Using the Model Perennial Grass *Brachypodium sylvaticum* to Engineer Resistance to Multiple Abiotic Stresses

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Abstract

We are using the perennial model grass *Brachypodium sylvaticum* to identify combinations of transgenes that enhance tolerance to multiple, simultaneous abiotic stresses. The most successful transgene combinations will ultimately be used to create improved switchgrass (*Panicum virgatum* L.) cultivars. To further develop *B. sylvaticum* as a perennial model grass, and facilitate our planned transcriptional profiling, we are sequencing and annotating the genome. We have generated ~40x genome coverage using PacBio sequencing of the largest possible size selected libraries (18, 22, 25 kb). Our initial assembly using only long-read sequence contained 320 Mb of sequence with an N50 contig length of 315 kb and an N95 contig length of 40 kb. This assembly consists of 2,430 contigs, the largest of which was 1.6 Mb. The estimated genome size based on c-values is 340 Mb indicating that about 20 Mb of presumably repetitive DNA remains yet unassembled. Significantly, this assembly is far superior to an assembly created from paired-end short-read sequence, ~100x genome coverage. The short-read-only assembly contained only 226 Mb of sequence in 19k contigs. To aid the assembly of the scaffolds into chromosome-scale assemblies we produced an F_2 mapping population and have genotyped 480 individuals using a genotype by sequence approach.

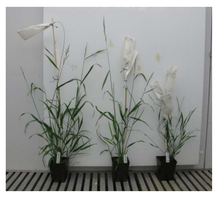
One of the reasons for using *B. sylvaticum* as a model system is to determine if the transgenes adversely affect perenniality and winter hardiness. Toward this goal, we examined the freezing tolerance of wild type *B. sylvaticum* lines to determine the optimal conditions for testing the freezing tolerance of the transgenics. A survey of seven accessions noted significant natural variation in freezing tolerance. Seedling or adult Ain-1 plants, the line used for transformation, survived an 8 hour challenge down to -6 ºC and 50% survived a challenge down to -9 ºC. Thus, we will be able to easily determine if the transgenes compromise freezing tolerance.

In the effort to develop biotechnological tools for perennial grass improvement, we have completed the transformation of *B. sylvaticum* with constructs containing 20 genes shown to be associated with enhanced abiotic stress tolerance in monocots. In addition, we have transformed plants with 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 generated single copy insert T_1 lines for all constructs and the generation and bulking of homozygous T_2 lines is well underway. In addition to our *B. sylvaticum* transgenics, we transformed *B. distachyon* with many of the same genes. Some of the transgenic *B. distachyon* plants subjected to a combined stress of both drought and salinity were able to produced higher yields than wild type plants. Our results indicate a great potential for the development of grasses with improved performance and yield in water-limited areas.

B. Sylvaticum as a model system

B. sylvaticum plants are compact, self-fertile, diploid, possess a relatively small genome (~340Mb), can go from seed to seed in 3 months and are perennial. (A) Three accessions at flowering stage. The pots are 10 cm tall. (B) While *B. sylvaticum* is self-fertile, the flowers do open and shed abundant pollen. This facilitates studies focused on gene containment, fertility and the creation of controlled crosses. The outcrossing rate is ~5% under greenhouse conditions. (C) *B. sylvaticum* can be transformed very efficiently.

A



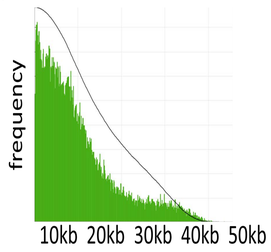
B

C

Experiment	Calluses co-cultivated	Transgenic plants	Efficiency
1	50	84	**168%**
2	64	10	**16%**
3	69	43	**62%**
4	236	51	**22%***
		average	**67%**

De novo genome assembly of *B. sylvaticum*

We have created a preliminary genome assembly containing only PacBio long read sequence. We generated 40x genome coverage from libraries created from DNA sheared to 20 kb before size selection (7 or 15kb). (A) Read length distribution from a typical SMRT cell. (B) Assembly statistics. Our next steps are to optimize the settings for the FALCON assembler to increase contig length, use a high-density genetic map to order contigs and polish the final assembly with 100X Illumina PE sequence.



Initial PacBio only genome assembly:
320Mb total assembled length
N50 contig length 315kb
N95 contig length of 40kb
2,430 contigs
Largest contig 1.6Mb
40X genome coverage

Genetic mapping of *B. sylvaticum*

QTL mapping and genome assembly resource

- *B. sylvaticum* inbred line, Ain-1, was crossed with the phenotypically and genetically divergent inbred line Sin-1
- 480 F2 progeny were grown and genotyped by sequencing
- Several phenotypes including height, flowering time and hairiness were scored
- Sin-1 mapping parent was sequenced and assembled using 100x coverage of 150bp paired-end Illumina reads
- Genetic map construction is in progress.

Generation of *B. sylvaticum* transgenic plants

We are using *B. sylvaticum* to optimize transgenic approaches to engineer tolerance to multiple abiotic stresses. Previously we identified 20 genes that enhance tolerance to salt, drought or heat in other plants. We have transformed all of these genes into *B. sylvaticum*, some under the control of multiple promoters. We will test the stress tolerance of these lines and conduct transcriptional profiling to identify transgenes that work through different mechanisms. We will also test the freezing tolerance and perenniality of the lines to identify unwanted side effects. Using all of this information, we will select combinations of transgenes that should enhance tolerance to multiple abiotic stresses. These combinations will be transformed into *B. sylvaticum* and characterized. The best combinations will ultimately be transformed into switchgrass. The table shows the current status of our transformation efforts. We have created transgenics and identified homozygous, single-insert lines for all constructs. Bulking seed for further characterization is underway.

Construct	T0 single copy insertion positive lines	T1homozygous germinating lines	T1 homozygous seeds to phenotype
SARK::DWF4	12	9	2
SARK::DWF5	5	5	4
SARK::HYD1	9	8	2
SARK::STR16	9	5	1
SARK::OsMADS57	11	8	1
SARK::OsWRKY47	7	7	4
Ubi::OsWRKY47	15	4	2
SARK::BAK1	7	4	1
SARK::BRI1a	14	12	2
SARK::BRI1b	13	9	4
SARK::BRIL300	16	10	4
SARK::BIN2	11	7	2
SARK::ZIP1	13	1	
SARK::ZIP2	13	7	3
SARK::OsK24	7	4	2
SARK::OsK1	9	9	2
SARK-HB4	16	8	1
Ubi-HB4	9	3	
SARK::IPT	12	9	6
Ubi::NHX1	8	10	4
Ubi::HSR1	10	9	4
SARK::IPT-Ubi::HSR1	12	8	4
SARK:IPT-Ub:HSR-Ub:NHX1	16	10	4
SARK::IPT-Ubi::NHX1	13	11	3
Ubi:CWL-GFP	7	7	
pOL:WRKY47	9	3	
pOL:osk1	9	4	
Ubi::HSR1-Ubi::NHX1	15	8	
UBIL-XYN	19	3	
UBIL-BdCV	5	2	
SUM	331	199	62

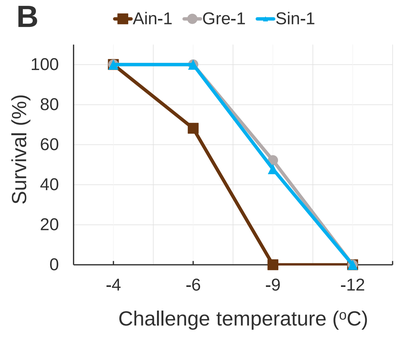
Freezing tolerance of *B. sylvaticum*

To ensure that our transgenic approaches to enhance abiotic stress tolerance do not compromise perenniality or hardiness, we will test the freezing tolerance and perenniality of the transgenic lines. Toward this goal, we have optimized test conditions. As expected, *B. sylvaticum* gains freezing tolerance after vernalization as shown in **(A)** where the plant vernalized for 4 days at 4ºC survived a challenge of - 4ºC for 8 hours and the non-vernalized plant died. We compared the freezing tolerance of seven natural accessions and noted significant natural variation. The freezing tolerance to 8 hours at various temperatures is shown in **(B)** for seedlings and in **(C)** for adult plants after flowering and seed set. Using these conditions we will be able to test the freezing tolerance of the transgenic lines.

A

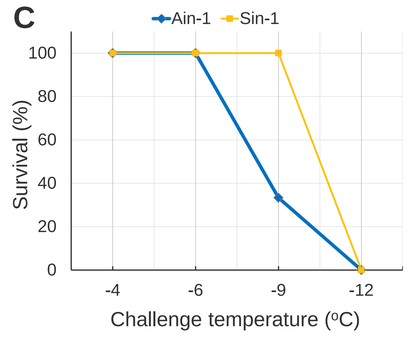
No vernalization 4 days @ 4ºC

B



Seedlings surviving 8 hours at the challenge temperature after 6 weeks of vernalization.

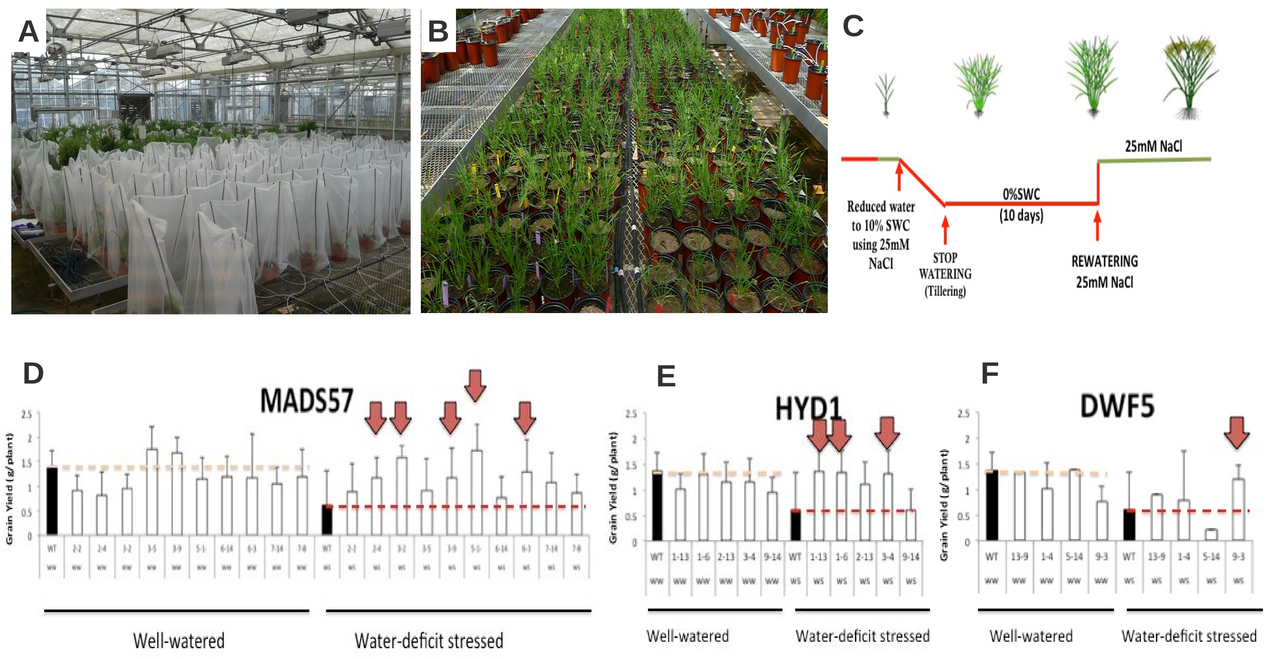
C



Mature plants surviving 8 hours at the challenge temperature after 6 weeks of vernalization.

Phenotypic analysis of transgenic *Brachypodium* plants

T0 and T1 transgenic *B. sylvaticum* transgenic plants growing in the greenhouse (**A**). Typical experiment for the assessment of stress tolerance of transgenic plants (**B**). Stress treatments of transgenic *B. distachyon* expressing Os*MADS57 (*transcription factor*),* or *OsHYD1 or OsDWF5 (*associated with Brassinosteroid synthesis and signaling) started at the 4th leaf stage. Plants were continuously irrigated with the saline solution (25 mM NaCl) to mimic conditions that are typical of poor quality soils and water. The typical electrical conductivity of the watering solution flowing through the pots at the end of the experiment was about 4.5-5.0 dS/m (equivalent to 45 mM NaCl). At the tillering stage, watering was halted for 10 days and resumed using a solution containing 25 mM NaCl until harvest (**C**). The expression of *OsMADS57* (**D**), *OsHYD1* (**E**) and *OsDWF5* (**F**) resulted in enhanced tolerance of the transgenic plants after a stress episode with yields similar to those displayed under normal watering conditions.



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