Echinochloa colona with Reported Resistance to Glyphosate Conferred by Aldo-Keto Reductase Also Contains a Pro-106-Thr EPSPS Target Site Mutation[OPEN]

Dear Editor,

Weeds can evolve resistance to glyphosate through modification of the herbicide target, 5-enolpyruvyl shikimate 3-phosphate synthase (*EPSPS*), primarily at the Pro-106 position or through increased production of *EPSPS*, both of which are referred to as target site resistance (TSR; Gaines et al., 2019). Weeds can also evolve resistance via differential interaction with glyphosate (reduced absorption or translocation, or increased metabolism of glyphosate), which is referred to as nontarget site resistance (NTSR). Recently Pan et al. (2019) reported field-evolved weed resistance to the herbicide glyphosate, putatively via an NTSR mechanism, of metabolism by aldo-keto reductase (AKR). AKR-mediated glyphosate metabolism has been previously reported, thus making AKR-based metabolism a possible route for genetic modification in glyphosate-resistant crop development (Agrawal et al., 2015; Vermanna et al., 2017). Pan et al. (2019), however, were the first to identify AKR as a route to evolution of herbicide resistance in field-treated weed species.

Preliminary work on the glyphosate-resistant *Echinochloa colona* population first identified by Gaines et al. (2012) on which the work of Pan et al. (2019) is based did not attribute glyphosate resistance to modification of the target site *EPSPS*, reduced absorption or translocation of glyphosate, or glyphosate metabolism (Goh et al., 2018). Resistant populations did not accumulate shikimate in the leaf tissue following glyphosate treatment to the level of susceptible plants, suggesting that either glyphosate did not reach the target site *EPSPS* or that the *EPSPS* target site had reduced binding affinity for glyphosate. The lack of reduced absorption or translocation, or increased metabolism in *E. colona* individuals, further supported a target site involvement in resistance mechanism. Thus, we hypothesized that a target site mutation had been missed by the original Goh et al. (2018) authors and was not examined using the transcriptome RNA sequencing data reported by Pan et al. (2019).

An *EPSPS* gene from *Digitaria insularis* was downloaded from NCBI (GenBank: KX108896) and used as a reference, as no *Echinochloa EPSPS* sequences were present in GenBank. Reads from the six different experiment accessions reported by Pan et al. (2019) within National Genomics Data Center, Beijing Institute of Genomics (http://bigd.big.ac.cn) PRJCA001826 (Table 1; CRX0654665 to CRX065470) were downloaded and mapped to the reference individually using CLC Genomics Workbench 20.0 (Qiagen). Variants were detected from the read mapping using the Basic Variant Detection tool with variants ignored with minimum nucleotide frequency of less than 35%. Nucleotide variants for *EPSPS* codons 101 to 110 were compiled based on read mapping and variant detection (Tables 2 and 3).

E. colona transcriptome reads (Table 1) mapped to an *EPSPS* reference revealed a Pro-106-Thr substitution in resistant read sets (Fig. 1; Table 2). Approximately 72% of reads that mapped in the three-glyphosate resistant read sets contained an ACA codon encoding a Thr at position 106 (data not shown). *E. colona* is a polyploid, which explains the ACA-Thr mutant allele on one subgenome, and the presence of the CCA-Pro allele in another subgenome. TSR-based glyphosate resistance in *E. colona* has been reported by Alarcon-Reverte et al. (2013). Alarcon-Reverte et al. (2013) reported 6.6 greater resistance in a Pro-106-Ser-containing *E. colona* biotype compared to susceptible biotype, which is comparable to the 5.6 to 8.6 more resistance compared to susceptible in the population first reported resistant by Gaines et al. (2012) and evaluated by Pan et al. (2019). Alarcon-Reverte et al. (2013) did not examine metabolism as a possible mechanism of herbicide resistance; therefore, it is possible that metabolic processes also contribute to resistance in these populations. We acknowledge that Pan et al. (2019) did observe increased metabolism in glyphosate-resistant *E. colona* compared to glyphosate-susceptible, which is contradictory to the initial findings of Goh et al. (2018) on this population. The ultra performace liquid chromatography tandem mass spectometry methodology used by Pan et al. (2019) likely yielded more reliable results than the C^{14}-based thin-layer chromatography methodology used by Goh et al. (2018). Based on data provided by Pan et al. (2019), glyphosate-resistant *E. colona* is metabolizing glyphosate and has higher *AKR* transcript abundance compared to glyphosate-susceptible types. However, because the shikimate pathway remains more functional in glyphosate-resistant *E. colona* due to the Pro-106-Thr containing *EPSPS*, greater metabolism could be occurring due to a stable physiological environment that

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Table 1. *Reads obtained from National Genomics Data Center, Beijing Institute of Genomics (https://bigd.big.ac.cn/gsa/browse/CRA002028) under accession number PRJCA001626 as reported by Pan et al. (2019)*

BioSample Accession	Experiment Accession	Run Accession	Sample Name	Archived File Name	Presumed Glyphosate Response
SAMC110955	CRX065465	CRR076662	baicao-R21	CRR076662_f1 CRR076662_r2	Resistant
SAMC110956	CRX065466	CRR076663	baicao-R22	CRR076663_f1 CRR076663_r2	Resistant
SAMC110957	CRX065467	CRR076664	baicao-R23	CRR076664_f1 CRR076664_r2	Resistant
SAMC110958	CRX065468	CRR076665	baicao-S17	CRR076665_f1 CRR076665_r2	Susceptible
SAMC110959	CRX065469	CRR076666	baicao-S18	CRR076666_f1 CRR076666_r2	Susceptible
SAMC110960	CRX065470	CRR076667	baicao-S19	CRR076667_f1 CRR076667_r2	Susceptible

Table 2. *Single nucleotide polymorphisms (SNPs) detected from read mapping of six experiment accessions of National Genome Data Center (https://bigd.big.ac.cn) Genome Sequence Archive BioProject PRJCA001826 to a* Digitaria insularis *EPSPS partial cds (NCBI accession KX108896) reference.*

Codon/ Amino Acid Number	Reference	CRX065465 (Resistant)	CRX065466 (Resistant)	CRX065467(Resistant)	CRX065468 (Susceptible)	CRX065469 (Susceptible)	CRX065470 (Susceptible)
	Codons Present						
	Translated Amino Acids						
101	GGA G	GGA G	GGA G	GGA G	GGA G	GGA G	GGA G
102	ACG T	ACT T	ACT T	ACT T	ACT T	ACT T	ACT T
103	GCA A	GCA A	GCA A	GCA A	GCA A	GCA A	GCA A
104	ATG M	ATG M	ATG M	ATG M	ATG M	ATG M	ATG M
105	CGT R	CGG R	CGG R	CGG R	CGG R	CGG R	CGG R
106	CCA P	CCA/ACA P/T	CCA/ACA P/T	CCA/ACA P/T	CCA P	CCA P	CCA P
107	TTG L	TTG/CCG L	TTG/CCG L	TTG/CCG L	TTG/CCG L	TTG/CCG L	TTG/CCG L
108	ACA T	ACA T	ACA T	ACA T	ACA T	ACA T	ACA T
109	GCG A	GCA A	GCA A	GCA A	GCA A	GCA A	GCA A
110	GCC A	GCC/GCT A	GCC/GCT A	GCC/GCT A	GCC/GCT A	GCC/GCT A	GCC/GCT A

Table 3. *Reads mapped and total reads count for paired-read data sets obtained from National Genome Data Center (https://bigd.big.ac.cn) Genome Sequence Archive BioProject PRJCA001826 as reported by Pan et al. (2019)*

Experiment Accession	Presumed Glyphosate Response	Reads Mapped to Reference EPSPS Partial CDS	Total Read Count
CRX065465	Resistant	1,107	60,961,185
CRX065466	Resistant	898	57,026,220
CRX065467	Resistant	934	70,192,858
CRX065468	Susceptible	276	62,463,728
CRX065469	Susceptible	310	65,997,636
CRX065470	Susceptible	329	73,336,968

is more conducive to xenobiotic metabolism when key processes are not being fully inhibited.

Secondary authors on Pan et al. (2019) have reported that EPSPS substitutions at Pro-106 are insufficient to confer resistance in *E. colona* specifically under some growth conditions (Han et al., 2016). Literature exists that supports the notion that *EPSPS* Pro-106 changes combined with secondary metabolic, translocative, or increased *EPSPS* expression increase the overall level of glyphosate resistance above that of target-site mutations alone (e.g. Bostamam et al., 2012; Bracamonte et al., 2016; Gherekhloo et al., 2017). It is our stance

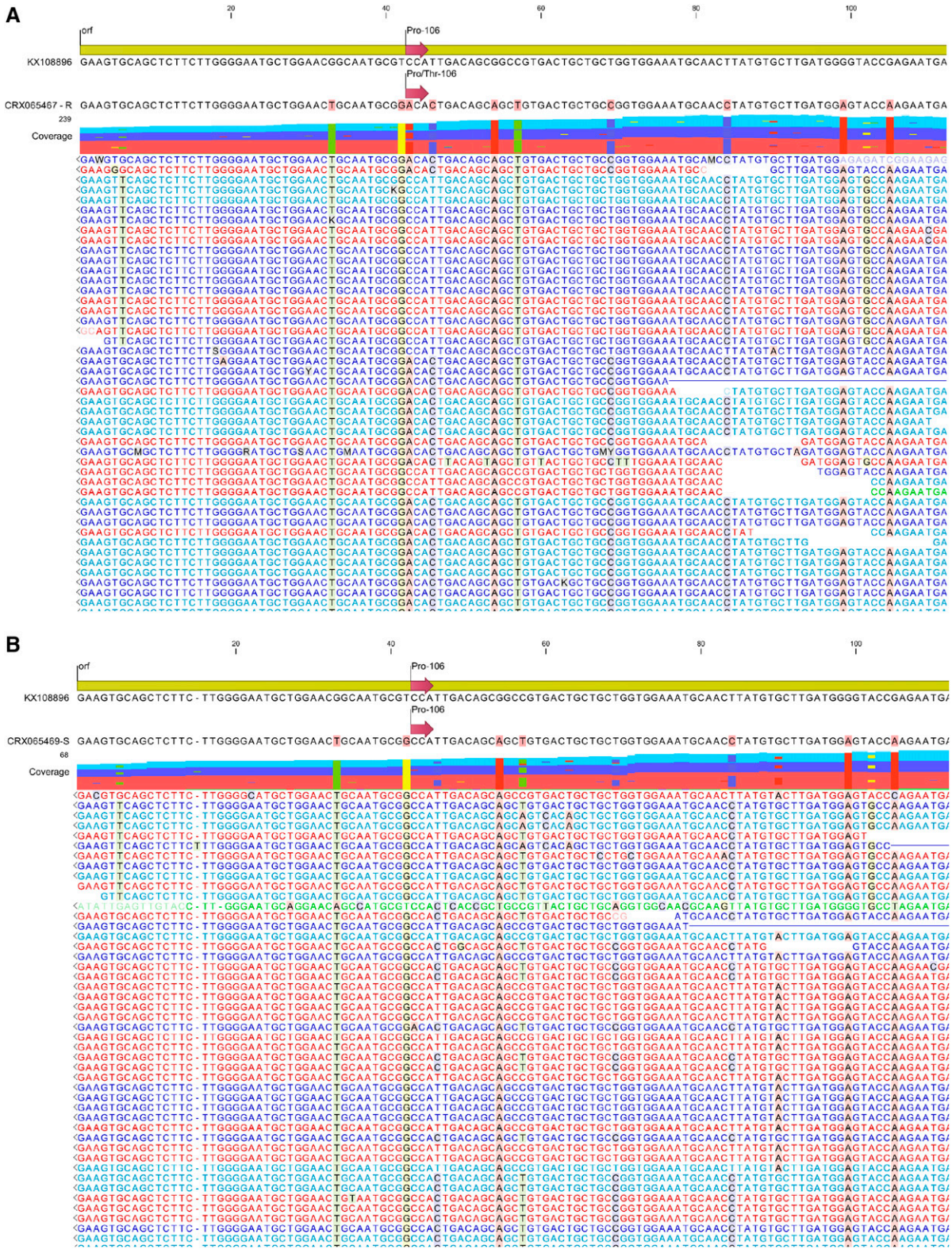

Figure 1. Graphical representation of Illumina sequencing reads for glyphosate-resistant *E. colona* (CRX065467; A) and glyphosate-susceptible *E. colona* (CRX065469; B) aligned to a partial coding sequence of *Digitaria insularis* as the reference *EPSPS*.

that Han et al. (2016) demonstrates that *EPSPS* Pro-106 amino acid changes do confer resistance and provide supporting evidence that combined target and NTSR mechanisms likely work in concert as others have reported.

We thus conclude that the glyphosate resistance in *E. colona* identified first by Gaines et al. (2012), evaluated by Goh et al. (2018), and on which AKR-based nontarget site glyphosate resistance evolution was asserted by Pan et al. (2019) can at least in part be explained by an undiscovered common *EPSPS* substitution in the resistant population. Our conclusion in no way should discount the work of previous research and the growing body of knowledge regarding AKR-based glyphosate metabolism or that AKR is playing a role in glyphosate resistance in this specific *E. colona* population; however, it should not be concluded that this *E. colona* population evolved resistance to glyphosate solely via AKR-based glyphosate metabolism.

It is our opinion that TSR can be easily overlooked, particularly in polyploid species. The current sequencing methodology to detect target site mutations is primarily PCR-based capillary sequencing of herbicide target genes. A reliance on PCR for identification of TSR may fail to adequately amplify all expressed copies of a given target site. *EPSPS* gene duplication has been demonstrated as a mechanism of resistance in *Amaranthus* spp., which further exacerbates the problem of determining if TSR is the result of gene duplication, target site mutation, or a combination of both mechanisms due to the high number of expressed gene copies that would need to be amplified (Gaines et al., 2010; Koo et al., 2018). Adequate sampling of target site loci with PCR-based methods is nontrivial, especially within weed species—the majority of which have little if any basic genomic information available, such as genome size, ploidy level, and chromosome number, let alone transcriptomic or genomic sequencing. PCR-based capillary sequencing of target site mutations obfuscates the presence of target site mutations in the presence of multiple target sites; this method as used by Goh et al. (2018) and the majority of research published to confirm TSR (even by ourselves) provides strong evidence for the presence TSR and weak evidence for its absence.

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