

106. Expanding the breeder's toolbox for perennial grasses: The use of the model perennial grass *Brachypodium sylvaticum* to identify combinations of transgenes conferring tolerance to multiple abiotic stresses

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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.

B. sylvaticum possesses all the traits necessary to serve as a model perennial grass. Despite its perennial nature, some accessions go from seed to seed in 3-5 months with no vernalization. It is self-fertile. It possesses a small genome size of approximately 350 Mb and a chromosome number of $2n=2x=18$. In addition, the close relationship between *B. sylvaticum* and *B. distachyon* allows leveraging the resources developed for *B. distachyon*. Unlike *B. distachyon*, *B. sylvaticum* flowers open, the stigmas exert before the anthers and the anthers shed copious amounts of pollen. These traits and its perennial nature are useful for studies that involve male/female sterility and many crosses.

We have developed a highly efficient transformation system, based on our *B. distachyon* transformation protocol. We have almost completed the transformation of *B. sylvaticum* with constructs comprising 20 genes listed in our proposal (Peleg et al., 2011; Reguera et al., 2013). These genes have been shown to be associated with the enhanced tolerance of monocots to abiotic stress. We have developed a highly efficient transformation system for *B. sylvaticum*, modifying our *B. distachyon* transformation protocol (average transformation efficiency = 55%). In most cases we used the stress-inducible SARK promoter (Delatorre et al., 2012). In some cases (i.e. WRKY47, HB4, NHX1, HSR1), where the constitutive expression is sought we also transformed under the control of the maize ubiquitin promoter sequence (Ubi). In a few cases, we will express the gene-of-interest under more restrictive promoter, in order to avoid secondary effects that could influence growth. In these cases (i.e., WRKY47 and the kinases OsK1 and OsK24) we will use a chemically-induced promoter that allows for tight control of gene expression. In addition, we have made constructs containing a combination of genes (i.e. SARK::IPT- Ubi::HSR1::Ubi::NHX1) in order to simultaneously overexpress genes associated with drought + heat tolerance + salt tolerance. We will make additional combination constructs after we have characterized the single gene constructs. We generated a large number of independent T0 lines for these constructs and are now in the process of generating T1 and T2 generations.

Enhanced stress tolerance of the transgenic plants will be assessed in the greenhouse. Wild type and transgenic plants will be grown in controlled greenhouse conditions and plants will be characterized

for response to a combination of water deficit, heat and salinity stress. Leaf material will be sampled periodically for the measurement of chlorophyll, starch, total and reducing sugars, and protein contents. Net assimilation rates, stomatal conductance and quantum efficiency will be obtained from analysis of gas exchange, during and after stress. Expression analysis of the different transgenes will be performed by quantitative PCR (endogenous transcription elongation factor (*TEF*) will be used as control).

To aid our planned RNA-Seq studies of the *B. sylvaticum* transgenics, we will sequence the genome of *B. sylvaticum*. In preparation, we have inbred the target line, Ain-1, through seven generations of single seed descent to decrease heterozygosity. We are now extracting DNA for sequencing at the UC Davis genomics center. In addition, we have created mapping populations and have harvested F₂ seeds. We will use genotype by sequence to create a high resolution genetic map based on ~500 F₂ individuals. Also, since *B. sylvaticum* is perennial, we can maintain the F₂ lines as an immortal, fully mapped segregating population. Interestingly, F₁ plants from independent crosses between Ain-1 and three other accessions, all exhibited a high degree of hybrid vigor.

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