied gene. The protein produced from this gene has also been used for protein complex purification, along with previously studied lignin biosynthesis enzymes. Initial results indicate that the Gb protein may interact with some cell wall components, suggesting a role in cell wall architecture.

Future Plans

For the remainder of the project, the focus for the mutant screening portion of the project will be on identifying the causal mutations behind the observed phenotypes. Two more mutants from the *ref3* enhancer screen will be mapped using NGS, one of high saccharification, the other of low saccharification. The mutant from the suppressor screen of the lignin mutant with impaired growth will also be mapped with the same procedure.

Transgenic *Arabidopsis* overexpressing the rice Gb protein will be analysed to test common function of homologues form different plants. Additionally, an antibody against *Arabidopsis* Gb protein is being prepared. This antibody will facilitate future studies of the Gb protein. The specificity of this antibody and its ability to recognise Gb homologues from different organisms will also be tested.

Publications

Patents: Modified Plants filed May 2012. Application number GB1208105.5.

Papers: Two papers are in preparation, one on the Gb mutant (in collaboration with Wout Boerjan) and one on the stacking of mutations in the large-scale mutant screen.

Talk: Claire Halpin at UKPlantSci 2013, Dundee 'Spinning straw into gold: Barley, biofuels and biosequestration' April 2013

Talk: Claire Halpin at Monogram Network Meeting 'Improving barley straw for bioenergy' March 2012

Talk: Chris McClellan at UK PlantSci 2012, Norwich, UK: 'Improving plants for the production of 2nd generation biofuels.' 18 April 2012

Poster: Kasia Rataj at 23rd International Conference on Arabidopsis Research, Vienna, Austria: 'Optimization of lignin content in Arabidopsis to facilitate biofuel production' 3-7 July 2012

Poster: Chris McClellan at UK PlantSci 2012, Norwich, UK: 'Genetic screening for improved saccharification properties in *Arabidopsis thaliana*' 18-19 April 2012

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