SUNLIBB

Sustainable Liquid Biofuels from Biomass Biorefining

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Deliverable 4.2
"Novel transgenic lines with improved saccharification in maize and Miscanthus"

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Sunlibb Deliverable 4.2: Maize and Miscanthus transgenics for enhanced saccharification potential

The beneficial enhanced saccharification efficiency of the naturally occurring 'brown midrib' (*bm*) mutants in maize and sorghum shows that disturbing lignin biosynthesis can be a good strategy for lignocellulosic crop improvement. In that perspective, the cultivation of these *bms* would form an improved supply of biomass for second generation biofuels. Unfortunately, these lines perform less well in the field and thus generate lower yields for both whole silage and grain. In order to compensate for the yield penalty of these mutants, it would be desirable to combine different beneficial traits to enhance saccharification in one and the same commercial line. This process of 'gene or trait stacking' is most easily studied by the use of transgenics made in a homozygous genetic background (a full inbred line). To explore the feasibility of improving lignocellulosic crops for second generation biofuels by means of genetic engineering, we initiated transformation experiments in a maize inbred line. This enables us to easily compare and combine different traits. Traits can be combined by crossing or by 'supertransformation' of a particular transgenic line.

As an initial target we chose the maize *CINNAMYL ALCOHOL DEHYDROGENASE2* (*ZmCAD2)* gene for down-regulation using a hairpin construct. The *ZmCAD2* gene is defective in the maize *bm1* mutant and is thus a good target. Also *Arabidopsis* and *Brachypodium* plants disrupted in the *CAD* gene display enhanced saccharification potential. We selected three highly-specific regions of the *ZmCAD2* gene (Figure 1), to avoid the downregulation of 'off-targets' and cloned them into the monocot-specific gateway vector pBb7GW-I-WG-UBI. This vector harbours a BASTA selectable marker and makes use of the maize UBIL promoter to drive the expression of the transgene.

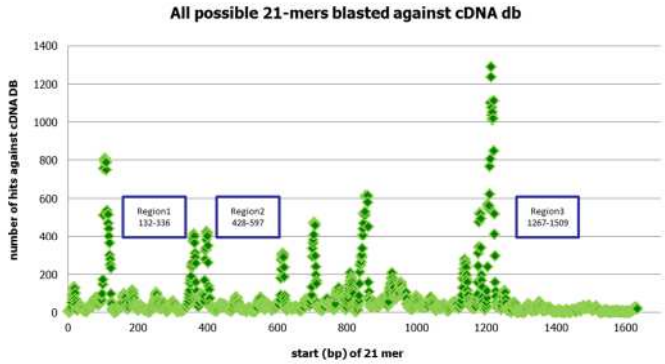

Figure 1. Selection of gene regions for specific down-regulation of the *ZmCAD2* gene

The generation of the first generation of transgenic seed takes approximately 7 months. We obtained in total 34 independent transformation events (8 lines for region 1, 17 lines for region 2 and 9 for region 3) for which we obtained heterozygous seed stocks. Heterozygous seed stocks are preferred since the segregating wild-type plants can be used as perfect controls. We chose to screen all lines for reduced CAD protein activity as this is expected upon successful down-regulation of the *ZmCAD2* transcript. The assay was performed according to Fornalé *et al*. (2012) and included the extraction of total protein from leaves of plants that were approximately 6 weeks old, and the measurement of the CAD activity by following the conversion of coniferyl alcohol into coniferyl aldehyde spectroscopically. As can be expected from an RNAi strategy, CAD activity was found to vary from no altered activity to merely 25 % residual activity (Figure 2). Based upon the screening test, one line per construct was selected for further analysis (103-01, 104-11 and 107-20).

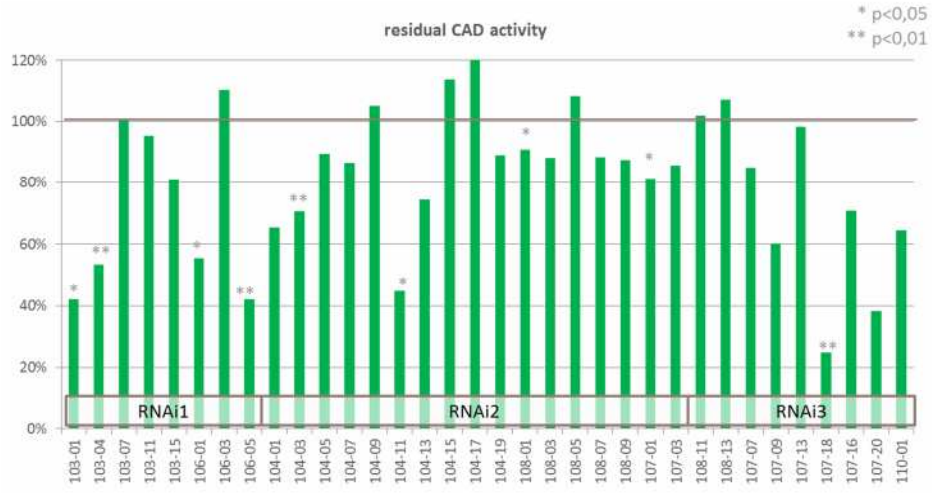

Figure 2. Independent transgenic lines screened for reduced CAD protein activity. CAD activity is expressed as residual activity compared to the segregating control plants per line

The reduction in CAD activity was confirmed in these lines in an independent experiment. Next, we performed an expression analysis to check whether the introduced construct was expressed and whether, at the transcript level, *ZmCAD2* was reduced compared to the wild-type. Genotyping of the segregating population was performed using an ammonium multiwall assay. For the analysis we harvested the bottom centimeter of leaf #4 of approximately 3-weeks-old plants and pooled 3 plants per biological replicate. We found that the transgene construct was indeed expressed only in the BASTA resistant samples. The *ZmCAD2* endogene, however, showed no reduced expression in the BASTA resistant plants as compared to the segregating wild-type plants. This contradicts the reduced CAD activity found earlier in these lines. To elucidate whether these lines showed a phenotype related to lignin and/or improved saccharification potential, we analyzed stems of fully mature senesced plants. We analyzed the stem material for total lignin with the acetylbromide method, for lignin composition with thioacidolysis and for saccharification potential. No changes were detected in total lignin content, which varied from 10 to 14 % of dry weight. In a saccharification experiment we loaded 0.015 FPU of Accellerase1500® (Genencor) into a 1 ml mixture containing 20mg of dry weight that was incubated at 50°C for 48h. We used ‘ethanol washed only’ biomass and also ‘ethanol washed and acid pretreated’ biomass. Samples were taken at 3, 6, 24 and 48h. Neither for the ‘washed only’ nor the ‘washed and acid pretreated’ biomass, could an enhanced glucose release upon enzymatic hydrolysis be detected (Figure 3). Alkaline pretreatment was also tested, but no differences were observed with this either.

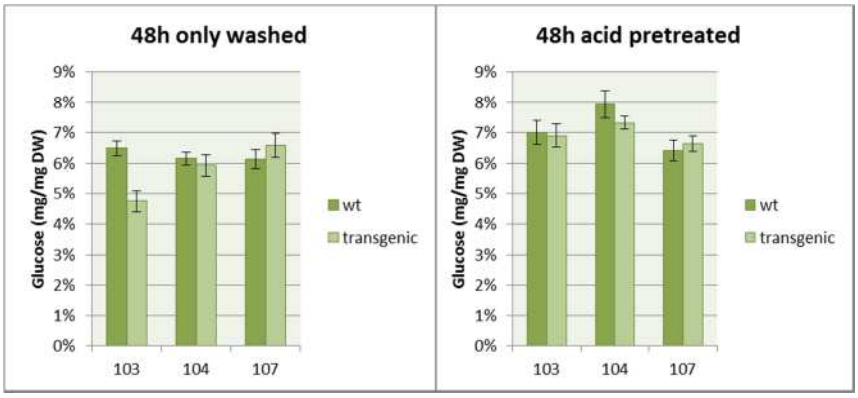

Figure 3. Saccharification efficiency. 48h measurements of 'only washed biomass' and 'washed and acid pretreated biomass' that was hydrolyzed using a 0.015 FPU loading of Accellerase1500® (Genencor)

We analyzed the lignin composition using the thioacidolysis method. We quantified the monomers H, G and S that constitute the lignin polymer and found that lignin in a maize stem is built up of 1% of H, 22% of G and 77% of S. This is in contrast to a typical dicot plant which has more or less the same frequency of G and S units in the lignin polymer. However, no differences in H/G/S lignin composition were observed. Besides the monolignols, also the corresponding aldehydes can be quantified using thioacidolysis. We expected an increase in aldehydes in lines impaired in CAD activity because the enzyme converts the aldehydes into alcohols in the last step of the phenylpropanoid pathway. This has been observed in the *bm1* maize mutant and also in *Arabidopsis* and *Brachypodium* plants disrupted in the *CAD* gene. However, in the transgenic lines we did not see this expected accumulation. We concluded the analysis by performing saccharification assays using different pretreatments, but did not see any significant changes. An expression study of the hairpin construct showed that the transgene is expressed in both leaf and internode tissue. However, the target gene, *CAD*, was not down-regulated in young leaf tissue and only mildly (approx. 20%) in elongating internode tissue.

We therefore conclude that the investigated approach, which is down-regulation using an RNAi approach using the *UBIL* promoter was not successful in creating a *cad* mutant phenotype. The endogenous *CAD* transcript level was not down-regulated enough to cause a loss-of-function phenotype. Nevertheless, we can conclude that the transformation platform itself is very efficient and successful in producing transgenic lines that are resistant to the BASTA herbicide. At the time we started our experiments, only the maize *UBIL* promoter was available, which is known to be active mainly in meristematic tissue, which in our case is less suitable since we are trying to down-regulate a gene that is expressed in differentiated cells undergoing secondary thickening. Recently a set of *Brachypodium* promoters were tested in maize tissue and revealed that these promoters are 300 times stronger and possibly also active in mature tissues (Coussens *et al*. (2012).

Discussion

Given this unsuccessful attempt to generate improved biomass using a transgenic approach, we might wonder whether alternative strategies, such as the use of mutants, would be more efficient.

For bioenergy purposes, existing mutants in maize and sorghum comprise a series of *brown-midrib* (*bm*) mutants, of which some are known to have mutations in lignin biosynthesis genes. Initially, they were proposed as enhanced feedstock for animal feed, given their higher digestibility. These lines display enhanced glucose release upon enzymatic hydrolysis of the cell wall. Thus, these lines are

promising breeding material for bioenergy purposes,. However, although commercial lines are available using *bm* mutations, these lines perform less well in the field, with yield penalty being the main concern (summarized in Pedersen *et al*., 2005). This yield penalty can theoretically be overcome if i) we can efficiently down-regulate a given lignin biosynthesis gene in maize and ii), if we can achieve this in a tissue-specific way (such strategies have been shown to work in Arabidopsis – see Petersen *et al*. (2012) and Yang *et al*. (2013).

Although the transformation strategy to down-regulate lignin biosynthesis genes did not work, we still believe this strategy is worth pursuing. It is just a matter of time before we are able to efficiently down-regulate genes. For example, it is now possible to use stronger promoters to drive transgene expression than those available when we started the transformation. Secondly, we are currently investigating new biotechnological approaches such as the CRISPR/CAS system, that enables gene editing. This means that we will be able to modify the maize gene itself, with the advantage that the down-regulation of the target gene is not achieved through the expression of a transgene. Instead, the gene to be down-regulated is modified itself at the DNA level, and this modification is heritable. In addition, this system might become compatible with EU legislation for commercialization in agriculture.

If the transgenic approach to down-regulation of genes in maize does work in the end, we envisage being able to down-regulate lignin biosynthesis genes in specific lignifying cell types. This would completely avoid pleiotropic effects such as growth delay and biomass yield penalties. We are already investigating the use of tissue-specific promoters to drive transgenes.

Another reason why we believe the transgenic approach is still worth pursuing is that it allows us to stack multiple genes into a given genetic background, thus circumventing the many breeding cycles that would be needed with a conventional approach (Gressel, 2008). This way, we can stack genes that optimize organ size (Nelissen *et al*., 2012) with genes that improve cell wall processing efficiency. If we can demonstrate that a given gene knock-out in maize can improve biomass processing, a similar approach can also be followed in other biofuel crops such as switchgrass and Miscanthus, that is, species for which the natural mutants have not yet been identified or give a yield penalty.

Within the SUNLIBB project, P13 has attempted to develop a protocol for efficient transformation of *Miscanthus sinensis*. Initially P13 tried to develop such protocol with 2 *M. sinensis* lines, but after several attempts it resulted in a very low percentage (bellow 0.1%) of transformed plantlets. In a second attempt, a total of 60 genotypes were tested for callus formation and regeneration efficiencies. This step proved to be extremely time consuming, and together with the search for novel vectors for transformation, Agrobacterium strains and the testing out of suitable protocols, Miscanthus transformation proved not to be feasible within the timeframe of SUNLIBB. P13 is still working on the improvement of the protocol, based on a new patent filed for Miscanthus transformation, and using the vectors developed at VIB, which are based on *Brachypodium* promoters and which have already been successfully tested in maize (Coussens *et al*., 2012).

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