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Development of a gene containment system to minimize gene flow from transgenic switchgrass.

Agricultural cultivation of transgenic switchgrass (a wind-pollinated species) with improved traits raises the possibility that engineered traits may move into natural populations with potential unintended environmental impacts. To develop a means to control transgene escape to wild plant populations we designed and evaluated a transgene containment system to block transmission of transgenes via pollen in the model perennial grass *Brachypodium sylvaticum* and switchgrass (*Panicum virgatum*). Using *Agrobacterium*-mediated transformation, we generated transgenic plants that express Barnase (a toxic protein) under the control of four rice pollen-specific promoters. Their expression in transgenic plants is expected to ablate the transgenic pollen. Independent transgenic lines for each construct were evaluated by pollen staining and/or genetic segregation analyses. Alexander's staining revealed that, relative to wildtype plants, >50% of the pollen collected from the *Brachypodium* T_0 transgenic plants was dead or deformed. Analysis of self-pollenated T_1 *B. sylvaticum* progeny showed that transgene heritability was 1:1, consistent with the expected segregation frequency for a male lethal gene, supporting the conclusion that successful ablation of transgenic pollen was achieved in these transgenic plants. Based on those results, we generated and characterized more than forty independent switchgrass Alamo A4 transgenic plants carrying the gene containment constructs successfully used in Brachypodium, for their effectiveness at mediating transgenic switchgrass pollen ablation.

Two construct types, one with the *PS1* (*Pollen Specific 1*) promoter and the other with the *PS2* (*Pollen Specific 2*) promoter produced transgenic switchgrass plants that grew and developed normally and produced morphological normal flowering panicles. The other two constructs containing the *PS3* and *OsGEX2* pollen-specific promoters produced transgenic switchgrass plants with morphological and/or developmental vegetative growth defects, suggesting that Barnase expression was not limited to switchgrass pollen.

To assess the viability of the transgenic pollen generated from the *PS1* and *PS2*-barnase switchgrass plants, pollen was again stained using a modified Alexander's staining method. Almost all of the tested *PS1* and *PS2* transgenic switchgrass plants produced a significantly higher frequency of abnormal staining pollen (blue arrows shown in **Figure 1a**) than wild type plants, where approximately 85% of the pollen grains uniformly stained red (fuchsia arrow in **Figure 1a**). Stained pollen samples containing 1000 to 2000 grains were also visibly scored for the distribution of the number of normal grains verses the number of ablated/abnormal stained pollen grains. Data from seven of the *PS2-barnase* and eight of the *PS1-barnase* lines is shown in **Figure 1b** and **1c**. Since the tested T_0 transgenic plants are hemizygous (but may contain multiple transgenic loci), 50% or more of the normally viable pollen is expected to be ablated and scored as abnormal. Thus, it is expected that the transgenic plants will produced some viable (presumably nontrangenic) pollen. The results are consistent with most of the transgenic plants

(except PS2-13) producing between 80% and 95% abnormal pollen. All of the transgenic lines also produced some (between 5% and 15%) normally staining pollen.

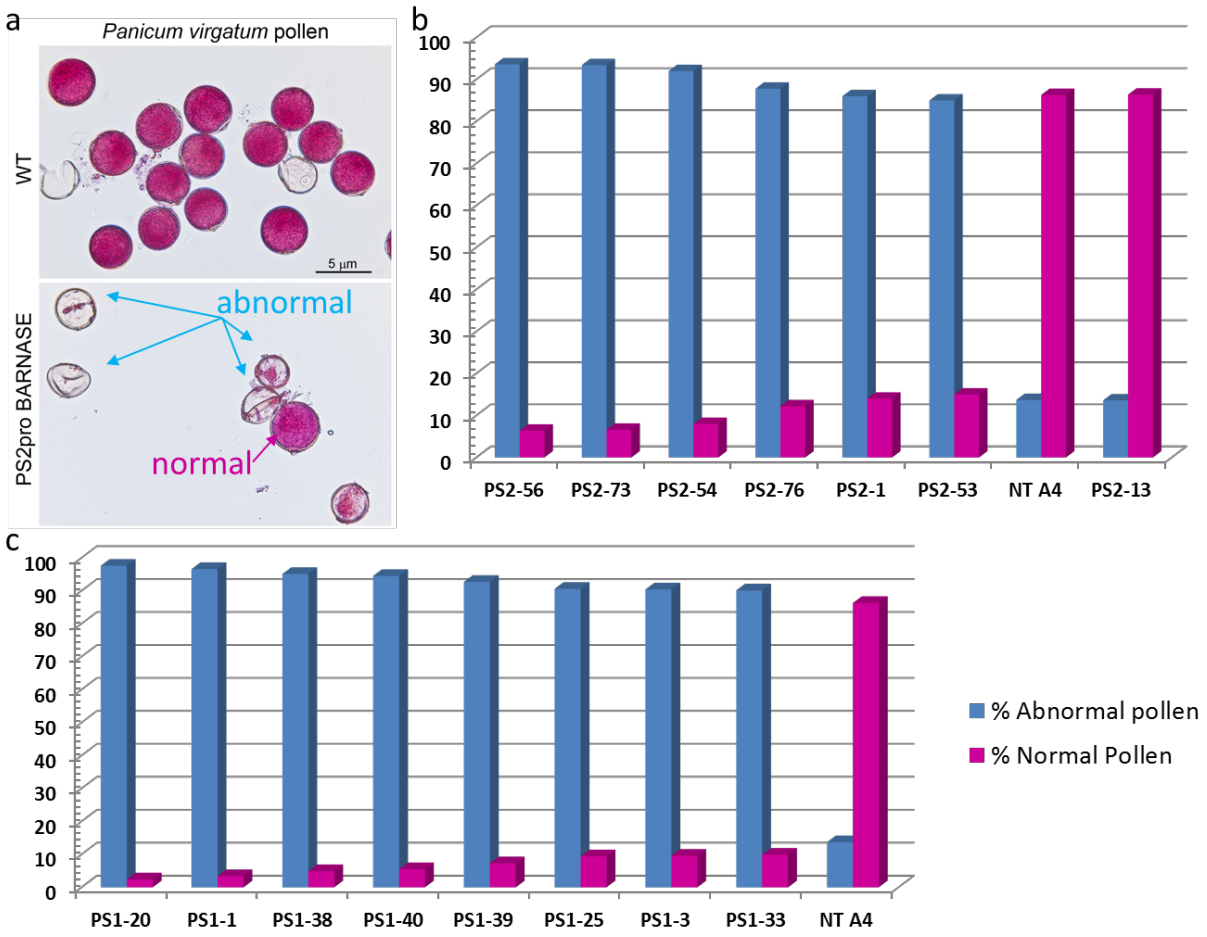

Fig. 1. Switchgrass pollen was collected from mature anthers and incubated in Alexander's stain. **a**). Example microscopic images from wildtype (WT) and *PS2-barnase* (PS2pro BARNASE) transgenic stained pollen. **b**). The quantified percentage of abnormal pollen (blue bars) and normal pollen (fuchsia bars) for seven *PS2-barnase* transgenic events and one wildtype switchgrass Alamo A4 plant (NT A4). **c**). The quantified percentage of abnormal and normal pollen for eight *PS1-barnase* transgenic events and one wildtype plant is shown.

To genetically validate the conclusion that the transgenic pollen is being ablated, we performed crosses between the *PS1* and *PS2* transgenic plants and recipient nontransgenic switchgrass plants. Since switchgrass is self-incompatible, all of the seed generated on the wildtype plant panicle is expected to be derived from pollination mediated by the transgenic plant pollen donor (i.e. hybrid progeny). The hybrid seed produced was harvested and germinated on antibiotic selection to screen for any progeny that contain the introduced transgene selection marker (indicating pollen-mediate transgene transmission). Normal transgene transmission would be expected to produce 50% or more of the progeny that are resistant to hygromycin, the antibiotic selection marker used to identify the transgenic switchgrass events. One *PS1-barnase* transgenic event has not produced any transgenic hybrid progeny that are antibiotic resistant and two other events showed transgene heritability at levels below 8% and 5%. The testing of additional

transgenic events and additional progeny from crosses is being performed to identify the best performing transgene containment construct and events that reduce or eliminate detectable transgene transmission via pollen.

Development of a CENH3-based method for creating doubled haploid grasses and demonstrate it in switchgrass.

Switchgrass neo-octaploid lines

The induction of neo-octaploids from the high-biomass *'Liberty'* switchgrass cultivar has been accomplished by seedling treatment with colchicine. A paper describing this population has been published in the journal *Bioenergy Research* (Yoon et al. 2017). Seedling treatment to induce polyploidy can be effective for breeding purposes where genetic exchange between different cytotypes is desired. In this study the tetraploid cultivar 'Liberty' was chosen because it is of recent hybrid upland/lowland origin, has superior yields, and is adapted to hardiness zones that are considered suitable for upland cultivars which are frequently octaploid.

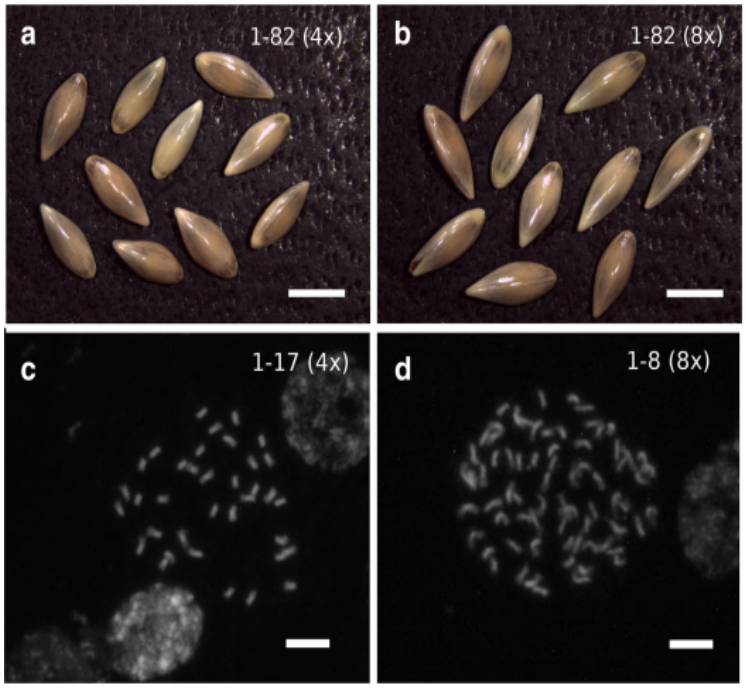

Fig. 2. Seed size and representative chromosome squashes from tetraploid and octaploid samples of Liberty switchgrass. Identical scale photographs of seed samples from **a** tetraploid and **b** octaploid sectors of maternal 1-82, a single genotype treated with colchicine at the 2–3 leaf seedling stage (scale bar 1 mm). Different genotypes treated with colchicine at the 2–3 leaf seedling stage were used to prepare root tip squashes 6 months after treatment. **c** Line 1-17 with 36 individual chromosomes visible. **d** Line 1-8 with approximately 70 individual

Initially treated individuals were largely cytochimeras containing 4x and 8x sectors. However, individual octaploid tillers were identified by flow cytometry and allowed to intercross to generate a 100% octaploid population. Seed harvests indicated that seed derived from octaploid sectors was 19% larger compared to tetraploid sectors and that fertility in these sectors was significantly lower (Fig. 2). Nonchimeric octaploid progeny of the treated individuals were confirmed to have approximately twice the number of chromosomes of tetraploid lines although there was high variability in these counts. Induced octaploids had larger pollen and leaf cell size. Guard cell density was found to be significantly lower in induced octaploid compared to tetraploid individuals. An earlier publication found no substantial differences in guard cell density in some naturally occurring octaploid and tetraploid switchgrass populations. Our results showing significant differences should have implications with respect to gas exchange rates and photosynthetic capacity.

This population has been clonally propagated and sent to ARS collaborators at the University of Lincoln, Nebraska. Intercrosses with other octaploid populations are being conducted as is further phenotypic analysis. One potential benefit is that the larger seed size will improve seedling vigor and stand establishment, as these traits are strongly correlated in

switchgrass and other perennial grasses.

Toward generation of haploid inducer lines via Centromere Mediated Genome Elimination

Haploid technology has been effectively used in a wide variety of plants, advancing many aspects of breeding and practical genetics. Haploid plants, whether derived from a diploid or a polyploid, have *half* the chromosome number of the euploid form. If the haploid genome is doubled (either spontaneously or via the application of anti-mitotics like colchicine) the resulting doubled haploid lines are completely homozygous. This creates inbred lines in a single generation eliminating the need for many generations of selfing. Furthermore, since switchgrass and many of the grasses proposed as energy crops are self-incompatible, efficient haploid-inducing lines would enable the production of recombinant inbred population, a currently unobtainable goal. Development of this transformative technology would open novel opportunities for the genetic improvement of switchgrass.

The technical approach to create haploid inducing switchgrass lines has been to eliminate the existing wild-type copies of CenH3 through genome editing while introducing via a crossing strategy or transgenesis one of several modified CenH3 versions intended to complement the defect that have been demonstrated in several other plant species to cause aberrant mitoses and whole genome elimination. This is thought to arise through missegregation and loss of chromosomes whose centromeres are tagged with the modified CenH3 during early embryogenesis. Initial attempts to engineer a non-transgenic genome edited Brachypodium and switchgrass CenH3(-) lines has resulted in measurable target mutagenesis in CenH3 genes using either TALON or CRISPR/Cas9 constructs. However, this activity has not led to the isolation of completely homozygous individuals (See previous reports). This failure is likely due to deleterious effects during plant regeneration or subsequent lethal gametophytic effects. CenH3 is a critical component of faithful chromosome segregation and it is likely that many mutations were not tolerated. Our latest strategy has been to transform *Brachypodium sylvaticum* and switchgrass with binary plant transformation vectors containing an expression cassette with several sgRNA's separated by tRNA spacers that are complementary to different regions of the CenH3 gene(s). These also carry a gene expression cassette that is designed to express one of several modified versions of CenH3 suspected of triggering genome elimination that are lacking complementarity to the sgRNA's and that are thus "immune" to editing (Table 1).

Table 1. Transformation Summary of *Brachypodium sylvaticum* and switchgrass. Each row indicates an individual transformation experiment. The individual sgRNA's were different between species but remained the same within a species for the different experiments. The different complement gene cassettes were either for control purposes (blue) or were engineered to disrupt CenH3 activity (orange). Tailswap indicates that the N-terminus of CenH3 has been swapped with the N-terminus of Histone H3, or the N-terminus of CenH3 from either *Zea mays* (Zm) or Arabidopsis (At).

Gene Construct	Species	No. sgRNA	Complement	Recovered Lines
1	B. sylvaticum	0	Immunized BdCenH3-WT	16
2	B. sylvaticum	4	Immunized BdCenH3-WT	17
3	B. sylvaticum	4	GFP-BdCenH3	0

4	B. sylvaticum	4	GFP-Tailswap BdCenH3	20
5	switchgrass	0	Immunized PviCenH3.1-WT	3
6	switchgrass	8	Immunized PviCenH3.1-WT	17
7	switchgrass	8	Immunized PviCenH3.2-WT	0
8	switchgrass	8	PviCenH3.1-G75E	6
9	switchgrass	8	PviCenH3.1-L122F	2
10	switchgrass	8	GFP-PviCenH3.1	1
11	switchgrass	8	Zm-Tailswap PviCenH3.1	13
12	switchgrass	8	At-Tailswap PviCenH3.1	7
13	switchgrass	8	GFP-Tailswap PviCenH3.1	2

We have recovered several confirmed transgenic lines so far, but these are still semi-characterized. We have sequenced PCR amplicons from the target site regions in multiple tillers to determine if the T0 plants contain target-site mutations and we are vegetatively propagating them in order to isolate bi-allelic knockouts through the continuous presence and activity of CRISPR/Cas9. Thus far, we have confirmed frameshift mutations in 3 out of 20 transgenic lines from construct #4, but these three are heterozygous and two out of the three appear to be infertile. All the lines will be vegetatively propagated and screened at least one more time for target-site mutations. Individual tillers from all of the T0 switchgrass lines have been harvested for seed collection and several tillers will be screened for target site mutations as above by amplicon sequencing. The same general strategy will be taken and if there are promising lines, these will be pursued.

Use of the model perennial grass *Brachypodium sylvaticum* to identify combinations of transgenes that confer tolerance to multiple abiotic stresses.

We examined wild-type inbred lines from different geographic regions to characterize stress-susceptible and stress-tolerant traits, typical of perennial grasses. We examined wild-type inbreed lines from different geographic regions and we characterized a stress-sensitive (Ain1, from Ain Duram, Tunisia) and a stress-tolerant (Osl1, from Oslo, Norway) *B. sylvaticum* accessions. We tested the salt tolerance of Ain1 and Osl1 (Sade et al., 2018, Plamt Mol. Biol. 96:305-314) and freezing tolerance (Fig. 3) (Sade et al., in preparation for submission to Global Change Biol.). Our results showed that Osl1 displayed a higher tolerance to salinity and freezing than Ain1. We developed methods for the genetic transformation of *B. sylvaticum* Ain1 and used the non-transformed Ain 1 as the wild-type line (see below).

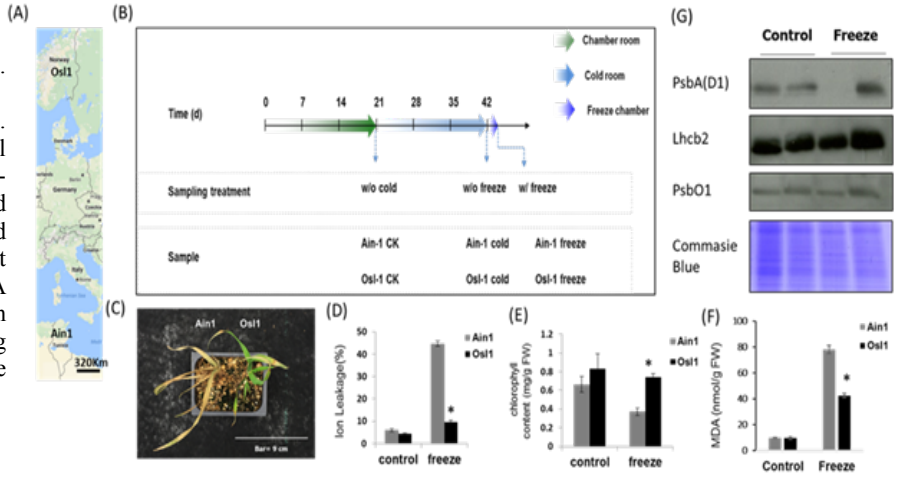

Fig. 3. Effect of freezing on two B. Sylvaticum accessions (Ain1 and Osl1). (A) Geographical habitat of the B. Sylvaticum accessions (B) Experimental setup (C) Ain1 and Osl1 phenotype post-stress (D) Ain1 and Osl1 Ion leakage and (E) Chlorophyll content (F) Lipid Peroxidation (MDA) and (G) Immunoblot analysis of PSII subunits Lhcb2, PsbA (D1), and PsbO1. Values are Mean ± SE (n = 3-12). The data were analyzed using Student's t test. Asterisks indicate significant differences ($P \leq 0.05$).

The generation of transgenic *B. sylvaticum* expressing the genes (20 genes) listed in the original proposal was completed (Table 2). A q-PCR-based method for the selection of single transgene copy lines (that eliminates the need of Southern blots and shortens considerably the time and efforts needed for obtaining homozygous

Construct	T0 single copy insertion positive lines	T1homozygous germinating lines	T2 homozygous lines with seeds
SARK::DWF4	12	10	10
SARK::DWF5	5	5	5
SARK::HYD1	9	8	8
SARK::STR16	9	9	7
SARK::OsMADS57	11	8	5
SARK::OsWRKY47	7	7	7
Ubi::OsWRKY47	15	9	9
SARK::BAK1	7	7	7
SARK::BRI1a	14	12	12
SARK::BRI1b	13	9	9
SARK::BRIL300	16	10	10
SARK::BIN2	11	8	8
SARK::ZIP1	13	6	6
SARK::ZIP2	13	8	8
SARK::OsK24	7	4	4
SARK::OsK1	9	9	7
SARK-HB4	16	13	13
Ubi-HB4	9	4	3
SARK::IPT	12	9	7
Ubi::NHX1	8	8	8
Ubi::HSR1	10	9	9
SARK::IPT-Ubi::HSR1	12	8	8
SARK:IPT-Ub:HSR-Ub:NHX1	16	11	11
SARK::IPT-Ubi::NHX1	13	11	11
Ubi:CWL-GFP	7	7	6
pOL:WRKY47	9	5	5
pOL:osk1	9	6	6
Ubi::HSR1-Ubi::NHX1	15	10	10
UBIL-XYN	19	9	9
SARK-XYN	5	5	3
Ubi:STR	6	3	-

Table 2: List of transgenic plants expressing stress-associated Genes and gene combinations.

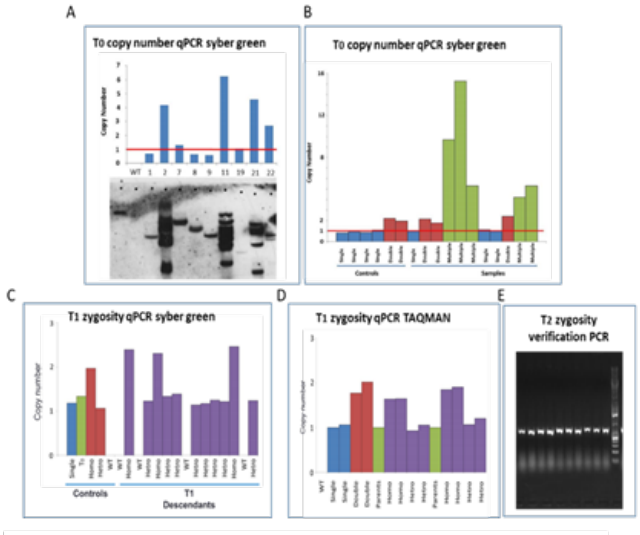

Fig. 4. High-throughput genotyping method.

transgenic lines) was developed. Basically, we used a specific amplicon of *hph* (encoding hygromycin B phosphotransferase) to confirm the presence of single, double or multiple inserts (Figs. 4 A,B). To increase the accuracy of the technique, the amount of DNA between the samples was normalized, a single copy of a *B. sylvaticum* gene was used as an internal control. The q-PCR-based method can be used for screening zygocity as early as in the T1 generation. The method was validated using Southern blots (Fig. 4A), an independent PCR method (TAQMAN, Fig. 4D) and PCR analysis of T2-generation plants (Fig. 4E).

Stress treatments and phenotypical analyses of *Brachypodium sylvaticum* transgenic plants.

Several stress treatments were developed and tested (Fig. 5): Normal irrigation (100% soil water content- SWC) (Fig 5A). For water-deficit stress treatments, the plants were fully irrigated until the 4th leaf stage and watering was stopped (Fig 5A) until harvest. For salinity stress treatments, the plants were fully irrigated until the 4th leaf stage, and the salt concentration of the watering solution was gradually increased to 200 mM NaCl. These salinity levels were maintained until harvest (Fig. 5A). A clear penalty of all the stress treatments was visible in wild-type untransformed *Brachypodium* plants (Fig. 5B). For the characterization of stress-specific genes/pathways it is important to express genes-of-interest using stress-specific promoters, in

order to avoid the influence of pleiotropic effects. We cloned and characterized a stress-inducible promoter (pSARK). We tested the induction of the promoter activity in transgenic B. sylvaticum Ain1 plants and showed that this promoter displayed low basal activity when the plants were grown under control conditions, but the expression was induced (GUS activity) when the transgenic plants were exposed to drought and salinity conditions (Fig. 6). On the other hand, plants expressing the constitutive promoter (UBQ) displayed GUS activity in all conditions.

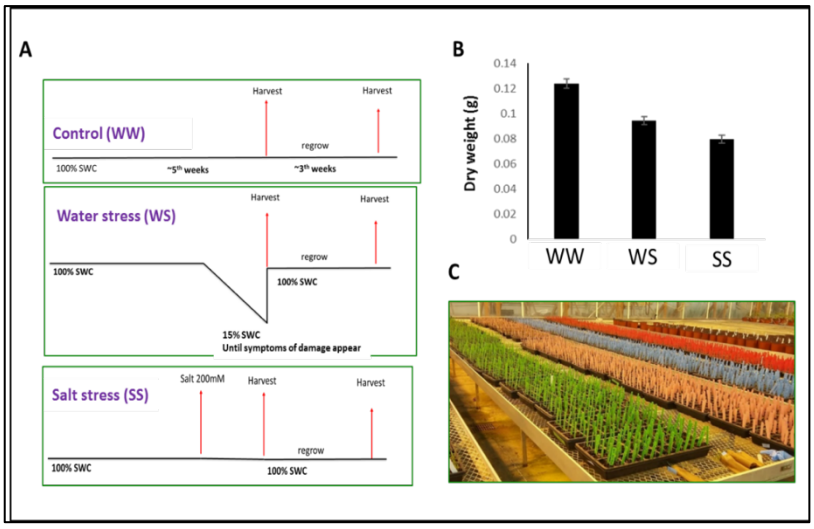

Fig. 5. Stress treatments. (A) Control (WW); Water stress (WS) and salt stress (SS); (B) yield penalty (dry weight) induced by the different stress treatments.(C) Typical stress experiment

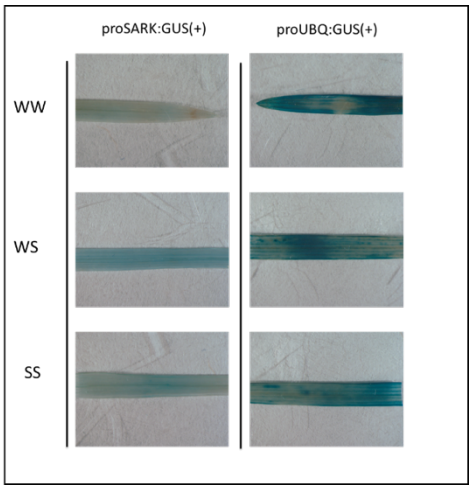

Fig. 6. Characterization of a stress inducible (*SARK)* and a constitutive promoter (UBQ) in *B. sylvaticum* transgenic plants under control (WW) water deficient (WS) and salt stress (SS)

We completed the genotypical and phenotypical characterization of all the transgenic lines under different abiotic stresses. The expression of several genes resulted in enhanced tolerance to water deficit and salinity (*OSK1, BRIL-300,SARK-IPT, STR16,* etc.). All these lines are available to all researchers in the field. A typical experiment for the assessment of stress tolerance of transgenic plants is described below (transgenic *B. sylvaticum* expressing *pSARK::BRIL-300). BRIL300* (LOC_OS11g06780) is a member of the Receptor-Like Cytoplasmic Kinases (RLCK) family, encoding a putative BRASSINOSTEROID INSENSITIVE 1-LIKE precursor involved in BRs signaling. Wild type and two independent transgenic *B. sylvaticum* lines(*BRIL300 1-1; BRIL300 19-3*) displayed enhanced growth (Fig. 7A) and higher biomass accumulation (Fig. 7B) after exposure to water-deficit stress (WS) or salinity (SS). We also tested the effects of *BRIL300* expression on perenniality. Following growth under stress conditions, leaves were harvested, plants were re-watered and were grown for 3 weeks (to mimic multiple year-harvesting). Growth of the transgenic lines was similar to that of WT plants, indicating that perenniality was not altered in the transgenic plants (Fig. 7C). To identify processes associated with *BRIL300* expression, we performed gene expression profiling of WT and *SARK:BRIL300* plants grown under control, water-deficit and salinity stress conditions (Fig. 8). Venn diagrams of the RNASeq data obtained from WT and SARK:BRIL300 at WW, WS and SS conditions revealed a stronger response of WT plants to stress conditions. For example, under salt stress (SS) , WT plant showed 3875 differentially expressed genes (DGE) while SARK:BRIL300 plants only displayed 1015 DGE (Fig. 8A). A similar trend was seen in water-deficit stress (WS) (i.e., 4230 DGE in WT vs 1179 in

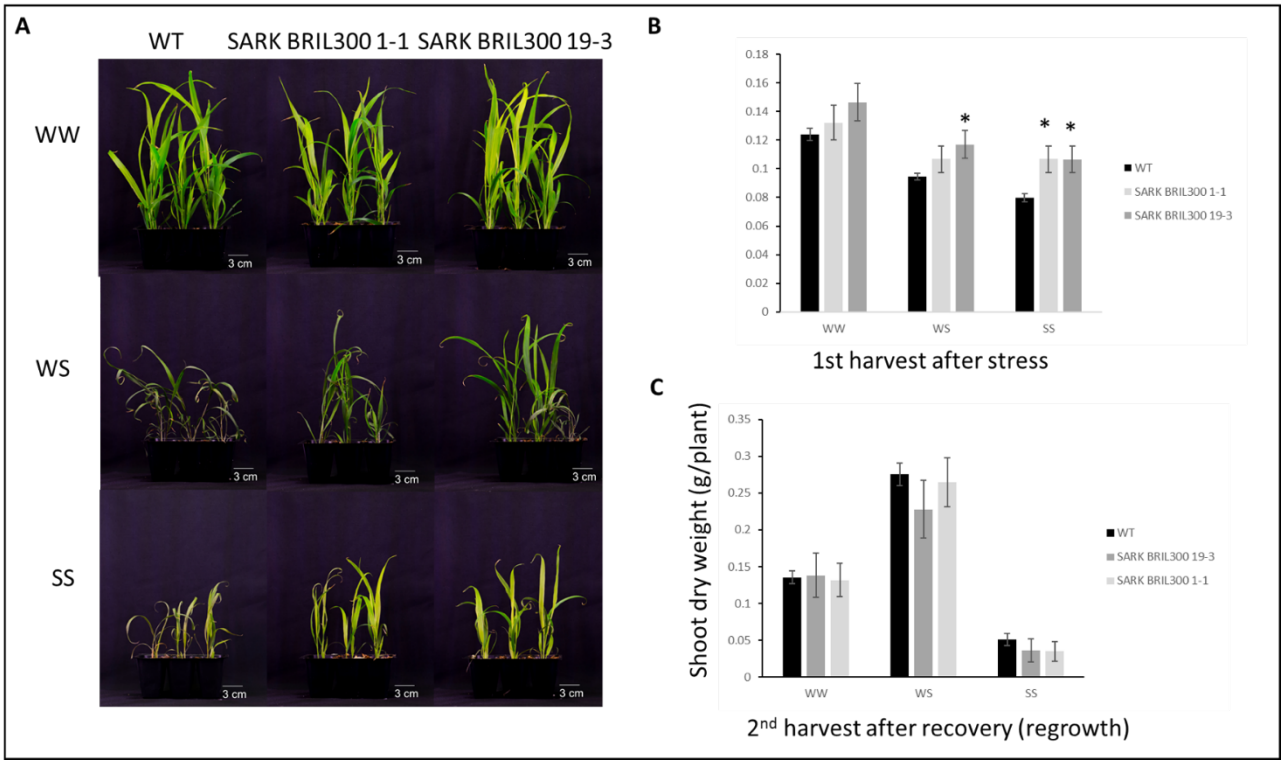

Fig. 7. (A) Phenotype of stress experiment; (B) growth of WT and transgenic B. sylvaticum plants expressing a BRIL300 construct. (C) re- growth of WT and transgenic B. sylvaticum plants expressing a BRIL300. Values are the Mean +/- SE (n = 16). Asterisks indicate significant differences ($P < 0.05$) between transgenic lines and WT.

SARK:BRIL300; Fig 8B). We examined the GO enrichment of genes displaying a differential expression response to stress and mapped the DGE into the KEGG database (data not shown). The data is being analyzed and it will be made available to the research community through scientific papers and incorporation to databases (Phytozome, JGI; KBase, DOE; PlaNet, (aranet.mpimp-golm.mpg.de), etc.).

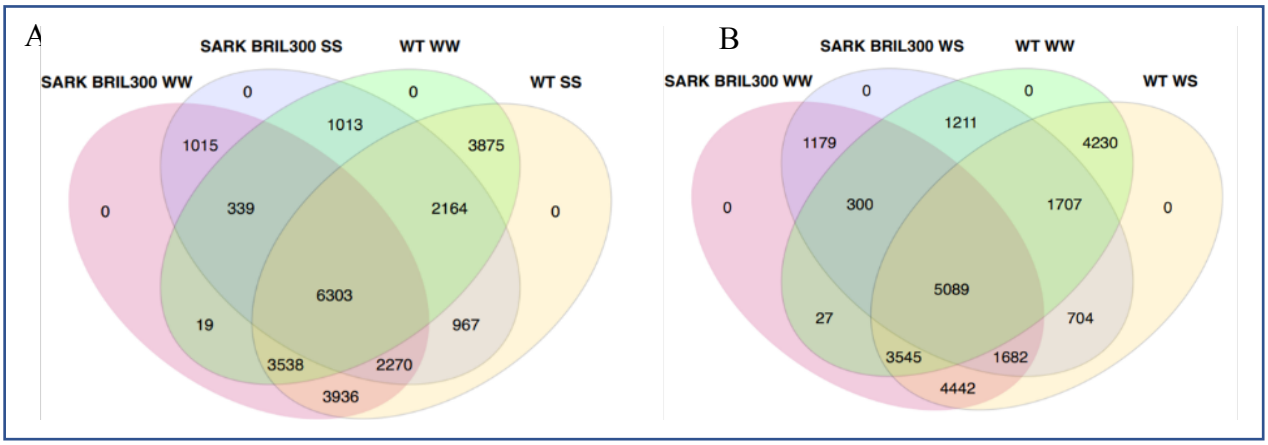

Fig. 8. Venn diagrams of *B. sylvaticum* WT and SARK BRIL300 and corresponding treatments
Samples corresponding to 3-5 biological replicates from two genotypes (WT and SARK BRIL300) and two different treatments were analyzed (either well water (WW) , water stress (WS) or salt stress (SS)- represented by different color-codes.

Sylvaticum genome assembly and annotation

To provide an essential resource for the use of B. sylvaticum as a perennial model system, we sequenced the genome of the line used for transformation, Ain-1. We created a highly contiguous genome assembly from 80X of PacBio sequence. The final assembly contained 1,117 contigs with a contig N50 of 876,844. A high density genetic map was created from an F2 mapping population consisting of 288 individuals. The genetic map was used to define 9 major linkage groups and anchor contigs within 9 psuedomolecules yielding 629 scaffolds and a scaffold N50 of 4 Mb. 99.98 percent of the total genome sequence was included in the 9 *B. Sylvaticum* psuedomolecules.

To discover details about how *B. sylvaticum* responsed to abiotic stresses, we used RNA-Seq to characterize the transcriptome in a variety of tissues/treatments (e.g. drought, heat and salt stress). Treatments included: freezing, cold acclimation, seedling tissues (root, leaf, crown), adult tissues (leaf, root, crown, node, internode), three forms of nitrogen, developing seeds and flowers at various stages. During this work we included samples that will allow *B. sylvaticum* to be compared to other grasses being characterized in the JGI gene atlas project. All of the data has been collected and analysis is ongoing.

To aid the annotation of the genome, we created a transcriptome assembly from 16 of the gene atlas samples. Approximately 300 million 150bp PE Illumina RNA-Seq reads mapped to the *B. sylvaticum* genome assembly and were assembled into 183,172 redundant cDNA contigs with a mean length of 607bp and max length of 8,086bp. In addition, we had 38,102 high-quality non-redundant consensus isoforms from PacBio single molecule sequencing with mean length of 1,234bp and max of 10,779bp. We used this transcriptome information to provide information about specific genes of interest to this project. The annotation identified 48,863 gene loci. Interestingly, the number of genes and gene density was higher than in *B. distachyon* or *B. stacei* indicating that the increased genome size was mostly due to additional genes and not simply an increase in repetitive DNA as we had expected. We worked with JGI's Phytozome team to incorporate the B. sylvaticum into Phytozome where is can be used by the research community (Fig 9).

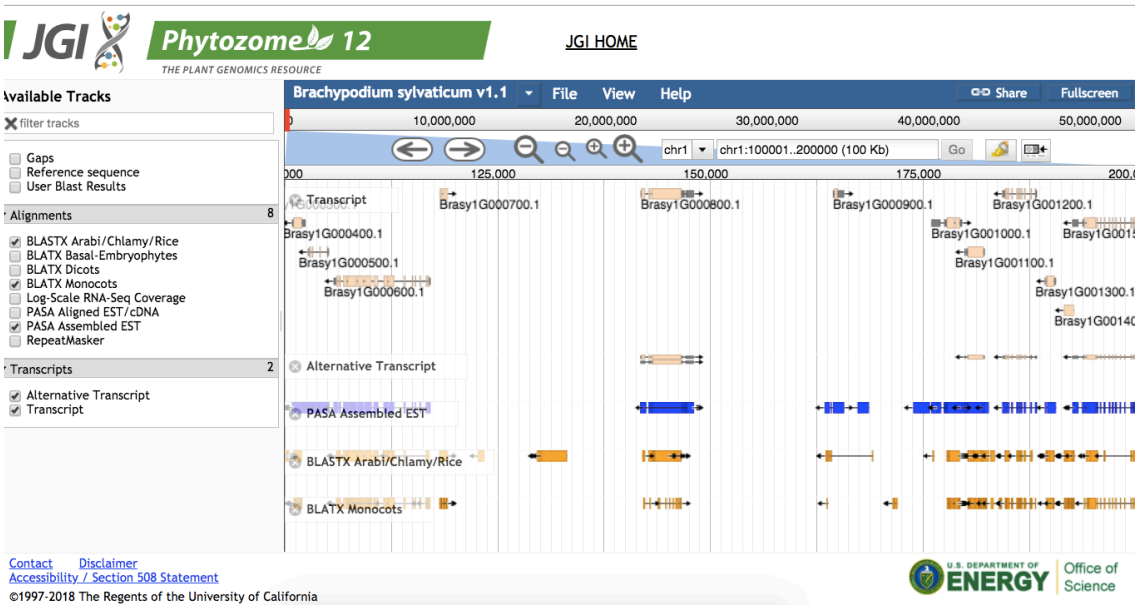

Fig. 9. Brachypodium sylvaticum portal at Phytozome.

Publications:

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Sade N, Wilhelmi MM, Tobias CM, Thilmony R, , Blumwald E, Vogel JP (2019). Comparative genomics and transcriptomics reveal fundamental differences between annual and perennial grasses. **Genome Biology** (to be submitted 1/5/2019).