Novel mutants optimised for lignin, growth, and biofuel production via re-mutagenesis

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Abstract

This project aims to find novel genes involved in lignin biosynthesis and to alter cell wall properties for more efficient biofuel production. Two approaches are being used towards this end. The first involves re-mutagenising existing mutants in monolignol biosynthesis genes and screening for suppressors or enhancers of these mutants. Two rounds of mutagenesis have been carried out, with 71 potential mutants identified from the first screen. This screen involved searching for plants with restored growth after mutagenising a stunted lignin gene mutant. Using lignin analysis and saccharification assays, these mutants are being characterised to identify targets for gene mapping. Once the genes responsible for the observable phenotype have been identified, the genes will be characterised for molecular function. Screening plants from the second round of mutagenesis (performed on a lignin mutant with normal growth) is underway using a high throughput saccharification assay. A third round of mutagenesis on yet another lignin mutants is also planned.

The second approach to find novel genes in the lignin biosynthesis process involves gene co-expression analysis. Genes involved in common processes or pathways may be transcriptionally coordinated and have similar expression profiles. We retrieved a list of 255 genes which co-express with known monolignol biosynthetic genes using three co-expression tools. Mutants in at least three of these genes exhibit reduced lignin accumulation or improved sugar release from inflorescence stems. The potential function of these co-expressed genes on cell wall development and lignification will be further investigated.

Introduction

The aim of this project is to identify novel genes involved in the lignin biosynthetic process 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. New lignin-related genes will also be identified by tandem-affinity purification of proteins associated with known monolignol biosynthesis enzymes. Genes identified with these strategies will be good candidates for manipulation in crops used in biofuel production.

Background

Although the monolignol biosynthesis pathway is well-characterized in the model plant *Arabidopsis thaliana*, we know relatively little about how monolignol monomers integrate into plant cell walls and cross-link with other polymers in the wall, although two laccases have recently been identified as being involved in lignin synthesis [1]. Standard screens for phenotypes that relate to lignin defects, such as irregular xylem (*irx*) and reduced epidermal fluorescence (*ref*) have yielded initial mutants that are promising for biofuel applications [2]. However, more research is needed to obtain the necessary range and specificity of mutants for lignin engineering. To find novel genes involved in lignin biosynthesis, we are using suppressor and enhancer mutagenesis 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 lignin biosynthesis genes using publicly available data sets from microarray expression experiments. Previously, co-expression experiments have been used successfully to explore novel genes involved in these some cell wall pathways and processes [3]. However, similar co-expression analysis on the monolignol biosynthesis pathway has not been reported yet.

Results

In the past year, significant progress has been made towards identifying novel genes involved in lignin production. Two approaches have been utilised towards identifying these genes in the model plant *Arabidopsis thaliana*. The first approach, a forward genetics approach, is to mutagenise existing Arabidopsis lignin mutants and screen for plants that have characteristics that make them better suited for biofuel production. Two such screens have been performed so far. The first 'suppressor' mutagenesis screen was performed on a lignin mutant which, while it exhibits improved saccharification properties, has reduced plant size, making it less suitable for optimal biofuel production. Seeds of this mutant were treated with the mutagen ethylmethanesulphonate (EMS), and allowed to grow for two generations. Seeds of the resulting M_2 generation were planted and the plants screened for increased size. 90,000 plants were screened in this manner, and potential mutants were genotyped to confirm the original 'founder' mutation. In all, 71 mutants have been identified for further study. However, many of these mutants have been found to express the gene which is knocked-out in the founder mutant, even though the founder mutation is still present in these plants. Four mutants which do not express the founder gene, yet have partially or fully restored plant size (Figure 1), have been selected for further analysis.

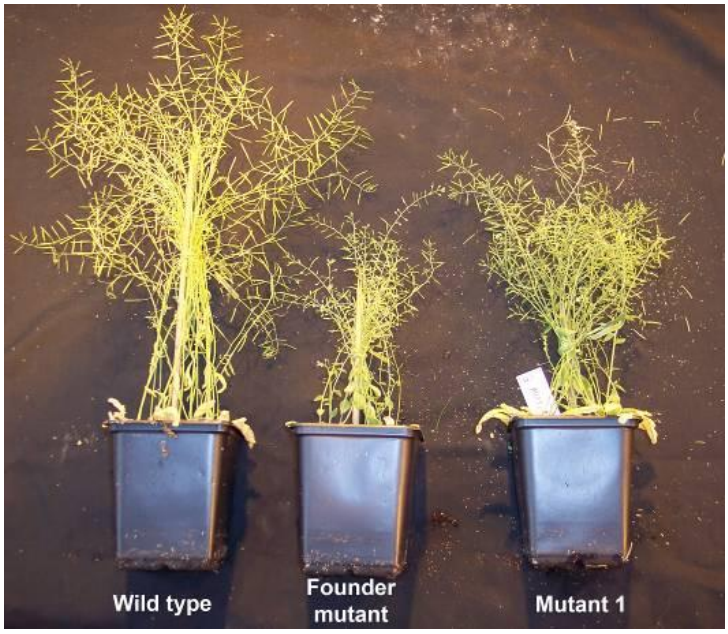

Figure 1: One potential mutant with partially restored plant size compared to the founder mutant, which is stunted. Plants were grown for eight weeks and photographed.

These suppressor mutants are being further characterised for lignin content and saccharification properties to confirm that they retain the original phenotypes that make the founder mutant a candidate for genetic manipulation. Mutants have been analysed for lignin content using the acetyl bromide assay [4]. Two mutants analysed in this manner still have reduced lignin content when compared to wild type, although variation in the assay is high (Figure 2). To confirm the results of the acetyl bromide assay, a second assay to determine lignin content, the Klason assay, will be performed on all mutants.

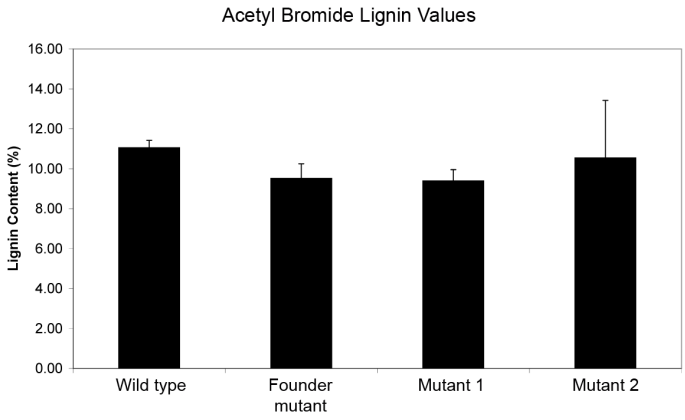

Figure 2: Lignin content of potential mutants measured by the acetyl bromide assay. Data bars represent the average of three biological replicates. Error bars represent standard deviation.

In addition to measuring lignin content of mutants, the saccharification properties of dry stems from potential mutants were analysed using a high-throughput semi-automated assay developed at the University of York [5]. The saccharification properties of two mutants analysed with this assay remain like those of the founder mutant which has an improved yield of released sugars compared to the wild type (Figure 3).

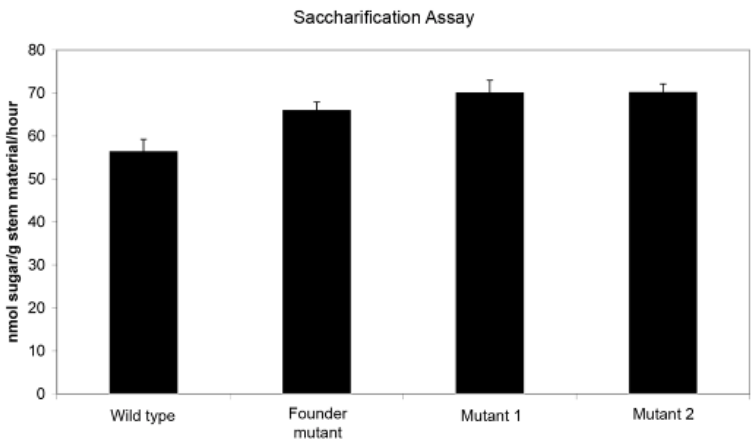

Figure 3: Measurement of reducing sugars produced from dry stems of potential mutants when analysed in a saccharification assay. Data points represent three biological replicates, with four technical replicates per biological replicate. Error bars represent standard error of the mean.

These suppressor mutants, which show increased plant size when compared to the founder mutant and still possess the improved saccharification properties and reduced lignin content of the founder mutant, could be valuable towards the production of plants that can be converted to biofuels more efficiently. To identify the genes mutated in these mutants, mapping crosses have been performed, where the mutant in the Columbia (Col-0) background has been crossed to another ecotype of Arabidopsis, Landsberg erecta (Ler). The F_2 progeny of these crosses will be used to identify the segment of the Arabidopsis genome that contains the mutation responsible for the increased size phenotype. By performing this mapping, the gene(s) responsible can be identified and targeted for further study.

An ‘enhancer’ mutagenic screen in Arabidopsis is also currently being performed. A second, different founder mutant, that has altered lignin but grows normally, has been mutagenised with EMS and progeny grown for two generations. The yield of sugars on saccharification are increased in the founder mutant compared to the wild type, but a greater improvement could produce even more benefit for biofuel production. To find mutants with such an additional improvement, the re-mutagenised population will be screened using the high-throughput saccharification assay at the University of York [5]. Currently, approximately 15,000 plants are in the process of screening, with the potential to screen more when growth space and assay time allow.

A third mutant has recently been selected for mutagenesis screening. This mutant has reduced lignin and a very significant improvement in sugar release in the saccharification assay but grows much less vigorously than wild type plants. This ‘founder’ mutant will be used in a suppressor screen to identify further mutations that maintain the cell wall and saccharification changes but restore a normal growth phenotype, similar to the original screen. Currently, seed for this mutant has been bulked and mutagenesis will proceed shortly.

The second approach utilised to identify novel genes involved in lignin production is gene co-expression analysis. In order to retrieve potential candidates involved in lignification, we performed individual co-expression analyses with known monolignol biosynthetic genes. In total, 255 genes were retrieved, with some of them shared between different analyses; 102 of them were chosen for further investigation. To investigate the potential biological function of these genes, we searched the Nottingham Arabidopsis Stock Centre (NASC) for available T-DNA insertion mutants in these genes. We have obtained 66 homozygous mutants and have confirmed them with PCR-based genotyping methods.

Protein complexes have been indicated to be involved in many important processes in plants. To explore any potential protein complex involving in lignification, three known lignin-related genes were chosen as baits to trap any potential protein complex using two tandem affinity purification systems. Up to date, six tag-fusion constructs for three bait genes have been developed. We will use these construct to generate related Arabidopsis transgenics which will be used to purify potential lignin-related protein complexes.

Progress

By identifying mutants and genes potentially involved in the production of lignin, these genes can be targeted for genetic manipulation (either by conventional breeding or biotechnology approaches) in biofuel crop plants. We have generated several mutants that reveal genes yet to be identified that could be used in this manner. We have also used co-expression analysis as an alternative route to identify useful novel genes that can alter lignin or saccharification properties. Plants modified in these genes, or in combinations of these genes, would be able to grow normally, but would contain alterations in their cell wall properties that would allow for a greater conversion of biomass to fuel production. We are currently exploring the prospect of filing a joint patent application on the use of one such gene with Prof Wout Boerjan prior to any public disclosure of the results.

Future Plans

Current plans are to map and characterise the suppressor mutations found on the basis of restored plant size from the first suppressor screen. We will also finish saccharification assays to perform the second ‘enhancer’ screen for plants with improved biofuel properties. A third EMS mutagenesis experiment using a known lignin mutants will be implemented to identify further suppressor mutations that maintain the cell wall and saccharification changes of the founder mutant but restore a normal growth phenotype. Lignin content and composition of mutants picked up from co-expression analysis will be analyzed.
Transgenics harbouring tagged-bait fusion constructs will be developed and be used to purify potential protein complexes involved in lignification.

Publications

No formal publications as yet. However the PI, Claire Halpin, has made several presentations where the GCEP research has been highlighted including:

1. 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.

2. BBSRC/South East Asia Workshop on Biofuels (February 2011, Hanoi, Vietnam)

3. College of Life Sciences, University of Dundee, Annual Symposium, Crieff Hydro Hotel, Scotland, March 2011)

4. GCEP symposium, Palo Alto, USA, Sept 2010

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