105. Expanding the breeder's toolbox for perennial grasses: Engineered doubled-haploid and gene containment systems

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Project Goals: The project aims at using a systems-based approach to develop new breeding tools for perennial grasses and apply these tools towards the improvement of switchgrass (*Panicum virgatum* L.). Our objectives are: (1) Accelerate conventional breeding using the fast generation of doubled haploid lines (developing a CENH3-based method in switchgrass); (2) Use the model perennial grass *Brachypodium sylvaticum* to identify combinations of transgenes that confer tolerance to multiple abiotic stresses; (3) Develop a gene containment system to minimize gene flow from transgenic switchgrass; (4) Create transgenic switchgrass plants containing the best combinations of transgenes identified in objective 2 and the gene containment system from objective 3; (5) Evaluate the best transgenic switchgrass plants from objective 4 in field trials.

Switchgrass is a large, perennial, outcrossing species native to most of North America. It carries wind-pollinated flowers and fields can produce substantial pollen during flowering. Small seeds which exhibit dormancy can be unintentionally transported by farm operations. These features which are not unique to switchgrass may improve individual fitness and preserve genetic diversity but also present difficult dilemmas for modern crop improvement by creating natural and artificial conduits for gene flow into the environment. This issue, along with concerns of the public and stakeholders with genetic diversity and maintaining differentiation among multiple potential end-uses are driving our work to develop genetic isolation technologies which will mitigate gene flow.

Evolving, complex, overlapping regulatory frameworks cover the release of engineered, transgenic switchgrass. To meaningfully evaluate new lines, field studies will need to be performed under strict oversight and gene containment measures will likely be required in some form. Our strategy employs a two component system that when combined in an F1 hybrid, will activate reproductive containment. The general design utilizes two types of transformation constructs that function together using recombinase-mediated excision.

The first component consists of a plant expression vector that may harbor four or more transgenes: (1) a 'gene of interest'; (2) a selectable marker; (3) the *Cre* recombinase; and (4) *Barnase* which encodes a ribonuclease that is toxic when expressed in plant cells. Transgenic plants generated with this construct will express the gene of interest, but the transgenic pollen produced from these plants will be inviable due to pollen-specific Barnase expression. We have demonstrated that the rice *Pollen Specific3* (*PS3*) promoter is active in switchgrass pollen and we are using this promoter to control Barnase expression. These hemizygous transgenic plants can be used as the female parent for generating hybrid switchgrass seed.

The second component of the proposed strategy utilizes plant expression vectors that are capable of harboring a second gene of interest, selectable marker, and the cytotoxic *Diphtheria Toxin A* (*DTA*) gene. Expression of DTA is initially blocked by the presence of a *lox*-flanked triple terminator 'stuffer' sequence (35Sterm, nosTerm and ocsTerm). This allows transgenic plants generated with these vectors to be fully fertile (due to the stuffer-mediated blocking of DTA expression). When these fertile plants are crossed with transgenic plants containing the first component (used as the female parent), hybrid plants containing both components will be generated. The hybrids constitutively

expressing the Cre recombinase will undergo site-specific recombination that excises the stuffer region flanked by the directly oriented *lox* sites. Following stuffer excision, the *DTA* open reading frame will be fused to the upstream pollen-specific promoter separated only by a single 34bp *lox* sequence, thus transgene expression and reproductive ablation will be activated. Initial testing of the hybrid transgene containment system will be performed using hemizygous transgenic lines for crossing. Hybrid genotypes containing both constructs will be identified with seedling selection and molecular screening.

Our gene containment strategy would function far more efficiently with availability of homozygous-inbred lines because these can be crossed to produce 100% hybrid seed containing both gene containment constructs. Fully homozygous lines are also advantageous for breeding, particularly for exploiting heterosis and major QTL or advancement of new transgenic lines. Rapid creation of inbreds via doubling of haploid lines is feasible in some species, but haploids are difficult to obtain in most species including switchgrass. We are currently exploring the potential of centromere-mediated genome elimination to efficiently produce haploids. This approach has been successfully applied to Arabidopsis, but not to other plant species. In Arabidopsis it was observed that substitution of wild-type CenH3 for one of several mutant versions can lead to creation of semi-fertile haploid-inducer lines. These are believed to act through an early embryonic defect resulting in mis-segregation and elimination of chromosomes derived from the haploid inducer line. Mature haploid embryos or young plants can subsequently be artificially doubled by chemical treatment to produce completely homozygous diploid lines. Switchgrass genotypes that we work with are tetraploid, and we have found that switchgrass contains two copies of *CenH3* which we have designated *PviCenH3-1* and *-2*. We are now attempting to create functional knockouts of both genes using TALENs. These lines will be assessed for haploid induction rates. As this is new and unproven technology for switchgrass, we are also attempting a parallel approach using *Brachypodium distachyon*. Evidence in support of haploid induction can be achieved more quickly in this species. To date we have targeted 10 separate loci using TALEN pairs designed to induce double strand DNA breaks. Each pair is coordinately transcribed via a single maize ubiquitin promoter and is separated by a T2A translational skipping sequence. Three pairs target *PviCenH3-1*, three target *PviCenH3-2*, and four target *BdiCenH3*.