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Research Article

Benzobicyclon efficacy is affected by plant growth stage, *HPPD Inhibitor Sensitive 1* (*HIS1*) expression and zygosity in weedy rice (*Oryza sativa*)

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Abstract

Benzobicyclon tolerance in rice (*Oryza sativa* L.) is dependent on the presence of a functional *HIS1* gene, but the level of sensitivity might vary among different cultivars. Greenhouse, laboratory, and field experiments were conducted to further explore the role of *HIS1* in cultivated rice tolerance and to exploit findings toward optimizing benzobicyclon activity on weedy rice (unwanted rice; *Oryza sativa* L.). In a heredity experiment, benzobicyclon tolerance was confirmed to be a semidominant trait conferred by *HIS1* based on the intermediate response (ED$_{50}$ values) of *HIS1* heterozygous F_1 plants. The spatial–temporal expression of *HIS1* was next studied in tissue types (blades, sheaths, and whorls) across tolerant cultivars (‘Roy J’, ‘Diamond’, ‘LaKast’, ‘CLXL745’, and ‘XL753’) and growth stages (2- to 3- compared with 5- to 6-leaf). The relative expression of *HIS1* was tissue specific and highest in whorls, followed by blades and then sheaths. Minimal differences in expression across cultivars and growth stages were observed. Furthermore, *HIS1* was not largely upregulated at 6 h after benzobicyclon treatment. In the same experiment, cultivar tolerance to benzobicyclon at the label rate of 371 g ha^{-1} was found to be growth stage dependent. Plant growth was reduced by ~35% when rice plants were at the 2- to 3- compared with 5- to 6-leaf growth stages. These results show that differences in benzobicyclon tolerance among *HIS1* homozygous cultivars is likely not directly correlated with the expression of *HIS1*. In this research a model was proposed and supported by a field proof of concept study, indicating benzobicyclon efficacy on weedy rice is a function of *HIS1* zygosity by growth stage at application. Prior research indicates *HIS1* is the dominant allele in weedy rice accessions in Arkansas, and thus, based on our model, benzobicyclon should be applied to weedy rice with $\leq$2 leaves for suppression.

Introduction

Benzobicyclon is a 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicide that has activity on a wide range of aquatic, grass, sedge, and broadleaf weeds in flooded rice (*Oryza sativa* L.) fields (Sekino et al. 2008). Tolerance in rice can vary depending on the genetic background of each cultivar, but in general, *japonica* cultivars exhibit tolerance to benzobicyclon, while most but not all *indica* cultivars are sensitive (Kwon et al. 2012; Lv et al. 2021; Maeda et al. 2019; Young et al. 2018). The *HPPD Inhibitor Sensitive 1* (*HIS1*) gene is responsible for tolerance in rice, which is conferred through hydroxylation of benzobicyclon hydrolysate (active parent) into a less phytotoxic compound and not benzobicyclon (proform) (Maeda et al. 2019). Sensitivity in *indica* cultivars is either the result of a loss-of-function frameshift mutation (28-bp deletion) resulting in truncated protein (Maeda et al. 2019) or a missense mutation located in the region of the putative substrate pocket that encodes for a glycine instead of a valine at the 286th (Val-286-Gly) amino acid position (Lv et al. 2021). Hereafter, a less than fully functional *HIS1* allele with either mutation will be referred to as *his1*.

Although the presence or absence of *HIS1* in rice is the major contributing factor in determining sensitivity, the spatial–temporal expression profile of *HIS1* and its role in tolerance is less understood. Based on the Rice Expression Profile Database, RiceXPro (Sato et al. 2013), *HIS1* is not a constitutively expressed gene; however, it was preferentially expressed in leaf blades and sheaths with little to no expression detected in roots (Maeda et al. 2019; Xia et al. 2017). Ancillary data from Maeda and coauthors (2019) implicated *HIS1* as a limiting factor in tolerance, as ‘Nipponbare’ (*japonica*) was tolerant to benzobicyclon at 0.3 μM but severely bleached at 0.5

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µM; however, when *HIS1* was constitutively expressed by the 35S promoter in transgenic lines, no bleaching symptomology appeared to be prevalent at 0.5 µM. On this point, research has shown crop response to benzobicyclon can vary among *japonica* cultivars, albeit minimally. For example, Kwon et al. (2012) observed the phytotoxic ratings for 18 transplanted *japonica* cultivars (~25-d old) ranged from 0 to 2 out of a 9 scale after applying benzobicyclon at 400 g ha^{-1}. These differences could potentially be explained by differential gene expression, but this needs to be explored further.

In Arkansas, weedy rice (unwanted rice; *Oryza sativa* L.) is a problematic weed, and growers are currently heavily reliant on herbicide-resistant rice technology (e.g., Clearfield® and Max-Ace®) for its control (FIFRA 2021; Hardke 2021; Young et al. 2018). In this paper, weedy rice is considered unwanted rice, including red rice or off-types from previous plantings of hybrid rice. Benzobicyclon at the label rate of 371 g ha^{-1} has shown some potential to be a complementary piece for a grower's weedy rice control program in both conventional and herbicide-resistant rice. Unfortunately, numerous weedy rice accessions in Arkansas are already segregating for *HIS1*, and this is mostly likely the consequence of gene flow with the cultivated *japonica* rice grown in Arkansas (Brabham et al. 2020; Chen et al. 2004; Shivrain et al. 2009; Young et al. 2018). Thus, the success of benzobicyclon in a particular field will be highly dependent on the accession's *HIS1* allele frequency; however, preliminary research suggests plant growth stage at the time of benzobicyclon application may have a role in determining weedy rice sensitivity and even crop tolerance regardless of *HIS1* genotype (Castner et al. 2020; Patterson et al. 2020). We hypothesize that *HIS1* expression is growth stage dependent, but more research is needed to understand the genetic contribution of *HIS1* to benzobicyclon sensitivity. In this paper, we used different rice cultivars, either *indica* ('Purple Marker' and 'Rondo') or *japonica* ('CLXL745', 'Diamond', 'Jupiter', 'LaKast', 'Roy J', and 'XL753') to explore three major objectives: 1) to confirm the inheritance of *HIS1* and the tolerance of heterozygotes to benzobicyclon, 2) to characterize the spatial–temporal gene expression profile of *HIS1* and its role in tolerance, and 3) to use these data to build a model toward optimizing the utility of benzobicyclon as a weedy rice control tool.

Material and Methods

Response of HIS1 Heterozygous Plants to Benzobicyclon

Plant Material and Growth Conditions

F_1s of reciprocal crossed Roy J (*HIS1*/*HIS1*) × Rondo (*his1*/*his1*) and Jupiter (*HIS1*/*HIS1*) × Rondo (*his1*/*his1*) rice varieties were created at the Rice Research and Extension Center near Stuttgart, AR. The *his1* allele in Rondo harbors the 28-bp deletion.

Rice seeds from these crosses were germinated in plastic trays containing potting mix (Sunshine premix No. 1®, Sun Gro Horticulture, Agawam, MA, USA) at the Altheimer Laboratory in Fayetteville, AR. Seedlings at the 1-leaf stage were then transplanted into plastic buckets (7.5 L, 24 cm in diameter) filled to three-quarter capacity with sieved Pembroke silt loam (fine-silty, mixed, active, mesic Mollic Paleudalfs) soil (pH of 6.6 and 2.4% organic matter). Plants were maintained under greenhouse conditions at a 32/22 C day/night temperature regime and a 16-h photoperiod consisting of natural lighting supplemented with halide lights. Plants were fertilized weekly following the manufacturer's instructions (Miracle-Gro® Water All Purpose Plant Food, Scotts Miracle-Gro, Marysville, OH, USA). When plants reached the 5-leaf growth stage, a 5-cm flood was established 24 h before benzobicyclon treatment and maintained until the end of the experiment.

Dose–Response Experiments

To assess the susceptibility of F_1 progeny to benzobicyclon, dose–response assays were carried out. Experiments were conducted as a completely randomized experimental design. Each bucket contained four plants, with one plant from each reciprocal crossed F_1 and its respective parents. The experiment was repeated in time; however, the number of replicates for each genotype at each rate varied from 6 to 12 because of limited availability of F_1 plants. Benzobicyclon was applied at 0, 23, 46, 93, 185, 371 (1×), 742, and 1,484 g ha^{-1}. Methylated seed oil (MSO; Leci-Tech, Loveland Products, Loveland, CO, USA) was added to all treatments at 1% v/v to optimize performance of the herbicide (Brabham et al. 2019). Treatments were applied using a research track sprayer equipped with two 1100067 flat-fan nozzles and calibrated to delivered 187 L ha^{-1}. At 35 d after treatment (DAT), aboveground biomass was collected and oven-dried for 3 d at 66 C to obtain dry weight. Dose–response curves for dry weights were generated in R (R Core Team 2020) using the DRC package (Ritz et al. 2015). Based upon the Akaike information criterion (AIC) and lack of fit, dry weight data for all genotypes were simultaneously fit using the following four-parameter log-logistic model:

$$Y = c + \left\{ \frac{(d - c)}{(1 + exp\{b[log(x) - log(e)]\})} \right\}$$

where Y represents dry weight, e is the ED_{50} (estimated dose required to reduce growth by 50%), c and d represent the lower and upper asymptotes, b represents the relative slope around e, and x represents the applied rate. The *EDcomp* function was used to compare the benzobicyclon response of F_1 plants to parental lines based on ED_{50} values.

Spatial–Temporal HIS1 Expression and Growth Stage Sensitivity to Benzobicyclon

A completely randomized mixed level-four factorial experiment was initiated to determine the spatial–temporal expression of *HIS1* and its role in benzobicyclon tolerance. The four main factors were: cultivar, growth stage, tissue type, and timing after application. The five *japonica* varieties included in this study were the inbred cultivars Diamond, LaKast, and Roy J, plus two hybrid cultivars CLXL745 and XL753 (Supplemental Figure S1). Seeds of each hybrid (~20 seeds) and inbred (~30 seeds) cultivar were seeded directly onto soil in plastic buckets as described earlier, so the final plant stand, after the six plants were removed for RNA extraction, better mimicked field-like densities recommended by the University of Arkansas (Hardke 2021). To establish a difference in growth stages (second factor), a second planting was started (separate buckets) once plants from the first planting had 2 leaves. At the time of tissue collection and benzobicyclon application, cultivars from the first planting had 5 to 6 leaves (height range over runs was 33 to 45 cm for hybrids and 30 to 38 cm for inbreds), while plants in the second planting had 2 to 3 leaves (height range over runs was 22 to 28 cm for hybrids and 18 to 24 cm for inbreds). Plants were started in the greenhouse, and once the seedlings reached the spiking growth stage, were moved outdoors for the remainder of the experimental run. Plants were fertilized weekly with Miracle-Gro® following the manufacturer's instructions. All experiments were conducted outside at the Altheimer Laboratory (36.098782°N, 94.179015°W) in 2018 between early May and September.

Once plants reached the desired leaf growth stage, a 5-cm-depth flood was established 96 to 120 h before tissue collection and benzobicyclon treatment and maintained until the end of the experiment (28 DAT). One day before benzobicyclon applications, three plants from each cultivar by growth stage were harvested (cut at the soil line) and dissected into leaf blades, sheaths, and whorls (third factor). The top 2 leaves with a visible leaf collar were dissected into blade and sheath. Afterward, any remaining tissue was removed from the plant to reveal the "whorl." The whorl contained the growing point and any unfurled tissue with no visible collar, with particular attention paid to selecting plants with a relatively new emerging unfurled leaf. Approximately 24 h later, benzobicyclon at 371 g ha^{-1} plus MSO (1% v/v) was sprayed broadcast as described earlier. At 6 h after herbicide treatment (timing after application, fourth factor), three plants from each cultivar by growth stage were collected and dissected as described earlier. Tissue from three plants in the nontreated controls were also collected to account for any time of day difference in expression. For all collection time points and factor levels, tissue from the three subsamples was pooled and immediately frozen in liquid nitrogen and maintained at −80 C until use. At 28 DAT, plant height and subsequent dry weight from three plants per replication were recorded. The experiment was repeated three times, and each run had three replicates; however, gene expression data were only determined for the first two runs.

Quantitative Real-Time PCR (qPCR)

Tissue was homogenized in liquid nitrogen using a mortar and pestle, and RNA was extracted using the phenol and guanidine isothiocyanate extraction method (TRIzol Reagent, Invitrogen, Carlsbad, CA, USA). Before use, RNA quality and quantity were checked with a spectrophotometer (NanoDrop 2000c, Thermo Scientific, Waltham, MA, USA) and agarose gel (1%) electrophoresis. Isolated RNA was treated with DNase 1 to remove any DNA contamination, then first-strand complementary DNA (cDNA) was synthesized from 1 μg total RNA according to the iScript cDNA kit (Bio-Rad, Hercules, CA, USA). Primers used for *HIS1* amplification were forward 5′- ACTCAACGAAGCTCCTGCATTT-3′ and reverse 5′-GAAGAATCGTCAAGAGGGTGCC-3′. *Elongation factor 1 alpha* (*EF1*) was utilized as the reference gene, and primer sequences were forward 5′-GTCATTGGCCACGTCGACTC-3′ and reverse 5′-TGTTCATCTCAGCGGCTTCC-3′ (Caldana et al. 2007). Primer efficiencies for *HIS1* and *EF1* across cultivars and tissue type ranged from 92% to 96%. A qPCR reaction mix consisted of 7.5 μl of SYBR Green Master Mix (Bio-Rad), 0.9 μl of a forward and reverse primer mix (final concentration per primer 0.3 μM), 1.5 μl of cDNA, and 5.1 μl water to make the total reaction 15 μl. PCR conditions were 50 C for 2 min; 95 C for 2 min; and 40 cycles of 95 C for 15 s, 57 C for 15 s, and 72 C for 1 min. A melt curve was added for quality analysis. Three biological and technical replicates were used per factor per run.

Before analysis, *HIS1* expression was normalized to *EF1* (ΔCt), and these values were used for ANOVA. Data met the basic assumptions of ANOVA, and no interactions with run as a factor were significant. Thus, data were pooled over runs. For any significant interactions, data were transformed into $2^{-\Delta\Delta Ct}$ fold change values (Livak and Schmittgen 2001) and were standardized to *HIS1* expression in Roy J (baseline cultivar used in most of our benzobicyclon experiments) or blade tissue (expression believed to be highest based on data from Kato et al. [2015]). Plant height and dry weight data for both treated and nontreated plants were standardized to the average value of the nontreated within a growth stage. Data met the basic assumptions of ANOVA, and no run interactions were detected, allowing data to be pooled across runs. Nontreated controls were included in this analysis. All analyses were performed in JMP (JMP v. 16, Cary, NC, USA), and Tukey's HSD test was utilized to separate means at $\alpha = 0.05$.

Weedy Rice Control Model Proof of Concept Study

To test the functionality of our weedy rice control model, proof of concept field experiments were initiated in 2019 at the Pine Tree Research Station near Colt, AR (hereafter referred to as the Pine Tree location; 35.126042°N, 90.9637°W) and at the Rice Research and Extension Center near Stuttgart, AR (hereafter referred to as the Stuttgart location; 34.466036°N, 91.402936°W). Field experiments were conducted on a Dewitt silt-loam soil (fine, smectitic, thermic Typic Albaqualfs) at Stuttgart and on a Calloway silt loam (fine-silty, mixed, active, thermic Aquic Fraglossudalfs) at Pine Tree. The soil at Stuttgart had a pH of 6.0 and organic matter content of 1.8% with 8.4, 71.4, and 20.2% sand, silt, and clay, respectively. The soil at Pine Tree had a pH of 7.5 and organic matter content of 1.3% with 10.6, 68.6, and 20.8% sand, silt, and clay, respectively. Experiments were setup as a quasi–two factor randomized complete block design with three replications but without nontreated controls. Factor A was weedy rice accession or cultivated rice line, and factor B was growth stage at application. A total of 16 weedy rice accessions were screened in this study, with 15 accessions collected in Arkansas (Brabham et al. 2020; Young et al. 2018) and XL745 F_2 plants (considered *HIS1/HIS1* weedy rice). Roy J × Purple Marker F_1 plants (*HIS1/his1*), the susceptible cultivars Purple Marker and Rondo (*his1/his1*), and the tolerant cultivar LaKast (*HIS1/HIS1*) were included as genotypic internal standards. The *HIS1* allele frequency for the 15 accessions was previously characterized by Brabham et al. (2020). Therein, accessions 9, 10, 12, 24, and 25 had contrasting data between observed field sensitivity and predicted *HIS1* genotype. Plants from these accessions were re-genotyped for the presence or absence of a 28-bp deletion in the *HIS1* gene using the assay provided in Maeda et al. (2019).

Each experiment and subsequent blocks were contained within a single bay (21 by 18 m wide). A 10-row drill was used to create furrows and plot widths. An individual plot was 1.5 by 1.5 m wide with a 1-m alley between plots. A block was four plots long and two wide (two tractor passes), and there were three total blocks. Plots in the right column of a block contained accessions/cultivars at the first planting date and the parallel plot in the left column contained the second planting date. Five different accessions/cultivars were hand planted into every other row within a plot (50 seeds row^{-1}), and the grouping of accessions/cultivars changed across blocks. To maintain experiments weed free, the Pine Tree location received an application of florpyrauxifen-benzyl (30 g ha^{-1}) plus cyhalofop-butyl (313 g ha^{-1}) and Stuttgart location received an application of quinclorac (420 g ha^{-1}). A day before benzobicyclon application, an 8- to10-cm flood was established, with the Stuttgart trial flooded on May 29, and Pine Tree on June 12. At the time of treatment, accessions/cultivars at Pine Tree were at the 4- to 5-leaf growth stage in the first planting and 1- to 2-leaf stage in the second planting.

Table 1. Parameter estimates for benzobicyclon dose–response curves and estimated dose required for 50% growth reduction (ED_{50}) of parental and F_1 families at 35 d after treatment.

	Dry weight: Model parameter estimates (±SEM)[a]				
Genotype	*b*	*c*	*d*	ED_{50}	SI[b]
				g ha^{-1}	
Rondo (sensitive)	1.19 (0.15)	0.15 (0.11)	2.94 (0.1)	54 (8)	—
Jupiter (tolerant)	2.43 (0.50)	0.22 (0.06)	1.98 (0.08)	171 (16)	3.2
Roy J (tolerant)	2.80 (1)	0.34 (0.06)	1.44 (0.07)	190 (28)	3.5
Jupiter × Rondo	1.29 (0.15)	0.34 (0.11)	3.78 (0.09)	105 (9)	1.9*
Roy J × Rondo	1.86 (0.29)	0.49 (0.08)	3.17 (0.09)	111 (8)	2.0*

[a] Dose–response curves for dry weight data were generated using a four-parameter log-logistic model. There was no significant difference between reciprocally crossed F_1 families from the same parentage and pooled. SEM, standard error of the mean.
[b] SI, Selectivity index calculated as ED_{50} (Rondo) divided by the ED_{50} of the different rice genotypes, respectively.
An asterisk (*) indicates values are significantly different from both parental ED_{50} values at $\alpha = 0.05$.

At Stuttgart, plants in the first planting had 1 to 2 tillers and were at the 3- to 4-leaf growth stage in the second planting. Benzobicyclon at 371 g ha^{-1} plus MSO (1% v/v) was applied to the entire area of the bay using a CO_2-pressurized backpack sprayer equipped with six AIXR110015 nozzles and calibrated to deliver 140 L ha^{-1}. Percent control (injury) ratings were taken at 28 DAT using a 0% to 100% scale (where 0% and 100% corresponded to no control and complete control, respectively). Control of weedy rice or injury to cultivated rice was mainly a result of chlorosis or bleaching of plant tissue that eventually led to plant death when severe.

Results and Discussion

Response of HIS1 Heterozygous Plants to Benzobicyclon

Benzobicyclon tolerance in rice is conferred by a major semidominant to dominant gene, *HIS1* (Kato et al. 2015; Maeda et al. 2019); however, the sensitivity of heterozygous *HIS1* plants in comparison to parental lines has not been characterized in a controlled manner. Here, two different tropical *japonica* cultivars, Roy J and Jupiter (*HIS1/HIS1*) were reciprocally crossed to a known sensitive *indica* cultivar Rondo (*his1/his1*) to create F_1 heterozygous populations. There was no significant difference between reciprocally crossed F_1 families from the same parentage, as expected from a nuclear-encoded gene, and data were pooled across reciprocally crossed F_1 families (data not shown).

Rondo was highly sensitivity to benzobicyclon, with an ED_{50} value of 54 g ha^{-1}, while Jupiter and Roy J were 3.2 and 3.5 times more tolerant, respectively, based on their ED_{50} values (Table 1; Supplemental Figure S2). Both F_1 families were significantly different from their respective maternal and paternal cultivars. The intermediate response confirms *HIS1* is a semidominant gene. For example, the ED_{50} value for reciprocally crossed Roy J × Rondo F_1 plants, was 111 g ha^{-1} and corresponded to 2 and 0.6 times more tolerance in comparison to the parental lines of Rondo and Roy J, respectively. Overall, the ED_{50} values for Rondo, Jupiter, and Roy J and the average of F_1 families corresponded to roughly 1/6, 1/2, and 3/10 of the benzobicyclon label rate of 371 g ha^{-1} (FIFRA 2021). In the field, both Jupiter and Roy J (*HIS1/HIS1*) exhibited excellent crop tolerance to benzobicyclon at 494 g ha^{-1} (Young et al. 2018), and thus the lack of tolerance in the greenhouse to even a label rate in our study was perplexing. We cannot sufficiently explain this observation, but our overall object for this experiment was still met, as benzobicyclon sensitivity was confirmed to be controlled by *HIS1*, which is a single semidominant gene.

Spatial–Temporal HIS1 Expression and Growth Stage Sensitivity to Benzobicyclon

HIS1 is not a constitutively expressed gene (Maeda et al. 2019), but expression of the gene in the whorl and how overall expression contributes to benzobicyclon tolerance has yet to be thoroughly explored. Here, we captured and compared the expression profile of *HIS1* among five *japonica* inbred and hybrid cultivars, growth stages (2- to 3-leaf compared with 5- to 6-leaf plants), tissue types (leaf blade, sheath, and whorl), and time after benzobicyclon application. Based on ΔCt values, *HIS1* expression within a tissue type for each cultivar at the 2- to 3-leaf compared with 5- to 6-leaf plants was not significantly different; therefore, data were pooled ($P = 0.11$).

Before benzobicyclon application and averaged over cultivars, there was a clear difference in *HIS1* expression among the tested tissue types ($P > 0.0001$). When standardized to expression in leaf blades, relative expression of *HIS1* in whorls was nearly 2-fold higher (1.9 ΔΔCt) and 2-fold less in the sheaths (0.5 ΔΔCt) (Figure 1A). Expression in roots was not investigated, because previous research (Maeda et al. 2019) as well as findings in our pilot study (data not shown) indicated *HIS1* expression in roots is minimal. Higher expression in the whorl is favorable, because benzobicyclon hydrolysate has ideal physicochemical properties for phloem mobility (Brabham et al. 2019).

In general, relative expression patterns across cultivars within a tissue type were similar, even though a significant interaction was detected ($P = 0.01$). Relative expression values among cultivars within a tissue type ranged from 0.76- to 1.74-fold when standardized to the respective expression in Roy J (Figure 1B). The only significant differences between cultivars was detected in sheath tissue, where the relative expression of *HIS1* in the inbred cultivar LaKast was nearly 2-fold less than in the hybrid XL753. The relative expression of *HIS1* at 6 h after application was measured only in the whorl tissue of the five cultivars at the 5- to 6-leaf stage. Again, there was no cultivar by treatment interaction ($P = 0.90$), but the main effect of treatment was significant ($P = 0.027$). *HIS1* expression in treated plants was 1.5-fold higher in comparison to the nontreated at 6 h after benzobicyclon application; however, this is probably biologically insignificant (<2-fold) (Figure 1C).

Unlike gene expression patterns, the response of a cultivar to benzobicyclon was dependent on the growth stage at application. However, there was no significant difference between the five cultivars within a growth stage based on percent height reduction ($P = 0.60$) and dry reduction data ($P = 0.76$). At 28 DAT, plants at the 5- to 6-leaf stage at application were not significantly different from the nontreated based on height data, but a slight significant

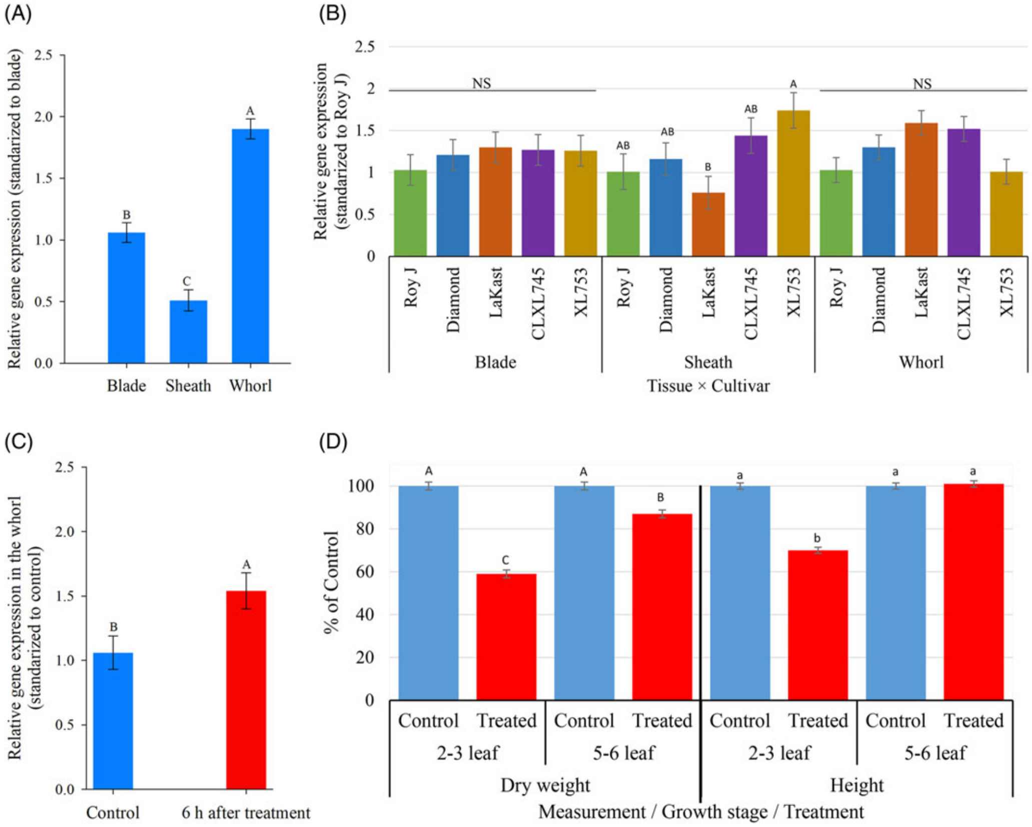

Figure 1. Relative gene expression of *HIS1* in different tissue types averaged over cultivars and growth stages and standardized to expression in blade tissue (A); comparison of relative *HIS1* expression within a tissue type across rice cultivars. Results are averaged over growth stages and standards to *HIS1* expression in Roy J within a tissue type (B). Change in relative *HIS1* expression in whorl tissue averaged over cultivars at the 5- to 6-leaf stage at 6 h after treatment with benzobicyclon (371 g ha^{-1}) (C). Comparison of dry weight and height accumulation of rice cultivars at the 2- to 3-leaf and 5- to 6-leaf growth stages at 28 d after treatment with benzobicyclon at 371 g ha^{-1}. No significant interaction between cultivars and growth stage was detected, and data were pooled accordingly (D). A and B show *HIS1* gene expression before benzobicyclon application, while C shows *HIS1* gene expression after application. Different letters on bars within a group indicate a significant difference ($\alpha = 0.05$). Bars represent means ± SEM.

reduction in dry weight (13%) was detected. In contrast, the height and dry weight of plants at the 2- to 3-leaf stage at application were reduced by 30% and 41%, respectively (Figure 1D). Based on these findings, difference in benzobicyclon tolerance among *HIS1* homozygous cultivars is probably not a function of expression, but a bigger cultivar sample size is needed to truly make this conclusion.

Weedy Rice Control Model and Proof of Concept Experiments

Prior research indicates benzobicyclon has the potential to provide rice growers in the southern United States with the added benefit of weedy rice suppression, but the degree to which this occurs can vary considerably (Brabham et al. 2020; Young et al. 2018). Therefore, a model was developed to predict the efficacy of benzobicyclon on weedy rice based on data from cultivated rice tolerance studies herein and in the literature. Our findings from greenhouse studies indicate benzobicyclon tolerance in cultivated rice is first and foremost dependent on *HIS1* zygosity followed by growth stage; thus, our model predicts weedy rice sensitivity to benzobicyclon is a function of *HIS1* zygosity by plant growth stage at application (Table 2). The model predicts benzobicyclon will provide moderate suppression to complete control of weedy rice plants at the ≤2-leaf growth stage regardless of genotype, while activity on older plants is completely dependent on *HIS1* zygosity.

To test our model, a proof of concept experiment was conducted in 2019 at two field locations (Pine Tree and Stuttgart) looking at weedy rice sensitivity to benzobicyclon at different growth stages. A total of 15 weedy rice accessions plus XL745 F_2 plants (considered *HIS1*/*HIS1* weedy rice) were tested. Roy J × Purple Marker F_1 plants (*HIS1*/*his1*), the susceptible cultivars Purple Marker and Rondo (*his1*/*his1*), and the tolerant cultivar LaKast (*HIS1*/*HIS1*) were included as internal standards. The *HIS1* allele frequency in a subsample of plants from each accession was previously determined (Brabham et al. 2020) or accessions were re-genotyped. In all but two accessions, *HIS1* was the dominant allele (Supplemental Table T1).

Data were grouped according to *HIS1* (*HIS1*/*HIS1*; *HIS1*/*his1*; *his1*/*his1*) genotyping, and average percent control or injury was calculated among groups. At Pine Tree, benzobicyclon efficacy (371 g ha^{-1}) on the genotyped *HIS1*/*HIS1* accessions at 28 DAT averaged from 69% to 8% control when plants were at the 1- to 2-leaf stage and 4- to 5-leaf stage at application, respectively. Roy J × Purple Marker F_1 plants together with weedy rice accession 7 were heterogeneously segregating for *HIS1*/*his1* alleles and displayed 77% and 9% control when plants were at the 1- to 2-leaf stage and 4- to 5-leaf stage at application, respectively. Accessions 18 and 20 were predominantly segregating for *his1*/*his1* (*HIS1* allele frequency ≤30%) and responded similarly to the susceptible standards Purple Marker and Rondo at both growth stages (98% and 93% of control, respectively) (Table 3).

Plants were at an advanced growth stage when applications were made at Stuttgart, and control was less than that observed

Table 2. A model for control of weedy rice in cultivated rice that is derived from lessons learned from crop tolerance research associated with *HIS1*. Weedy rice, considered unwanted rice, including red rice or off-types from previous plantings of hybrid rice, control is proposed to be a function of genotype by growth stage.[a]

	Plant growth stage at application	
Genotype	≤2 leaves	≥5 leaves
his1/his1	Control	Control
HIS1/his1	Severe suppression to control	Moderate to severe suppression
HIS1/HIS1	Moderate suppression	Tolerant

[a] Applicable only to U.S. Midsouth rice production practices where benzobicyclon is applied post-flood in a soluble concentrate formulation at 371 g ha^{-1} plus methylated seed oil (1% v/v).

Table 3. Results from a proof of concept experiment testing our benzobicyclon weedy rice control model in which weedy rice sensitivity is proposed to be a function of genotype by growth stage.[a]

		Pine Tree				Stuttgart			
		Growth stage							
Accession or cultivar	Genotype[b]	1- to 2-leaf		4- to 5-leaf		3- to 4-leaf		1- to 2-tillers	
		% control or injury[c]							
LaKast, XL745 F_2, 2, 5, 8, 9, 10, 12, 24, 25, 26, 32, 33, 36	*HIS1/HIS1*	69	(24)	8	(9)	24	(20)	4	(8)
Roy J × Purple Marker F_1, 7	*HIS1/his1*	77	(26)	9	(7)	37	(29)	11	(5)
Purple Marker, Rondo, 18, 20	*his1/his1*	98	(7)	93	(3)	93	(7)	82	(12)

[a] Trials were conducted in 2019 at the Pine Tree Research Station near Colt, AR, and at the Rice Research and Extension Center near Stuttgart, AR. The table contains the *HIS1* genotype and average percent control or injury of weedy rice accessions and rice cultivars treated with benzobicyclon (371 g ha^{-1}) at 28 d after treatment.
[b] The *HIS1* genotype for each weedy rice accession was previously determined in Brabham et al. (2020). Accessions 9, 10, 12, 24, and 25 were re-genotyped. An accession was categorized as *HIS1/HIS1*, *HIS1/his1*, or *his1/his1* if the *HIS1* allele frequency was ≥70%, 31%–69%, or ≤30%, respectively. XL745 F_2 was considered weedy rice. Purple Marker and Rondo were included as *his1* susceptible standards, while LaKast and Roy J × Purple Marker F_1 were the *HIS1* homozygous and heterozygous standards, respectively.
[c] Values correspond to the mean ($n = 3$), and numbers in parentheses corresponds to SD.

at Pine Tree. Overall, benzobicyclon efficacy (371 g ha^{-1}) at 28 DAT on weedy rice accessions ranged from 24% to 93% and 4% to 82% when plants were at the 3- to 4-leaf stage and 1- to 2-tiller stage at application, respectively. At the 3- to 4-leaf stage, the homozygous (*HIS1/HIS1*) LaKast group exhibited 24% control, while the heterozygous (*HIS1/his1*) standard Roy J × Purple Marker F_1 plants had an intermediate response (37% injury). At the 1- to 2-tiller stage, control of accessions or injury to cultivars genotyped as *HIS1/HIS1* or *HIS1/his1* did not exceed 12%. Again, the sensitivity of accessions 18 and 20 (*HIS1* allele frequency ≤30%) and the susceptible standard cultivars Purple Marker and Rondo was independent of growth stage, and control averaged 82% (Table 3).

Data from the proof of concept field experiments appear to provide supporting evidence for our model. As predicted, *his1/his1* accessions/cultivars were highly sensitive to benzobicyclon at 371 g ha^{-1} regardless of growth stage, and predominantly *HIS1* accessions or *HIS1/HIS1* cultivars (e.g., LaKast) at the ≥5-leaf growth stage were tolerant. These statements are supported by evidence in the literature (Akasaka et al. 2011; Kwon et al. 2012; Lv et al. 2021; Maeda et al. 2019; Young et al. 2017). For *HIS1/his1* accessions/cultivars, the model predicted severe suppression to complete control at ≤2-leaf growth stage, which was observed at the Pine Tree location, but the model would indicate greater control at the 5-leaf growth stage. For instance, benzobicyclon activity on Roy J × Purple Marker F_1 plants and accession 7 (mixed *HIS1/his1* population) dropped to less than 15% once plants had ≥ 4 leaves at application in both trial locations. This result contrasts with work from Akasaka et al. (2011) and Kwon et al. (2012), who observed moderate to significant benzobicyclon injury on transplanted *indica* × *japonica* cultivated rice.

Moderate suppression of homozygous *HIS1* plants at the ≤2-leaf growth stage was predicted and observed at the Pine Tree location, where weedy rice accessions and cultivars at the 1- to 2-leaf stage were moderately to severely suppressed (69% control). We believe the increased sensitivity is related to overall *HIS1* expression but not expression pattern, and we therefore theorize that it is possible to "overload" the *HIS1* enzyme in smaller plants. Anecdotal evidence from our gene expression experiment indicated *HIS1* is probably not a highly expressed gene, as Ct values for *HIS1* ranged from 29 to 31 (*EF1* averaged 20). This statement can be strengthened substantially by RNA-seq data at RiceXPro (Sato et al. 2013) and the Rice Expression Database (Xia et al. 2017) indicating *HIS1* (Loc_Os02g17940) is a low to at most moderately expressed gene (even in comparison to *HPPD* gene Loc_Os02g07160). Thus, we hypothesize *HIS1/HIS1* weedy rice plants will be at least suppressed at ≤2-leaf growth stage; this would be exacerbated in the field by the flood and competition from the 5-leaf rice crop.

In closing, the model we present currently only applies to drill-seeded rice production practices in the U.S. Midsouth, where benzobicyclon is applied post-flood in a soluble concentrate formulation at 371 g ha^{-1} to the rice crop at the 5-leaf growth stage or beyond. It should also be assumed that all weedy rice accessions in the U.S. Midsouth are segregating for *HIS1*, and it is probably the dominant allele, as previous screening efforts have indicated (Brabham et al. 2020; Young et al. 2018). Therefore, in general, benzobicyclon should not be expected to provide acceptable weedy rice control, but based on our model, benzobicyclon can provide some degree of suppression on plants with ≤2 leaves at the time of application regardless of the *HIS1* genotype. Further research is needed to validate our benzobicyclon weedy rice control model.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2022.17

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No conflicts of interest have been declared.

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