Advances in
Biotechnology & Microbiology
ISSN 2474-7637

Juniper Publishers
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Opinion
Volume 6 Issue 2 – September 2017
DOI: 10.19080/AIBM.2017.06.555685

Adv Biotech & Micro

Cellulase Production in Transgenic Plants: Molecular Pharming with a Twist

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Submission: September 22, 2017**; Published:** September 25, 2017

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Opinion

The current cost of the degradation of biomass into sugars has hampered its development. The two greatest hurdles to overcome in terms of expense can be attributed to the pretreatment of biomass and the requirement of cellulolytic enzymes (cellulases). One way to circumvent the latter is by engineering plants which themselves can serve as production platforms for cellulases. The mass production of cellulases in plants would greatly diminish cost barriers facing the lignocellulosic biofuel industry. Other advantages of cellulase molecular farming include the ease of scalability, the generation of post-translation modifications that are eukaryotic in nature and the ability of plant tissues to be altered in such a way that chemical pretreatment is reduced. The hydrolysis of cellulosic biomass into fermentable sugars requires three different types of enzymes acting synergistically; they are endoglucanase, exocellobiohydrolase and β-glucosidase. This opinion piece describes the molecular farming of cellulase in host systems such as corn, rice, sugarcane, and tobacco. Plants with cell walls that have been tailored to be more effective for degradation are also described. The piece concludes with a projection of the use of transgenic plants specifically designed for biofuel production.

Cellulase E1 from the bacterium *Acidothermus cellulolyticus* has been the most widespread example of an endoglucanase that is produced in transgenic plants. The rationale for generating E1 in plants is that it is a hyperthermophilic enzyme with an optimal temperature of activity of 81 °C. As a result, E1 would be inactive at temperatures favoring plant growth, but could become activated when the plant tissue was subjected to the more elevated temperatures that are required for biofuel production. The use of thermophilic cellulases with optimal temperatures greater than 60 °C causes little damage to the host plants, and the transgenic plants more resemble their conventional non-transformed counterparts with respect to morphology and development. Tobacco was the first plant generated that expressed E1; in this case, E1 was targeted to the chloroplast. To date, many different transgenic crop types expressing E1 have been produced, however, they can differ widely with respect to transgene expression levels and effect on plant development. For example, E1 transgenic maize and tobacco cell walls exhibited significant morphological differences from each other as well as their respective non-transgenic parents. In general, the presence of a biologically active E1 during cell wall synthesis could play a role by creating gaps in the cell wall or through reducing cellulose crystallinity, both important steps for increased enzyme accessibility.

To date, many transgenic crops that have been generated to produce cellulases are model plants for which transformation strategies are well established. For example, endoglucanase Cel6A of Thermobifida fusca, a thermophilic bacterium, has been transformed into alfalfa, tobacco, and potato. The enzyme retained its activity and exhibited no negative phenotype on whole plants. Transgenic maize expressing CBH1 from *Trichoderma reesei* showed no yield or growth performance difference compared to wild type plants under field conditions (Garda et al., 2015).

Rice offers a tremendous source of biomass, and the production of cellulases in rice straw could prove to be a valuable use of the residual tissue for biofuel after the grain is harvested. The cellulase endo-1,4-β-glucanase V gene from *T. reesei* has been generated in the plastids of transgenic rice plants. Expression of protein in the plastids rather than the cytoplasm is believed to reduce its potential for degradation by cellular enzymes and to improve its level of accumulation. Another strategy used has been to overexpress cellulose under the control of a senescence –inducible promoter, such as STAY GREEN (SGR), a gene involved in the induction of chlorophyll degradation. The use of an inducible promoter reduces stunting and other forms of growth retardation found with constitutive promoters. Transgenic rice crops expressing cellulase under the control of a developmental stage-specific promoter such as SGR were unaffected with respect to plant growth and development but displayed enhanced saccharification properties, suggesting that this could be a viable approach for the improvement of biofuel production.

Sugarcane has also been engineered to express cellulases. The fiber from sugarcane (begasse) has great potential as a biomass source for tropical countries. For example, a synthetically-designed endoglucanase (psEG) expressed from a constitutive maize promoter was developed in the chloroplast, ER and vacuoles of sugarcane cells. Chloroplast-derived endoglucanase was found to be the optimal organelle for the greatest expression. In another study, transgenic corn stover leaf expressing a cellobiohydrolase was extracted and applied to pretreated begasse and the effect of glucan conversion was compared to the presence or absence of a commercial cellulase mixture that was added to the substrate. Activity was doubled in the presence of transgenic extracts expressing cellulase, indicating that transgenic crops present an economically feasible opportunity for biofuel development.

Even more intriguing is the use of gene stacking as a means to generate transgenic plants expressing multiple cellulases that can act synergistically on a particular substrate. For example, the Foot-and-mouth disease virus (FMDV) 2A self-cleaving peptide can be placed between two proteins which, when expressed as a larger polyprotein containing several cellulase open reading frames, can mediate self-cleavage into individual cellulases. Polyproteins consisting of β-glucosidase (BglB) and endoglucanase (Cel5A) from *Thermotoga maritima,* xylanase (XylII) from Trichoderma reesei, and exoglucanase (E3) from *Thermobifida fusca* linked to the FMDV 2A sequence were coexpressed in tobacco plant chloroplasts and demonstrated to act synergistically to hydrolyze cellulose at higher levels than any individually expressed cellulase. The fact that these cellulases could remain stable in lyophilized leaf tissue that was stored for long time periods indicates that this procedure could be an attractive means by which to improve the efficiency of biofuel production. In another example, the cellulases E2 and E3, as well as a fused version of these two proteins were constitutively expressed in tobacco, and the transgenic plants which were generated exhibited normal growth. Transgenic plants that expressed cellulases in the apoplast exhibited the greatest levels of enzyme activity, although all subcellular compartments exhibited similar mRNA abundance.

To overcome resistance of cellulose to hydrolysis in general, some researchers have engineered the plant cell walls themselves so that they can more easily be hydrolyzed into glucose subunits. For example, plants expressing antisense constructs directed against lignin-biosynthetic genes were found to produce decreased amounts of lignin, which enhanced the efficiency of hydrolysis of the cell wall. In another strategy, plant cell walls underwent an increase in hydrolysis to glucose residues by altering the expression of the regulators of secondary cell wall synthesis NST1 and NST3. Mutant plants produced cell walls that were less crystalline, had less lignin accumulation and were twice as susceptible to hydrolysis as wild type plants.

The use of plant biomass in the form of dedicated biofuel crops or residual agricultural crop waste offers tremendous potential as a renewable energy. However, the efficient degradation of recalcitrant cellulose remains a principle bottleneck for making this technology economically sustainable. The generation of microbial cellulase degrading enzymes in transgenic plants, as described in this paper, could offer one possible solution. Much of the work that has been accomplished to date concerns the use of annual crops which are well established in the research lab. Future steps will include the generation of transgenic perennial crops such as switchgrass and poplar that express celluloytic enzymes or have cell walls that are modified to improve glucose production. Transgenic cellulases that function successfully in plants will decrease the amount of commercial cellulases required, and thus decrease the current costs associated with biofuel production substantially. The production of second generation biofuels that are price competitive with fossil-derived fuels will advance the effort to achieve global energy security.

DOI: 10.19080/AIBM.2017.06.555685

How to cite this article: Kathleen Hefferon. Cellulase Production in Transgenic Plants: Molecular Pharming with a Twist. Adv Biotech & Micro. 2017; 6(2): 555685. DOI: 10.19080/AIBM.2017.06.555685