Novel molecular markers for monitoring the gene flow from herbicide-resistant crops to closely related species

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INTRODUCTION

- Concerns about gene flow from sorghum to its weedy relatives (shattercane, SH; and johnsongrass, JG) have limited regulatory approval of genetically modified traits, such as herbicide resistance. This significantly limits sorghum production relative to corn [*Zea mays* L.], despite sorghum's greater drought tolerance.
- ALS-resistant (ALS-R) grain sorghum ('Inzen', DuPont-Pioneer) is in the final stages of commercialization. Inzen carries a target-site mutation in the ALS gene ($Trp_{574}Leu$) conferring resistance to imidazolinone and sulfonylurea herbicides.
- Inzen will inevitably outcross to SH and JG; thus, its unique alleles may serve as an effective landscape-level indicator of weed evolution responses when a new nuclear crop trait is highly adopted by growers, transfers to weedy relatives, and increases their fitness in typical production systems.
- Kompetitive allele-specific PCR (KASP) markers are a fluorescence-based SNP genotyping technology. Molecular inversion probes (MIPs) have been used in human genetics for many years to screen populations of cancer cells at rapidly evolving loci. MIPs can be multiplexed, and each one can amplify target sequences from 1-500 bp.
- The high-throughput, low-cost nature of these markers makes them especially suitable for large-scale population genetic studies.
- Here we present the current status of KASP and MIP marker development intended to monitor ALS allele variants and SSR loci in sorghum, SH, and JG as the foundation of a regional monitoring program for crop-to-weed gene flow and weed population genetic diversity following commercial deployment of Inzen sorghum.
- Similar methods could be deployed for any ecological risk monitoring study.

OBJECTIVE

Create inexpensive, high-throughput molecular markers capable of genotyping many individuals from weedy *Sorghum* populations to monitor ALS-R evolution.

MATERIALS AND METHODS

- KASP primers targeting a unique short-range haplotype of Inzen (Val560Ile + Trp574Leu) were used to screen samples of Inzen (ALS-R), a wild-type grain sorghum hybrid (87P06, DuPont-Pioneer), and SH and JG populations segregating for wild-type and ALS-R alleles (Werle et al, 2014) (**Figure 1** & **Table 1**).
- Libraries were prepared for 7 samples previously genotyped by KASP using a KAPA HyperPlus kit and KAPA A & B adapters. Size selection was optimized for 500-bp fragment lengths using KAPA Pure Beads. Adapter-ligated libraries were amplified in 8 cycles of PCR to generate 100-ng finished libraries. Samples were normalized such that JG DNA concentration was 2x that of sorghum and SH to assure equal coverage of its two parental genomes (*S. bicolor* and *S. propinquum*).
- Whole-genome sequence was generated on an Illumina NextSeqTM 500 using 75-bp paired-end reads. 1X coverage was obtained using a gap-tolerant aligner (GSNAP) to the reference genome (v3.1). Alignments at ALS are found in **Figure 2**.
- 41 SSRs (Billot et al, 2013; **Table 2**) used to genotype over 3300 accessions of wild and domestic sorghum from the global composite germplasm collection (GCGC) covering all ten chromosomes are being converted into MIPs.

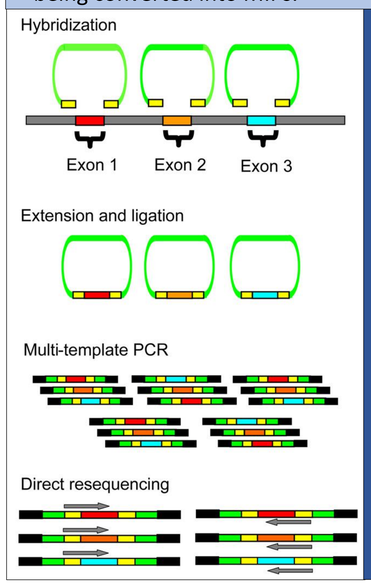

Figure 4. Schematic detailing molecular inversion probes using exon capture and direct resequencing. Each MIP is an oligonucleotide that includes a common linker (green) flanked by target-specific sequences (yellow) that hybridize with their respective targets (Exons 1-3). Targets are extended and ligated into the circular probe. Illumina adapters (black) are appended by multi-template, inverse PCR reaction, resulting in amplicons that are ready for direct sequencing. Since sequences of black, green, and yellow sections of the amplicon are known, removing them from the final sequence will result in only exon sequence.

ACKNOWLEDGMENTS

Many thanks to Dr. J. Schnable and Z. Liang for their assistance and Dr. D. Hyten for lab space and equipment for this project.

LITERATURE CITED

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2. Billot et al. (2013) Massive Sorghum Collection Genotyped with SSR Markers to Enhance Use of Global Genetic Resources. PLoS ONE 8(4); e59714. doi:10.1371/journal.pone.0059714
3. Werle, R. Resisistance to acetolactate synthase-inhibiting herbicides in shattercane and johnsongrass: Current status and future predictions. 2016. Dissertation, in Department of Agronomy & Horticulture. Lincoln, NE: University of Nebraska-Lincoln. pp. 136.

RESULTS & DISCUSSION

- The short-range Inzen *ALS* haplotype ($Val_{560}Ile$ + $Trp_{574}Leu$) was detected in only one population of JG (JG-35); however, no individual was homozygous, confirming the relative rarity of the Inzen *ALS-R* allele.
- 27 SSR primer pairs showed 100% homology with the reference genome, whereas the remaining 14 have SNPs or are not specific to single loci, and are under further development.
- In a regional monitoring program, KASP markers may be used to preliminarily determine if new ALS-R *in situ* weed populations have the short-range $Val_{560}Ile$ + $Trp_{574}Leu$ Inzen haplotype. If so, plants may be genotyped with MIPs targeting a longer-range Inzen ALS haplotype. Additional MIPs targeting SSRs will simultaneously document population genetic diversity while providing supporting evidence of gene flow.

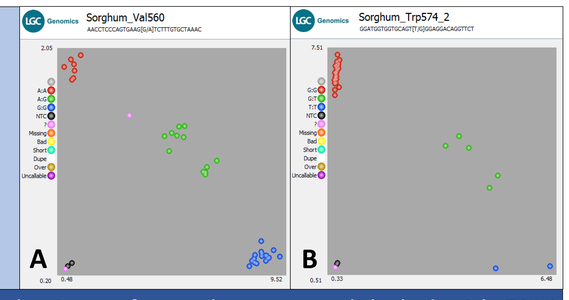

Figure 1. Scatterplots of fluorescence indexes of FAM and HEX fluorophores (*x* and *y* axes, respectively) in 40 samples of Inzen, grain sorghum, JG populations J-35, J-46, and J-40; and SH populations S-58 and S-63. JG and SH were segregating for ALS-resistance. **A (left):** $Val_{560}Ile$ mutation in Inzen that does not confer ALS-resistance. **B (right):** $Trp_{574}Leu$ mutation in Inzen that confers ALS-resistance.

Table 1. KASP Primers purchased from LGC Genomics. Forward primers for each assay are labeled with FAM and HEX fluorophores. Wildtype (WT) or Inzen alleles are indicated as FAM or HEX.

SNP ID	5' Primer (-FAM)	5' Primer (-HEX)	3' Primer (Common)	Inzen FAM	WT HEX
$Val_{560}Ile$	GTGCTGGTTGTTTAGCACAAAGAC	GGTGCTGGTTGTTTAGCACAAAGAT	GGAGCTAGCTATGATCCGAATTGAGAA	G	A
$Trp_{574}Leu$	CCTGGGGATGGTGGTGCAGTT	CTGGGGATGGTGGTGCAGTG	TATGTGTGTGCTCTATTGGCCTTATAGAA	T	G

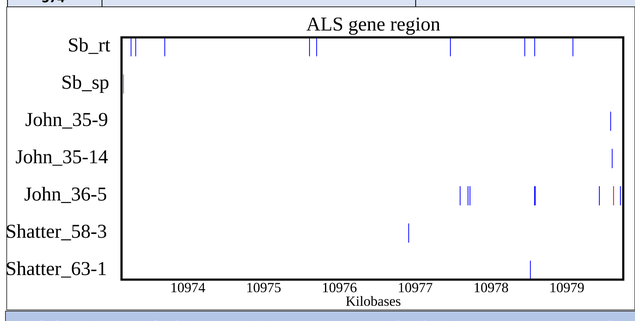

Figure 2. Alignment of the ALS region between Inzen (sb_rt; ALS-R), a wild-type hybrid (sb_sp), and individuals from JG (John_35-9, John_35-14, and John 36-5) and SH (Shatter_58-3 and Shatter_63-1) populations previously determined to be segregating for ALS-resistance by Werle et. al (2017). Blue bars denote a non-reference-genome SNP and red bars represent a heterozygous SNP.

Table 2. 5 of the 41 SSRs currently being converted to MIPs (Billot et al, 2013).

SSR marker	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Repeat	Chr	Allele Number	Gene diversity (*He*)	Observed heterozygosity (*Ho*)
gpsb067	TAGTCCATACACCTTTCA	TCTCTCACACACATTCTTC	(GT)10	8	15	0.681	0.032
gpsb123	ATAGATGTTGACGAAGCA	GTGGTATGGGACTGGA	(CA)7+(GA)5	8	14	0.720	0.030
mSbCIR223	CGTTCCAATGACTTTTCTTC	GCCAATGTGGTGTGATAAAT	(AC)6	2	10	0.703	0.023
mSbCIR238	AGAAGAAAAGGGGTAAGAGC	CGAGAAACAATTACATGAACC	(AC)26	2	27	0.859	0.027
mSbCIR240	GTTCTTGGCCCTACTGAAT	TCACCTGTAACCCTGTCTTC	(TG)9	8	35	0.746	0.034

CONCLUSION & FUTURE DIRECTIONS

- MIPs targeting *ALS*, which we previously *de novo* assembled across *Sorghum* species (Werle et al. 2017), are being developed to identify a longer haplotype unique to Inzen to distinguish between newly-evolved alleles in SH and JG due to herbicide selective pressure, and those conferred by crop-to-weed gene flow.
- Results from ecological risk monitoring studies will assist Federal regulatory agencies (USDA-APHIS) in making science-based decisions about the introduction of genetically-modified sorghum in the U.S., plant breeding companies interested in limiting gene flow from genetically modified sorghum to its wild relatives, and other researchers developing environmental stewardship plans for the management of potential genetically modified sorghum traits.
- We will be field-testing a portable, isothermal PCR prototype using KASP genotyping chemistry in 2017. This technology will be able to determine if weedy populations contain the short-range $Val_{560}Ile$ + $Trp_{574}Leu$ Inzen haplotype in as little as 30 minutes (**Figure 3**).
- If an Inzen allele is preliminarily detected, individuals will be collected and sequenced in the lab using MIPs to confirm gene flow from Inzen and the frequency of ALS-R alleles in the population.

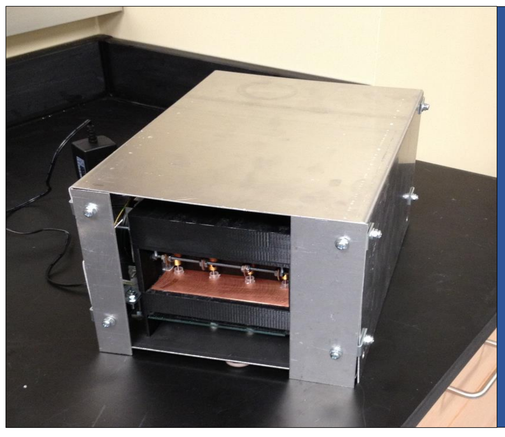

Figure 3. Field-deployable isothermal PCR prototype. Multiple leaf samples of JG and SH can be pooled into a large reaction volume (50 µL+) to simultaneously screen for the short-range Inzen haplotype. The cost savings, convenience, and portability of this technology are paramount to a large-scale ecological risk monitoring program.