

Adaptation of an invasive grass to agriculture: ecological and genomic evidence

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ABSTRACT

Species vary phenotypically and genetically across their environmental range limits, and this variation can influence ecological processes. Ecologically meaningful intraspecific variation might be particularly important in the context of agricultural weeds and exotic invaders, because intraspecific variation in these species might allow them to rapidly adapt to their unusually dynamic and variable environments. In a greenhouse study, we explored intraspecific variation in the size, rhizome production, and competitive ability of the global invader, Johnsongrass (*Sorghum halepense*), representing populations from agricultural and non-agricultural habitats across its introduced North American range. We also used these populations to explore the relationship between phenotypic variation and genomic endoreduplication responses to the common stresses herbicides, competition, and clipping. Endoreduplication occurs when plants increase their genome size by increasing their nuclear chromosome number, with some evidence showing correlations with stress response. We found that Johnsongrass plants from agricultural habitats were larger than plants from non-agricultural habitats, but there was no difference between habitats in either rhizome production or competitive ability. Two of the five herbicides we tested, primisulfuron and imazethapyr, had the strongest suppressive effects on Johnsongrass, and also stimulated the greatest rates of endoreduplication. Furthermore, agricultural populations showed higher levels of endoreduplication. We found no overall effect of competition on endoreduplication, although endoreduplication was higher for non-agricultural populations than agricultural populations. When competing with corn, but not with conspecifics, Johnsongrass roots increased endoreduplication by 13%. Clipping induced substantial endoreduplication, but there was no difference between agricultural and non-agricultural populations. Our results suggest that endoreduplication may play a role in some, but not all, stress responses in Johnsongrass. Furthermore, our results indicate that Johnsongrass has adapted in some phenotypic and genomic ways to agricultural habitats in North America. Such adaptation may play a role in this species' success as both an agricultural weed and an exotic invader.

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GENERAL AUDIENCE ABSTRACT

Agricultural weeds and exotic invasive plant species cause substantial economic and ecological impacts. Exploring the ways in which these species thrive, spread, and cope with different forms of stress contributes to our understanding of why these plants can be so successful and cause such damage. We studied how one of the world's worst weeds and invaders of natural systems, Johnsongrass (*Sorghum halepense*), responded via growth and by increases in the amount of DNA in its cells to different forms of stress. We also asked whether any Johnsongrass populations appeared to have adapted to agricultural habitats. We found that Johnsongrass plants grown from seed collected from agricultural fields produced larger plants than those from non-agricultural locations (e.g., roadsides), suggesting some degree of adaptation to agricultural environments by Johnsongrass. We also found that stress from sub-lethal doses of some herbicides suppressed the growth of Johnsongrass, simultaneously stimulating more cells with increased cell DNA content, a genomic process called endoreduplication. Interestingly, there were higher rates of endoreduplication in plants from agricultural populations in the herbicide experiment, suggesting that endoreduplication may help Johnsongrass cope with stress and that agricultural populations show adaptive increases in this genomic stress response. Competition from corn or other Johnsongrass plants suppressed target Johnsongrass plants, but did not stimulate more endoreduplication in the suppressed plants. Finally, clipping reduced the size of Johnsongrass substantially, and also stimulated increased rates of endoreduplication. In general, our results indicate that Johnsongrass has adapted in some ways to agricultural habitats in North America, and that this adaptation may play a role in this species' success as both an agricultural weed and an exotic invader.

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Chapter 1: Agricultural ecotypes of an invader produce larger plants and rhizomes than non-agricultural populations, but are not better competitors

Abstract

Members of a species vary genetically and phenotypically within their environmental range limits, but less is known about how within-species variation may influence community processes. Ecologically meaningful intraspecific variation might be particularly important in the context of anthropogenic impacts on natural systems, such as agriculture, because of how rapidly plants have adapted to agriculture. In a greenhouse study, we explored how agricultural and non-agricultural populations of invasive Johnsongrass (*Sorghum halepense*), collected from 20 sites throughout the US, varied in size, rhizome production, competitive ability, and response to fertilization. Johnsongrass size varied substantially among accessions, and plants from agricultural habitats were 9% larger than plants from non-agricultural habitats. Rhizome production also varied among accessions but did not differ among source habitats. Conspecific competition suppressed the aboveground mass of Johnsongrass by 32% and rhizome mass by 20%, whereas corn had weak to no effect on Johnsongrass. Fertilization increased growth equally for all accessions. Our results indicate habitat-specific differences in Johnsongrass size, though this did not correspond with greater competitive ability against either conspecifics or corn. Importantly, our results show that substantial intraspecific variation in competitive ability has arisen in an introduced species over a relatively short time. Also, species-specific competitive responses and changes in rhizome allocation suggests the possibility of adaptation by Johnsongrass to corn, an agricultural species with which it commonly occurs.

Introduction

Environmental variation and biotic interactions drive genetic variation both within and among populations across the ranges of most species. Clausen et al. (1941) described among-population variation in a series of classic studies; showing that populations may consist of “ecotypes” which are locally adapted and functionally distinct (also see Albert et al. 2010, Atwater et al. 2015). Ecotypic diversity is generally associated with adaptation to variation in the abiotic environment. For example, Clausen et al.’s (1941) experiment along very large elevation gradients described ecotypes that were highly adapted to their particular positions on the abiotic gradient. Joshi et al. (2001) also conducted reciprocal transplants and found that ecotypes grew best where they were collected. Since Joshi et al. (2001) found that local ecotypes outperformed all non-local ecotypes, they argued that such local adaptation indicated the importance of maintaining intraspecific diversity in general.

There is a great deal of evidence for intraspecific variation between population of exotic invasive plant species in native and non-native ranges and also among populations within these ranges (Bohn et al. 2004; Bossdorf et al. 2005, Lavergne and Molofsky 2007, Maron 2006, Ridenour et al. 2008). These traits include size, growth rate, and defenses against herbivores (Siemann and Rogers 2001; DeWalt et al. 2004; Lafuma and Maurice 2007). Exotic invasive species also provide an interesting context in which to explore local adaptation. They are often distributed widely, experience a broader range of natural and anthropogenic stresses, and appear to show more variation in fitness among environments than native species (Davidson et al. 2011). Furthermore, there is evidence for small scale, rapid local adaptation by exotic species (Ledger et al. 2009; Godoy et al. 2001; Atwater et al. 2016). Finally, exotic invasive species have been shown to have disproportionately strong competitive effects relative to natives (Vila et

al. 2011), thus exploring variation among populations or ecotypes of these species is promising for the study of competition in general.

Similar opportunities for studying the ecological implications of intraspecific variation arise through the evolution of plants in response to agriculture. Over a relatively short history, agriculture has led to evolutionary responses by many plant species, most of which we now call “weeds” (Baker 1974; Young and Evans 1976; Bommarco et al. 2010; Ellstrand et al. 2010; Muller et al. 2011). A classic example is that of *Echinochloa crus-galli* var. *oryzicola* (barnyard grass), a problematic weed in rice paddies that has evolved to be visually similar to rice, but this variety does not look at all like a closely related conspecific variety that does not grow in paddies (Barrett 1983). Such crop mimicry is thought to be an adaptation to selection by visually-based hand weeding (Ellstrand et al. 2010). Bommarco et al. (2010) found evidence for rapid evolutionary change in spatially intermixed populations of *Cirsium arvense*. They found that competitive ability of this agricultural weed differed among populations from semi-natural, conventional farms, and organic farms, and suggested that some adaptation had occurred in response to agricultural conditions.

As described for exotic invasive species and agricultural weeds, intraspecific diversity can shed light on the way plants interact with each other. For example, competition is a key way in which plants interact but most of our understanding of competition comes from studies of *interspecific* interactions (Aschehoug et al. 2016). Thus, we know that competitive outcomes can vary substantially depending on the species identity of the competitors (Vellend et al. 2006). We also know that competitive interactions can exclude species from habitats that are otherwise suitable (Gurevitch 1989). But understanding similar roles of competitive interactions between ecotypes or among members of a population that vary genetically are far less understood. But

we do know that intraspecific variation can dramatically affect the outcome of competition. Atwater et al. (2015) compared the competitive effects and responses of different genotypes of native *Pseudoroegneria spicata* to the exotic invader *Centaurea stoebe*, and found wide variation among genotypes in two competition indices. They also found that the exotic invader had the potential to drive selection for stronger competitive *responses* by *P. spicata*, but not competitive *effects*: all of which was supported further in field experiments (Fletcher et al. 2016). In other studies of plant-plant interactions in the context of intraspecific diversity, individuals of unrelated *Ipomoea hederacea* grown in mixture allocated more biomass to roots and had lower levels of seed production than mixtures of more closely related individuals (Biernaskie 2011). In another study, Turkington (1989) found that genotypes of two different grasses that naturally coexist were less likely to outcompete each other than genotypes that did not coexist; suggesting that genotypic variation in populations might set the stage for coevolution in ways that sustain coexistence. Lankau (2009) reported that differences in intra- and interspecific competitive abilities created complex networks of competitive outcomes which altered the way that plant species coexist. Together these studies indicate that a greater understanding of intraspecific variation in competitive outcomes is crucial to gain a more holistic understanding of the processes structuring plant communities (Atwater 2012).

Since being introduced to the US in the late 1800s, *Sorghum halepense* (Johnsongrass), has become one of the world's worst weeds (Kearney and Peebles 1951). Globally, it is one the most successful and damaging agricultural pests, costing farmers tens of millions of dollars a year in management costs and yield loss (Pimentel 2014). In addition to being a damaging agricultural weed, Johnsongrass also strongly suppresses native species in grasslands (Rout et al. 2013). Introduced to North America during the Civil War as a forage species, Johnsongrass has

since successfully established throughout the United States (Sezen et al. 2016). Agricultural fields are commonly invaded by Johnsongrass, which can dramatically reduce corn yields (Orr et al. 1995). Its exceptionally broad distribution across diverse habitats, occurrence in a wide range of environmental conditions, its ability to outcross with conspecifics, and the potential for exotics in general to evolve rapidly in their new ranges (Lee 2002), make it likely that Johnsongrass may show intraspecific variation in important life history traits like competitive ability. Surprisingly, there is also evidence for genetic (Sezen et al. 2016) and phenotypic (Atwater et al. 2016) differences between agricultural and nonagricultural populations. Thus, the cosmopolitan nature of Johnsongrass, its documented intraspecific variation, and agricultural and ecological importance, make it an excellent species with which to explore important questions about within-species diversity. A better understanding of how habitat differentiation may produce predictable phenotypic and genotypic differences, as has been found in other species (Kane and Rieseberg 2008; Leimu and Fischer 2008; Atwater et al. 2015), could enhance our eco-evolutionary understanding of widespread invasions.

One such adaptive response might be competitive ability. Atwater et al. (2016) explored such adaptation to agricultural habitats using field experiments in which Johnsongrass was planted with corn at agricultural densities. Contrary to field observations, they found that corn very strongly suppressed Johnsongrass seedlings, but did not find differences in size or competitive ability of agricultural and non-agricultural populations. However, important questions regarding competitive dynamics remain unanswered, the answers to which may elucidate the mechanisms of success for such a widespread invader. Of particular interest is the level of intraspecific variation in competitive dynamics and rhizome production at the seedling stage, which often structures later community dynamics. Studying rhizome responses may be

particularly important because rhizomes can influence the outcome of competition (Huber-Sannwald et al. 2011) and because the rhizomes of Johnsongrass harbor endophytic bacteria that profoundly increase the growth and competitive ability of the weed (Rout et al. 2013). The rhizomes of Johnsongrass may also play an important role in its overwinter survival and general “weediness” (Paterson et al. 1995).

Based on the high levels of intraspecific diversity in Johnsongrass and differences in size between agricultural and non-agricultural populations; we further explored differences between agricultural and non-agricultural Johnsongrass populations in a greenhouse competition experiment. We measured the competitive response (ability to tolerate competition) of Johnsongrass to intra-specific (Johnsongrass vs itself) and inter-specific competition (Johnsongrass vs corn). We tested the following hypotheses: 1) Johnsongrass populations will vary in competitive response and in rhizome production; the latter of which is very important but poorly understood (Rout et al. 2013); 2) Johnsongrass from agricultural habitats will be suppressed less by corn than Johnsongrass from non-agricultural habitats; 3) rhizome production by Johnsongrass will be stimulated by competition, and 4) Johnsongrass from agricultural habitats and non-agricultural habitats will compete equally with conspecifics. Pursuing these hypotheses has the potential to yield insight into the extent of rapid evolution by Johnsongrass to agricultural habitats, and the extent of within-species variation in competition.

Materials and methods

Johnsongrass populations

Johnsongrass populations were sourced from accessions (individual plants) collected from 20 populations across 9 states in the fall of 2014 (see Supplementary Materials S1.). In order to reduce maternal effects, seeds used in this experiment were collected from plants allowed to grow and pollinate naturally, primarily through self-pollination, in a common garden at Kentland Research Farm, near McCoy, VA (37.19391 N, 80.57300 W). Original accessions were collected at sites designated by either agricultural or non-agricultural habitat types, detailed in Atwater et al. (2015). In brief, locations containing crops (i.e., corn, pumpkin, grain sorghum) were designated as “agricultural habitats”. Locations with no prior history of agriculture (i.e., roadside, old fields) constituted “non-agricultural habitats”. Environmental variables were not measured at the time of seed collection; however, agricultural and nonagricultural habitats differ greatly in regard to: substrate type, resource availability, disturbance, and community structure.

Greenhouse experiment

To overcome dormancy, all seeds were soaked for 4 hr in Clorox (10% sodium hypochlorite) bleach followed by a 1 hour water rinse. Johnsongrass seeds from 20 populations (30 accessions, Supplementary Materials S1) were then sown into separate 51 X 25 X 5 cm flats containing a sand/soil mixture (2:1) in June of 2015 (Table 1). Topsoil was collected from a local site in Blacksburg, Virginia. Conventional field corn (*Zea mays*) and a commercially acquired Johnsongrass variety used for weed research (hereafter referred to as a conspecific) were sown into separate flats two days after Johnsongrass to homogenize germination timing. Two weeks after germination, seedlings were transplanted into 246 cm³ pots containing a

sand/soil mixture and placed in a greenhouse located at Virginia Tech. Plants were grown under standard greenhouse conditions of 30/20°C day/night temperature, 16-h photoperiod, and watered three times daily with an automatic irrigation system. Plants were randomly assigned to one of the following competitive treatments: 1) Johnsongrass vs corn; 2) Johnsongrass vs conspecific 2; and 3) Johnsongrass alone. Growing each accession alone and with competition allowed us to assess the competitive responses of each accession.

Because agricultural habitats are fertilized, we hypothesized that agricultural populations would be more adapted to high soil fertility. To test this, all three treatments were replicated with an additional fertilization treatment achieved with 50 ml of Miracle-Gro, mixed at 1 g/L, applied on 15 July, 30 July, and 15 August 2015. All other pots were amended with a standard low level of fertilizer in order to maintain plant health under greenhouse conditions. All 30 accessions were represented in each competition and fertility treatment, with 7 replicates per treatment. Replicates (pots) were arranged into 7 blocks, with each block containing all possible treatment by accession combinations resulting in an initial total of 1260 pots.

Data collection and statistical analyses

Aboveground biomass and rhizomes were harvested early September 2015 from a total of 806 pots, the number remaining after seedling mortality during the experiment. Rhizome length was recorded at time of harvest and aboveground and rhizome biomass was dried to a constant mass and then weighed. Mixed-effects models fitted with restricted maximum-likelihood estimation were used to estimate Johnsongrass response to competition using aboveground biomass, rhizome biomass. Continuous response variables were transformed to meet normality assumptions (aboveground biomass: square root transformed; rhizome biomass: cube root

transformed). Johnsongrass accession habitat (agricultural; non-agricultural), accession nested within habitat, fertilizer (high fertilizer; low fertilizer), and competition (JG alone; JG vs. conspecific; JG vs. corn) were modeled as fixed effects and block as a random effect. We modeled all interactions among main effects and then used backward model selection, individually removing non-significant higher order interactions. Main effects were retained regardless of significance. Rhizome presence/absence was modeled as above, except that a generalized model was specified, with a logit link and a binomial distribution.

Additionally, we calculated the ability of Johnsongrass to tolerate competition from corn and a conspecific using the relative interaction index (RII; Armas et al. 2003), which is calculated as follows:

$$RII = [biomass\ in\ competition - biomass\ grown\ alone] / [biomass\ in\ competition + biomass\ grown\ alone]$$

Values of this metric are evenly distributed between 1 and -1. A negative value indicates a negative competitive interaction and a positive value indicates a positive competitive interaction (i.e. facilitation). We calculated RIIs using a mixed-effects model with transformed (square-root) aboveground biomass of Johnsongrass as the response variable, competition (JG alone vs. conspecific; JG alone vs. corn), habitat (agricultural; non-agricultural), fertilizer (high fertilizer; low fertilizer), accession nested within habitat as fixed effects, and block as a random effect. All analyses were performed in JMP 13 (SAS Institute).

Results

The high fertility treatment roughly doubled the aboveground biomass of Johnsongrass across all treatments, and modestly altered competitive outcomes (Table 1, Fig.1). The overall competitive effect of corn on Johnsongrass was negligible (Tukey HSD, $P=0.9906$, Fig.1), whereas conspecifics suppressed Johnsongrass by roughly 25% across all treatments (Tukey HSD, $P<.0001$, Fig. 1). There was substantial variation among accessions (Wald test, $P=0.0009$, Table 1), however there were no differences in competitive ability between agricultural and non-agricultural Johnsongrass (Mixed-effect model, $P=0.2566$, Table 1)

Overall, Johnsongrass plants in high soil fertility produced >50% more rhizome mass than plants in the low fertility treatment (Tukey HSD, $P<.0001$, Fig. 2). More Johnsongrass individuals produced rhizomes when growing with corn than when grown alone or with conspecifics (Table 2, Fig. 3). When grown alone or with conspecifics, approximately 35% of Johnsongrass plants produced rhizomes, but when grown with corn, more than 50% of the individuals produced rhizomes (X^2 test, $P<.0001$, Fig. 3). Variability among populations was high (Wald test, $P<0.0001$), but 43% non-agricultural plants produced rhizomes compared to 38% for plants from agricultural accessions (Fig. 3).

High soil fertility also increased mean rhizome mass across all treatments (Mixed-effect model, F ratio= 1359.26, $P<0.0001$, Table 1, Fig. 2). Variation among populations was high (Wald test, $P= 0.014$, Table 1) but there was no difference in rhizome mass for plants from agricultural and non-agricultural populations (Mixed effect model, habitat effect, F ratio= 0.17, $P=0.681$, Table 1). Competition increased Johnsongrass rhizome mass across all treatments, but the effects of conspecifics differed strikingly (Tukey HSD, $P=0.0002$, Fig.2). Conspecifics did not have a significant effect on Johnsongrass rhizome mass overall (Tukey HSD, $P=0.3554$), but

tended to increase rhizome mass at low fertilizer levels and decrease rhizome mass at high fertilizer levels (Tukey HSD, $P=0.013$, Fig 2). Johnsongrass competing with corn increased rhizome mass by roughly 10% compared to controls.

RII competition index demonstrated that both corn and the conspecific had competitive effects on Johnsongrass. Interestingly, conspecific competition suppressed the aboveground biomass of Johnsongrass roughly 2-3 times more than corn (Tukey HSD, $P<.0001$, Fig. 4). Competitive effects were weaker at high fertility (Table 3, not shown in Fig. 4). There was substantial variation among populations in competitive responses across corn and conspecifics combined (Mixed-effect model, F ratio= 8.13, $P<0.0001$, Table 3), and marginally greater resistance to competition by agricultural Johnsongrass accessions (F ratio= 3.66, $P=0.0561$, Table 3).

Discussion

We found some evidence for source population-level differentiation to anthropogenic, agricultural habitat by Johnsongrass, but we found no evidence that competitive outcomes were affected by this differentiation. For instance, we found that Johnsongrass plants from agricultural populations were significantly larger than those from non-agricultural populations. Using many of the same Johnsongrass populations, Atwater et al. (2016) found that agricultural and nonagricultural populations were genetically and phenotypically different, with their phenotypes closely associated with their home environment. They also found that non-agricultural populations were more broadly distributed in climatic and geographic space than agricultural populations. Non-agricultural populations demonstrated plasticity that suggested local adaptation to the local environments from which they were collected. In contrast, agricultural populations had plastic responses to environmental variability that suggested broader

ability to acclimate. Most importantly, as we found, Atwater et al. (2016) found that plants from agricultural habitats grew much larger than plants from non-agricultural habitats. However, in a subsequent study, Atwater et al. (2017) found equivocal evidence for the performance of agricultural and non-agricultural Johnsongrass, which strongly depended on the scale of the sourced accessions (i.e., national vs. local) and the competitive environment of the surrounding plant community. Thus, our study was designed to identify competitive responses in resource allocation (rhizomes and aboveground biomass) at a finer scale to elucidate whether Johnsongrass has indeed locally adapted to specific habitats and competitors, as some species have been shown to express particular traits in agricultural habitats (Baker 1974), including rapid growth (Kane and Baack 2007).

Previous work suggests that Johnsongrass may have evolved an “all-purpose agricultural genotype”, which may facilitate competing well with plants that express agricultural traits (Atwater et al. 2016). Our results partially support that hypothesis, as Johnsongrass from both habitats performed nearly as well in competition with corn as when growing alone. Mitskas et al. (2003) found that Johnsongrass grown from seeds and from rhizomes both strongly suppressed corn. They also found that Johnsongrass plants from rhizomes emerged earlier and were larger than plants from seed. In their experiment, corn did not affect Johnsongrass mass or stem number regardless of mode of reproduction. Similarly, using many of the same accessions in our study, Atwater et al. (2016) found that corn strongly suppressed seedling-transplanted Johnsongrass. However, in our greenhouse experiment, we found that Johnsongrass was only marginally suppressed by corn - though equally in agricultural and nonagricultural accessions, which does not support habitat-based adaptation.

One of our more striking findings was that Johnsongrass plants were more likely to

produce more and larger rhizomes when grown with corn than when grown alone or with conspecifics. In fact, conspecifics reduced rhizome production by Johnsongrass. Several potential mechanisms may explain this response. First, corn may have altered the physical environment in pots in ways that favored plants with more rhizomes. Second, conspecifics often have highly overlapping resource requirements (Chesson 2000), and thus the competitive effects of conspecific Johnsongrass on target Johnsongrass may have been particularly intense. Others have found that allocation to vegetative propagation, such as rhizomes, is favored in stable and competitive habitats (Chen et al. 2015), and perhaps such competition with corn stimulated rhizome production. However, our results indicate that corn was a weak competitor in the conditions of our experiment. Third, Johnsongrass may have “recognized” corn in a way that stimulated rhizome production (see Karban et al. 2000) if indeed high allocation to rhizomes improved Johnsongrass’ response to or effect on corn.

It is possible that increasing allocation to rhizomes improved the competitive tolerance of Johnsongrass to corn. Plants grown from seed can behave differently than plants grown from rhizomes even if they are the same genotype (Schmid and Weiner 1993) and clonal integration, such as through rhizome connections can affect competitive outcomes (Liu et al. 2016). But in the case of Johnsongrass, increased rhizome production may produce another form of competitive advantage. Rout et al. (2013) found that the rhizomes of Johnsongrass housed a diverse assemblage of bacterial endophytes, many of which provided beneficial functions for Johnsongrass that were related to nutrient acquisition - including atmospheric nitrogen fixation. When these bacterial mutualists were suppressed with antibiotics, there were substantial declines in plant growth and biomass, and rhizome production declined precipitously. Paterson et al. (1995) reported that highly rhizomatous Johnsongrass plants were more likely to survive for

extended periods and that rhizomes appeared to contribute to the weediness of Johnsongrass. Thus, it appears that rhizomes might have the potential to contribute to the competitive ability of Johnsongrass. If so, the stimulation of rhizome production by corn might be what made Johnsongrass a better competitor against corn in our experiments. This poses the question – why would Johnsongrass not always maximize the number of rhizomes? They might not if rhizome production incurs substantial costs in some environments. Additionally, rhizome production, which enhances local population persistence, may be suppressed if Johnsongrass recognized a conspecific. Thus, if Johnsongrass is capable of neighbor recognition, this would partially explain why rhizome production was favored with a “foreign” vs “self” neighbor. However, our experiments were performed in a greenhouse and we do not know how these data translate to the field.

Importantly, Johnsongrass interacted with neighbors in a species-specific way. Understanding the species-specific nature of plant-plant interactions contributes to our understanding of process that determine community structure (Hutto et al. 1996), and also informs the importance of biodiversity. For example, Bittebiere and Mony (2014) found that rhizomatous *Elytrigia repens* showed marked differences in trait expression that corresponded with the species composition of the particular experimental garden plots in which individuals were planted. They noted that trait responses were explained by the abundance of specific species in the community. Such interactions could have important implications for understanding the mechanisms that promote species coexistence and maintain plant diversity (Cavieres and Badano 2009).

Our results are relevant to understanding the role of intraspecific diversity in ecological processes. For example, Vellend et al. (2010) found genotypic variation among populations in

the ability of *Taraxacum officinale* to invade experimental communities of *Poa pratensis*. We have explored intraspecific variation in the context of competitive interactions because exotic plant species often have very strong competitive effects on native species (Vila et al. 2011). Understanding how within-species diversity affects the competitive ability of Johnsongrass may aid in the development of tools to better manage the agricultural effects of this weed and invasion into natural habitats. In all competitive metrics, there was large variation among Johnsongrass accessions, suggesting strong intra-specific variation in competitive dynamics. This is consistent with all of the other studies examining intraspecific diversity in Johnsongrass (Atwater et al. 2015, 2016). However, it is important to note that we have explored intraspecific diversity across the southern United States, not within population diversity. And it might be this among-population diversity that is important in most ecological contexts.

Johnsongrass is known to hybridize with cultivated *S. bicolor* (Paterson et al. 1995), which may not only have contributed to the unexpected large genetic diversity of Johnsongrass (Sezen et al. 2016), but may also have facilitated adaptation to agricultural environments. Adaptation of exotic species to anthropogenic environments, evolution of the wild populations that invade anthropogenic systems, and gene flow between species in agricultural and non-agricultural habitats has contributed to the evolution of plant species in ways that make some species almost completely dependent on agro-ecosystems, and other species that occupy both agricultural and non-agricultural habitats (Arnold 2004). Muller et al. (2011) point out that crop-related weeds have evolved through many processes, but most of these appear to have led to particular competitive impacts in agro-ecosystems. Evolutionary changes in agricultural weeds can be very rapid (Vigueira et al. 2013), and the habitat differentiation among Johnsongrass populations has probably occurred over a relatively short period of time (Vigueira et al. 2013).

As stated by Vigueira et al. (2013) “agricultural weeds compose a unique evolutionary state, neither wild nor domesticated, that has developed in parallel to crop domestication”. Thus, as study systems, agricultural weeds are subject to both natural and perhaps an unusual form of artificial selection. We did not find differences in competitive ability among agricultural and non-agricultural populations of Johnsongrass, but the differentiation among populations between these habitats in overall plants size (also see Atwater et al. 2015) may provide unusual insight into early, or nascent, selection for success in anthropogenic environments. Furthermore, the substantial variation in growth and competitive ability among populations of Johnsongrass has the potential to provide insight into the evolution of invasiveness that might arise in particular populations.

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Table 1. Mixed-effect models of competition, fertilizer, and the interaction between competition and fertilizer on aboveground and rhizome biomass. Significant effects ($P < 0.05$) are in bold.

Wald test was used to generate P values for random effects.

Response variable				
Effect type	Effect	<i>DF</i>	<i>F</i> ratio	<i>P</i> value
Aboveground biomass				
Fixed	habitat	1	8.15	0.2566
Fixed	competition	2	110.82	<.0001
Fixed	fertilizer	1	1359.26	<.0001
Fixed	competition x fertilizer	2	11.23	<.0001
Rhizome biomass				
Fixed	habitat	1	0.17	0.6817
Fixed	competition	2	8.70	0.0002
Fixed	fertilizer	1	88.29	<.0001
Fixed	competition x fertilizer	--	--	NS

Table 2. Effect Likelihood Ratio Tests. Results to ordinal logistic model assessing effects of habitat, fertilizer, and competition on presence-absence of Johnsongrass rhizomes.

Response variable				
Effect type	Effect	<i>DF</i>	L-R χ^2	<i>P</i> > χ^2
Rhizome presence/absence				
Fixed	habitat	1	1.4E-.05	0.1517
Fixed	competition	2	21.04	<.0001
Fixed	fertilizer	1	166.72	<.0001
Fixed	competition x fertilizer	2	10.28	0.0158

Table 3. Mixed-effect model results assessing effects of: fertilizer level, competition, habitat, on RIIs for the effect of conspecifics and corn on Johnsongrass aboveground biomass. Biomass data square root transformed in order to meet normality assumptions.

Response variable				
Effect type	Effect	<i>DF</i>	F Ratio	<i>P</i> value
Aboveground biomass				
Fixed	habitat	1	3.66	0.0666
Fixed	competition	1	63.13	<.0001
Fixed	fertilizer	1	809.31	<.0001
Fixed	competition x fertilizer	1	8.11	0.0046
Fixed	accession w/in habitat	--	8.13	<.0001
Random	block	--	--	0.3360

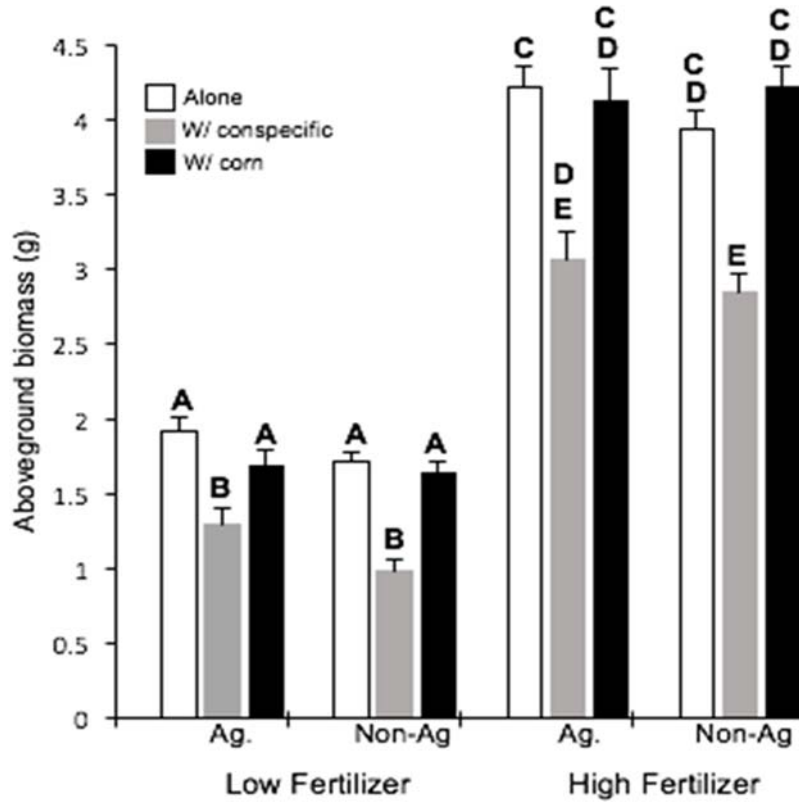


Figure 1. Aboveground biomass (Mean \pm 1 SE) of agricultural and non-agricultural Johnsongrass accessions grown alone, in competition with a conspecific, or in competition with corn grown at low and high soil fertility. Biomass means were calculated from square root transformed biomass to meet normality assumptions. See Table 1 for statistics.

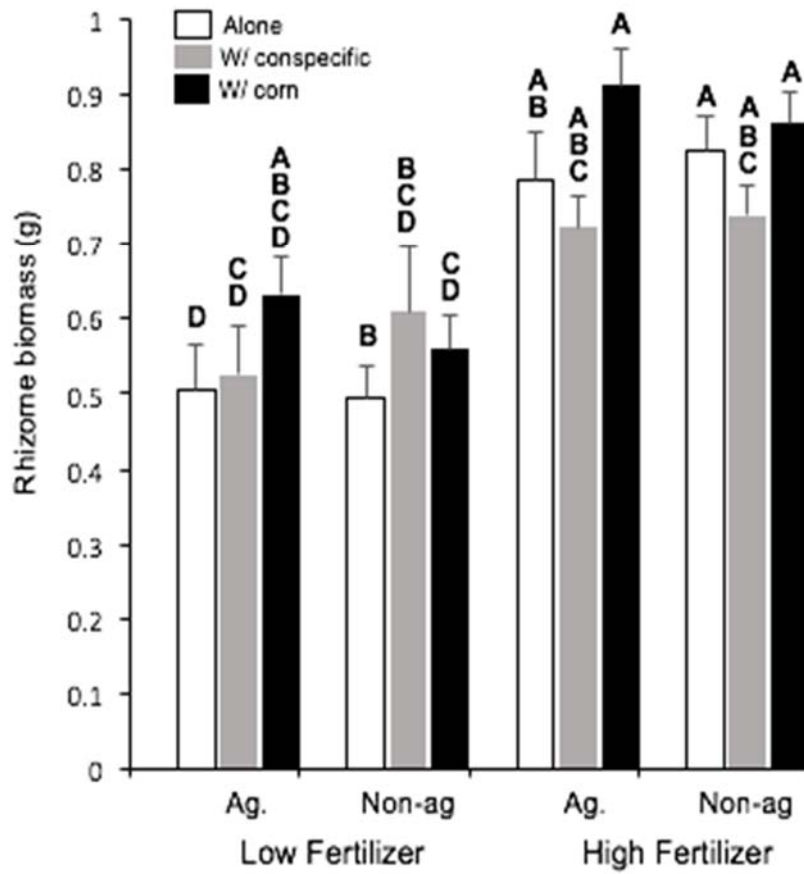


Figure 2. Rhizome biomass (mean \pm 1 SE) of agricultural and non-agricultural Johnsongrass accessions grown alone, in competition with a conspecific, or in competition with corn grown at low and high soil fertility. Biomass means shown here were calculated from cube root transformed biomass. See Table 1 for statistics.

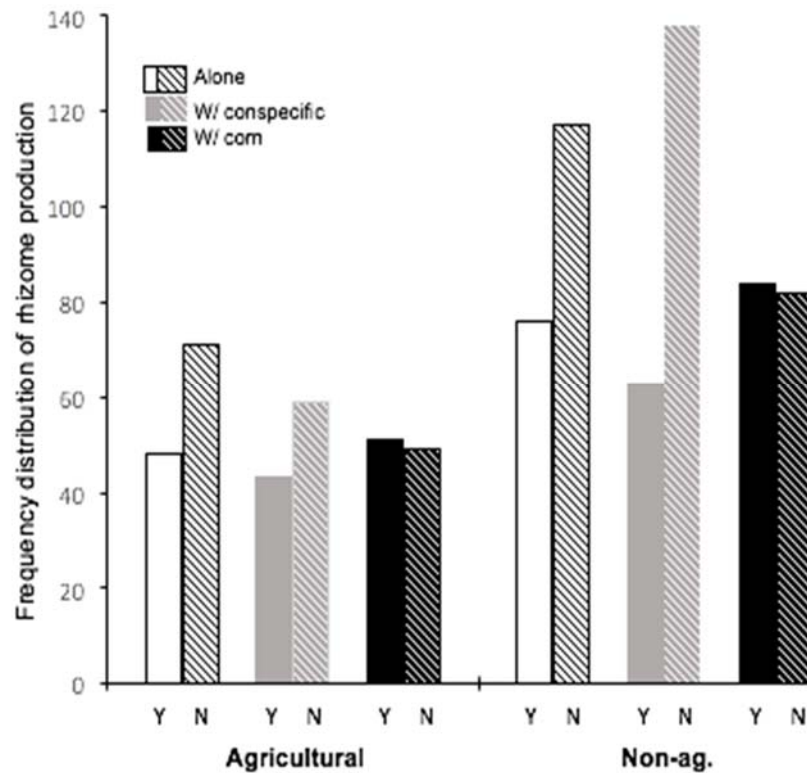


Figure 3. Bar graph illustrating rhizome presence-absence counts associated with competition treatment (alone; Johnsongrass vs. conspecific; Johnsongrass vs. corn) and “home” habitat (Agricultural; Non-ag.). Y=number of plants with rhizomes and N=number of plants without rhizomes. Nominal logistic model was used to assess count data and generate Chi-square frequencies (see Table 2).

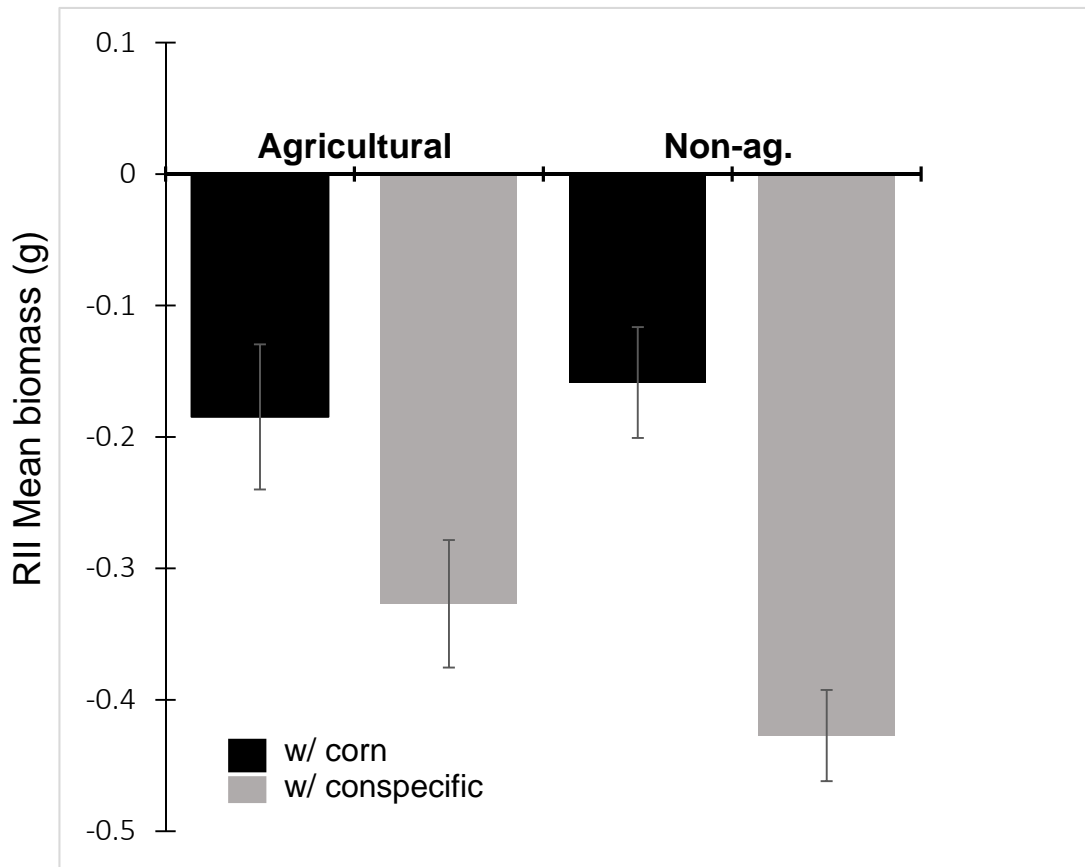


Figure 4. Relative Interaction Indices (mean \pm 1 SE) of aboveground biomass for response of Johnsongrass to competition with corn and conspecifics.

Supplementary materials

S1. Location and home habitat information for Johnsongrass accessions.

Accession ID	State	Latitude	Longitude	Elevation. (m)	Habitat
1	AL	34.2778	-86.8898	296	Non-Agricultural
2	AL	34.7521	-86.959	207	Non-Agricultural
3	AZ	32.2734	-110.9031	731	Non-Agricultural
4	AZ	33.1032	-111.9742	353	Non-Agricultural
5	AZ	33.1032	-111.9742	353	Non-Agricultural
6	CA	36.1990	-119.2519	92	Agricultural
7	CA	36.3088	-119.3806	92	Non-Agricultural
8	CA	36.2337	-119.3120	94	Non-Agricultural
9	CA	36.2337	-119.3120	94	Non-Agricultural
10	GA	32.8916	-84.3271	225	Non-Agricultural
12	GA	32.205	-82.3635	87	Non-Agricultural
13	GA	33.525	-83.4441	195	Agricultural
14	GA	34.4655	-84.6995	240	Non-Agricultural
15	KS	38.8833	-97.7344	408	Non-Agricultural
16	KS	38.7061	-97.4277	378	Agricultural
17	KS	38.7061	-97.4277	378	Agricultural
18	NM	34.3559	-103.0617	1262	Agricultural
19	SC	34.0306	-81.5883	140	Non-Agricultural
20	SC	33.8616	-80.8601	43	Non-Agricultural
21	SC	33.2906	-81.3373	78	Non-Agricultural
22	TX	31.06	-97.3422	200	Agricultural
23	TX	33.2797	-96.8927	171	Agricultural
24	TX	33.2797	-96.8927	171	Agricultural
25	TX	34.3308	-102.9762	1240	Non-Agricultural
26	TX	34.3308	-102.9762	1240	Non-Agricultural
27	TX	34.3155	-102.7736	1196	Non-Agricultural
28	TX	34.3155	-102.7736	1196	Non-Agricultural
29	VA	37.2114	-80.4876	NA	Agricultural
30	VA	37.2114	-80.4876	NA	Agricultural

Chapter 2

Plasticity in the DNA: exploring endoreduplication as a genetic mechanism of stress tolerance in a global invader

Abstract

Plants respond to stress through morphological, physiological and biochemical changes, and in some cases, these changes may correspond with a poorly understood form of genetic plasticity called endoreduplication. Since agricultural weeds and exotic invaders often encounter novel environments, it is particularly important to understand their responses to stress, and the mechanisms underlying those responses. We evaluated the performance and endoreduplication of Johnsongrass (*Sorghum halepense*), a globally important weed, from 23 populations representing agricultural and non-agricultural habitats distributed across its North American range, subjected to the common stresses of herbicides, competition, and mechanical clipping. Two of the five herbicides, primisulfuron and imazethapyr, had the strongest suppressive effects on Johnsongrass, and stimulated the greatest rates of endoreduplication, with agricultural populations having higher levels of endoreduplication. Johnsongrass shoot and root biomass in competition with corn (*Zea mays*) or conspecifics were strongly suppressed relative to controls, but competitive responses did not differ between agricultural and non-agricultural populations. There was no overall effect of competition on endoreduplication, though the proportion of cells showing endoreduplication was significantly (8%) higher for non-agricultural populations than agricultural populations. When separating the effects of corn from that of conspecifics, when competing with corn Johnsongrass roots increased endoreduplication by 13%. A single early clipping reduced Johnsongrass biomass by 10% and stimulated a 4% increase in endoreduplication. Multiple clippings reduced Johnsongrass aboveground biomass by ~40%, with a corresponding 35% increase in endoreduplication in shoots. Johnsongrass from different

habitats did not differ in either growth or genomic responses to clipping. In most cases, endoreduplication levels were inversely correlated with plant performance, suggesting a possible role for endoreduplication in some stress responses by Johnsongrass. To our knowledge, this is the first test for intraspecific variation in the possible role of genetic plasticity as a mechanism of stress tolerance.

Introduction

Plants regularly experience a variety of biotic and environmental stressors, in which they respond via changes in morphology, physiology, and biochemistry (Anjum et al. 2011). For example, plants exposed to drought stress often produce smaller thicker leaves and accumulate more of the osmotic regulator proline than well-watered conspecifics (Van Rensburg et al. 1993; Hoffman et al. 2005). In response to herbivory, many plant species induce chemical defenses (Kessler and Baldwin 2001; Kaplan et al. 2008). In concert with, or potentially underlying, morphological, physiological, and biochemical responses to stress, some plant species may cope with environmental stress through forms of genetic plasticity, including “endoreduplication” (Scholes and Paige 2015). Endoreduplication is the process of whole genome replication without cell division, which increases chromosome number (ploidy) and correlates with increases in cell size (Scholes and Paige 2011), with roughly 90% of all angiosperms thought to be capable of the process (Scholes and Paige 2015). Sometimes called “endopolyploidy”, this phenomenon has been shown in response to a wide range of abiotic stresses (Ceccarelli et al. 2006; Scholes and Paige 2015), and in a few cases, biotic stresses (see review by Scholes and Paige 2015). For example, Scholes and Paige (2011) found higher levels of endoreduplication following the removal of the apical meristem of *Arabidopsis thaliana*, which correlated with greater biomass and more fruit and seed production. Thus, increases in ploidy associated with stress may result in important changes in cell size, metabolic processes, and gene expression that may enhance the performance of plants in stressful conditions. However, so far there have been few studies of endoreduplication with respect to its ecological and evolutionary importance. Thus, the degree to which endoreduplication is related to tolerance of different forms of stress remains poorly understood.

Natural variation within species provides an excellent tool with which to explore the relationship between endoreduplication and different responses to stress by plants because such variation allows measurement of local adaptation, which is especially relevant for widespread species that experience many different types and magnitudes of stresses. Furthermore, understanding variation in ecological and evolutionary processes, such as endoreduplication, within a species might be exceptionally important in the context of anthropogenic impacts on natural systems. Over a relatively short time, the evolution of many plant species has been affected by agriculture. Most of these species we now call “weeds” (Baker 1974, Bommarco et al. 2010, Ellstrand et al. 2010, Muller et al. 2011). For example, Bommarco et al. (2010) found that traits indicative of competitive ability differed among populations of the agricultural weed *Cirsium arvense* from semi-natural, conventional farms, and organic farms. They suggested that some adaptation had occurred in response to agricultural conditions, which present stresses not naturally experienced (e.g., herbicides).

Exotic invasive species are often distributed very widely and experience a broad range of natural and anthropogenic stresses. Davidson et al. (2011) found that exotic species showed more variation in fitness among environments than native species. Thus, agricultural weeds and exotic invasive species present important opportunities for exploring intraspecific variation in morphological responses to different forms of stress, and potentially variation in the expression of genetic plasticity such as endoreduplication. The evolution of weeds in response to agriculture also provides an excellent opportunity to explore rapid adaptive changes in endoreduplication. In fact, Scholes and Paige (2015) note that one of the outstanding questions regarding endoreduplication is “under what conditions [is endoreduplication] initially under selection”? Thus, comparisons of phenotypic and genomic responses to stresses of plants from agricultural

habitats to those of plants from non-agricultural habitats provides an excellent opportunity to explore the potential of selection on the endoreduplication process.

Sorghum halepense (Johnsongrass) is a cosmopolitan invasive perennial C4 grass with substantial intraspecific diversity (Morrell et al. 2005; Sezen et al. 2016). Johnsongrass is unusual in that it is an aggressive agricultural weed and an exotic species that invades natural systems (Rout et al. 2013). It occurs in a wide range of climates and ecosystems globally (Atwater et al. 2016), but we lack a mechanistic understanding explaining its widespread success. Since being introduced to the US in the late 1800s, Johnsongrass has expanded from agricultural to non-agricultural habitats (Sezen et al. 2016), with these “ecotypes” differentiating genetically and phenotypically, while also responding differently to environmental variation (Atwater et al. 2016). Furthermore, non-agricultural populations are distributed across a broader climatic niche than agricultural populations. Interestingly, under common growing conditions, Atwater et al. (2016) found that plants from agricultural populations were larger than non-agricultural populations, suggesting that adaptation to agricultural or non-agricultural habitats might play a role in the ecology of Johnsongrass. Thus, Johnsongrass experiences a broad range of environmental and biotic stressors that vary widely across its introduced range that has led to tremendous phenotypic (Atwater et al. 2016) and genetic (Sezen et al. 2016) variation—making it an ideal study system to identify intraspecific variation for the role of endoreduplication in response to stress.

Weedy invaders such as Johnsongrass are commonly treated with herbicides, and since Johnsongrass is a top agricultural pest, herbicides are the primary means of control. Johnsongrass also commonly occurs in environments where physical damage such as mowing or herbivory is common, especially since Johnsongrass is extremely common along roadsides. Finally, as for

almost all plants, Johnsongrass is subjected to interspecific and intraspecific competition wherever it occurs. Additionally, one of the parental species of Johnsongrass (Morrell et al. 2005), *Sorghum bicolor*, has been shown to plastically increase its ploidy (Ceccarelli et al. 2006). Ceccarelli et al. (2006) found that roots of salt-tolerant *S. bicolor* individuals showed evidence of endoreduplication after they were exposed to salt (NaCl), whereas roots of non-salt-tolerant individuals of *S. bicolor* did not endoreduplicate in response to salt stress. Thus, endoreduplication may play a role in the response of Johnsongrass to stress.

In this context, we conducted three experiments in which genetically distinct populations of Johnsongrass, representing agricultural and nonagricultural habitats, were exposed to the stresses caused by herbicides, physical damage, and competition. We measured both growth and endoreduplication in response to these stresses. We hypothesized that 1) endoreduplication will be more common in Johnsongrass plants stressed by competition, physical damage, and herbicides than in unstressed plants, 2) Johnsongrass populations would vary in phenotypic and genomic stress responses, and 3) Johnsongrass populations from agricultural habitats and non-agricultural habitats would show distinct phenotypic and genomic stress responses.

Materials and Methods

Johnsongrass accessions

Johnsongrass accessions (see Supplementary Table S1) were collected in the fall of 2014 from 23 genetically distinct populations across nine states. Seeds used in this study were collected from an ongoing common garden to reduce maternal effects. Populations growing in active crops (i.e., corn, pumpkin, grain sorghum) were designated as “agricultural habitats” and populations from sites with no known recent history of agriculture (i.e., roadside, old fields) constituted

“non-agricultural habitats” (Atwater et al. 2016). Environmental variables were not measured at the time of seed collection; however, agricultural and nonagricultural habitats differ greatly in regard to substrate type, resource availability, disturbance, and community structure.

Stress experiments

Because of the logistical complexity, we conducted three different experiments at different times. These experiments did not share a single control, but instead there were controls embedded within each experiment.

Measurement of endoreduplication

Tissue collection

Fresh leaf tissue collected from newly emerged leaves following stress application was used for each of the stress studies (competition, herbicide, and clipping) to assess ploidy plasticity in response to stress. Fresh leaves are the most likely to demonstrate genomic responses because they formed after the stress (Dermastia et al. 2012). All stress treatments were applied to plants at 3.5 weeks of age (4 to 5 leaf stage) and applied at a level to severely stress plants without causing mortality. Plants were allowed to regrow after the stress treatments for 10 to 25 days. Due to the large number of treatments and accessions in each stress study, flow cytometry was limited to 3 replications per treatment for each accession. Tissue samples were weighed and later converted into dry mass and added into individual plant total biomass.

Flow cytometry

Preparation of plant tissues for nuclear flow cytometric analyses was the same for each experiment. A standard protocol was followed to prepare samples (see Galbraith et al. 1983).

Plant tissue from the newest available leaf tissue was collected, weighed, and approximately 0.5 g was allocated per sample. Leaf material was placed in a petri dish and chopped with a sharp razor blade to release nuclei. Fresh leaves of *Solanum lycopersicum* (tomato) were collected and approximately 0.5 g were co-chopped with Johnsongrass to serve as an internal standard. After chopping, 0.8 mL of DeLatts buffer (see DeLaat and Blaas 1984) containing cell constituents and 50 µg/mL RNase were passed through a 30-µm filter. Samples were centrifuged for 5 minutes at 200 rpm at 4°C. The supernatant was then removed and the pellet resuspended in 0.5 ml DeLaat's buffer and 200µl of propidium iodide staining solution (1µg/ul) was added. Samples incubated at room temperature for 20 minutes before being processed by flow cytometry by the Flow Cytometry Resource Library at Virginia Tech. Relative fluorescence was measured with the FL2 detector, and DNA content was quantified with FL2-area (integrated fluorescence) and displayed on histograms (Baldwin et al. 2013).

Endoreduplication was measured for each plant replicate as a continuous variable (log transformed), with the percentage of nuclei counted in each channel relative the JG control peak, and the percentage determined from the mean relative fluorescence or nuclear cell content. With endoreduplication as the response variable, we used home habitat (agricultural or nonagricultural), population nested within habitat, and the different stress treatments as categorical predictors in a mixed-model ANCOVA. Initial height and final height were included as covariates to test for linear relationships between phenotypic and genomic responses. The interaction between each factor was included in the analysis.

Herbicide experiment

Johnsongrass seeds from 41 accessions representing 23 populations (Supplementary Table S1) were sown into 5-cm pots containing a sand/soil mixture in March of 2016, and

thinned to one plant per pot. Topsoil was collected from a local site in Blacksburg, Virginia. Four herbicides most commonly used to control Johnsongrass (Table 1) were applied at two different rates to assess population variation in phenotypic characteristics and cellular ploidy levels. We conducted a pre-experiment at 10 and 50% of the 1x lethal rate on a common cultivated commercial Johnsongrass to determine the minimum inhibitory concentration (MIC) to stress plants without killing them, which was 0.5x. Weeds commonly experience sub-lethal herbicide exposure as a result of application drift, the surrounding plant canopy, and low application volumes.

From each accession, three replicates were planted for each herbicide and rate for a total of nine treatments (4 herbicides x 2 rates + controls). Seedlings were grown in a greenhouse in Blacksburg, VA under standard conditions of 30/20°C day/night temperature and a 16-h photoperiod. Plant height was recorded for each plant prior to herbicide application in order to serve as a covariate to account for variation in initial size. Herbicides were applied at the 2-4 leaf stage (~3.5 weeks post-emergence) with an automated spray chamber. All herbicides were applied with surfactants following label instructions (0.25% nonionic surfactant NIS; COC (1% + AMS (4.4 kg/A)). After treatment, plants were returned to the greenhouse, with pots and trays arranged randomly and re-randomized every few days to reduce variation in greenhouse conditions. Plants were rated visually using reference control plants on a scale from 0 (no visible damage) to 100% (dead) 2 and 4 weeks after treatment. Eight weeks after treatment, plant height was recorded for all plants and leaf samples were collected for endoreduplication analyses (see below for details). Aboveground biomass was collected following 7 days of drying in the greenhouse, followed by an additional 48 hours at 65°C.

The effects of herbicides on biomass were assessed using an ANCOVA with herbicide

treatments as a fixed categorical predictor, habitat (agricultural and non-agricultural) as a fixed categorical predictor, accession as a fixed categorical predictor nested within habitat, and interactions among the main factors. We used model selection in which insignificant interactions were removed in a stepwise fashion, and then the model was run again until only significant interactions remained. The heights of seedlings measured before clipping (“initial height”) were included as a covariate to account for initial size variation among accessions. ANCOVAs were followed with posthoc Tukey HSD tests. Analyses here and throughout were performed in JMP 13 (SAS Institute).

Competition experiment

Johnsongrass seed of 15 accessions from agricultural and non-agricultural populations was sown into separate 51 X 25 X 5 cm flats on July 15th 2016 containing sand/soil mixture (Supplementary Materials, Table 1). Corn and a commercially acquired *Sorghum halepense* (hereafter referred to as a conspecific) were sown into separate flats on July 18th to homogenize germination rates. As an agricultural weed, corn is a common competitor, and in non-agricultural communities, Johnsongrass commonly encounters other Johnsongrass. Two weeks after germination, seedlings were transplanted in D40H (656 ml) Deepots filled with a sand/soil (2:1) mix and placed in a greenhouse in Blacksburg, VA. Shoot and root lengths were measured and recorded for each seedling prior to transplanting to account for size variation. Plants were arranged in the following treatments: 1) Johnsongrass vs. corn, 2) Johnsongrass vs. conspecific, 3) Johnsongrass alone as a control, 4) conspecific alone as a control, 5) corn alone as a control. There were six replicates for each accession in each of these treatments and the controls. Plants were grown for six weeks, harvested, separated into roots and shoots, tissue samples collected for endoreplication, and then dried and weighed to a constant temperature.

A restricted maximum likelihood (REML) analysis of covariance (ANCOVA) test was performed including habitat (agricultural or nonagricultural) and competitive treatments (alone, vs. corn, and vs. conspecifics) as fixed categorical predictors. These tests were performed for both root mass and shoot mass. The root length of the longest root on each seedling was measured at the time of transplanting (“initial root length”) and included as a continuous predictor to account for inherent variation among accessions. The heights of seedlings measured before transplanting (“initial height”) were also included as a continuous predictor to account for variation among accessions. The interaction between habitat and competition treatments, and the interaction between each factor and each continuous effect was included in the analysis.

Clipping experiment

Pre-treated Johnsongrass seed of 27 accessions from 19 populations was sown into 51 x 25 x 5 cm separate flats on May 7th 2016 (Supplementary Table, S1). Ten days after germination, plants were transplanted into 5 cm³ filled with a sand/soil mix and placed in a greenhouse located in Blacksburg, VA. Plants were randomly assigned to one of three treatments: 1) unclipped controls; 2) one-time “early” clipping; 3) three-time “multiple” clipping. Multiple clipping occurred 21, 35, and 51 days after transplanting, where we removed ~90% of the aboveground biomass. Initial height was measured for every plant on June 7th before treatments began. Plants were harvested July 30-31, 86 days after sowing, when we measured height, sampled for endoreduplication, and dried aboveground material to a constant mass for 72 hours, and weighed. Single early clipping and multiple clipping treatments allowed us to examine population variation in response to two levels of stress from physical damage, a form of stress that would most commonly be caused by mowing or cutting. Clipping also roughly simulates herbivory, although the effects of clipping can be quite different than actual herbivory

(see Strauss and Agrawal 1999). The varied timing of the clippings was intended to allow regrowth in response to severe damage.

We tested effects of clipping on biomass and height using two separate restricted maximum likelihood (REML) analyses of covariance (ANCOVA) for each (early and multiple clip treatment). Clipping treatments were included in the model as a fixed categorical predictor; control vs. early clip in one, and control vs. multiple clip in the other. In each ANCOVA, habitat (agricultural and non-agricultural) was a fixed categorical predictor, accession was used as a fixed categorical predictor nested within habitat, and all interactions among main factors were determined. The heights of seedlings measured before clipping (“initial height”) were included as a covariate to account for variation among accessions. The ANCOVAs provided general effect tests of clipping treatments and populations, and the presence of interactions.

Results

Herbicide experiment

Biomass – Overall, herbicides reduced Johnsongrass biomass by >30% (ANCOVA, $P < .0001$, Table 2, Fig. 1A), though there was considerable variation among herbicides. Primsulfuron had the strongest suppressive effect on Johnsongrass, reducing aboveground biomass by >40% (Tukey HSD, $P < .0001$) in comparison to all other herbicides. Imazethapyr and glyphosate also reduced Johnsongrass aboveground biomass by 28% and 12%, respectively (Tukey HSD, $P < .0001$, Fig. 1A). Sethoxydim did not affect Johnsongrass biomass (Tukey HSD, $P > 0.5975$, Fig. 1A). Additionally, there was significant variation among accessions within a habitat (ANCOVA, $P = 0.0107$, Table 2). There was a significant interaction between habitat and herbicide treatment, with plants from agricultural habitats treated with primsulfuron and imazethapyr showing more tolerance, higher biomass, relative to plants from non-agricultural

populations, though all populations responded similarly to glyphosate and sethoxydim (ANCOVA, interaction effect, $P=0.0234$, Table 2).

Endoreduplication – Endoreduplication occurred in the controls and all treatments, but overall, endoreduplication increased by 11% in response to the herbicides (ANCOVA, $P<.0001$, Table 2, Fig. 1B), though there was significant variation among herbicides and habitats (Table 2, Fig. 1B). Plants from agricultural populations showed higher levels of endoreduplication than plants from non-agricultural populations in response to herbicides. This corresponded with the lower susceptibility to herbicides shown by agricultural populations. In addition, the herbicides that suppressed growth the most also induced the highest rates of endoreduplication. For example, primisulfuron, which had the strongest inhibitory effect on Johnsongrass, also stimulated the most endoreduplication. Imazethapyr, which also strongly suppressed Johnsongrass, also stimulated more endoreduplication than either glyphosate or sethoxydim, which had weak to no effects on the weed while not stimulating any increase in endoreduplication (Tukey HSD, $P=0.0071$, Fig. 1B). Across all herbicides, endoreduplication decreased as height increased ($R^2=0.339$; $P<0.00001$) (Figure 2). However, this relationship varied among herbicides. First, control plants showed a negative relationship between final height and endoreduplication rates, but height provided very little explanatory power ($R^2=0.03$; $P=0.024$). The two herbicides that suppressed Johnsongrass the most, primisulfuron ($R^2=.225$; $P<0.0001$) and imazethapyr ($R^2=0.227$; $P<0.0001$) showed the strongest relationships negative between height and endoreduplication. The herbicide that suppressed Johnsongrass to a lesser degree, glyphosate ($R^2=0.136$; $P<0.0001$), showed less of a relationship between height and endoreduplication. Finally, the herbicide that had no effect on Johnsongrass, sethoxydim ($R^2=0.007$; $P=0.387$) showed no relationship between height and endoreduplication.

Competition experiment

Root biomass – Competition from corn and conspecifics suppressed Johnsongrass root biomass both by ~45% (Table 3, Fig. 3A). There was significant variation among accessions within a habitat (ANCOVA, $P < .0001$, Table 3), but no overall difference between agricultural and non-agricultural habitats (Table 3).

Root endoreduplication – Despite strong competitive suppression of Johnsongrass by both corn and conspecifics, competition did not affect the proportion of cells showing endoreduplication, which occurred in the controls and all treatments (Table 3, Fig. 3B). There was no difference in endoreduplication responses between the two competitors, nor was there an effect of habitat. However, there was a weak interaction between habitat and competition, with agricultural populations showing slightly less endoreduplication than non-agricultural populations in competition with corn. Overall, endoreduplication rates in the roots of Johnsongrass was positively correlated with root length, but root length explained very little variation in endoreduplication ($R^2=0.072$; $P=0.0023$). Competition with conspecifics resulted in a significant relationship between final height and endoreduplication ($R^2=0.169$; $P=0.008$), but there was no such relationship for either corn ($R^2=0.072$; $P=0.088$) or in the controls ($R^2=0.003$; $P=0.705$) (Figure 4).

Shoot biomass – Competition from corn and conspecifics suppressed Johnsongrass shoot biomass by 50%, and as for roots, no differences between competitors (Table 3, Fig. 3C). Unlike root biomass, there was variation among accessions within a habitat, but no overall differences between the competitive responses of Johnsongrass between agricultural and non-agricultural habitats.

Shoot endoreduplication – Competition had no effect on the proportion of cells showing endoreduplication (Table 3, Fig. 3D), but there was significant variation among accessions in endoreduplication. Across competition treatments, the proportion of cells showing endoreduplication was 8% higher ($P=0.044$) for populations from non-agricultural habitats than populations from agricultural habitats.

Across all treatments, endoreduplication rates in Johnsongrass shoots was marginally negatively correlated with shoot height, but shoot height explained very little variation in endoreduplication ($R^2=0.029$; $P=0.054$) (Figure 4). Competition with corn resulted in a significant relationship between height and endoreduplication ($R^2=0.134$; $P=0.017$), but there was no relationship for either conspecifics ($R^2=0.00009$; $P=0.952$) or controls ($R^2=0.022$; $P=0.333$) for this relationship.

Early clipping experiment

Shoot biomass – Clipping Johnsongrass early (21 days after transplanting) reduced final biomass by 10% (Table 4, Fig. 5C). There was no difference in the final biomass of plants from agricultural and non-agricultural habitats, though there was considerable variation among accessions in final biomass.

Shoot endoreduplication – Clipping increased endoreduplication by 5% (Table 4, Fig. 5D), which occurred in the controls and the treatments. There was variation among accessions in endoreduplication, but there were no differences among plants from agricultural and non-agricultural populations. Furthermore, there were no significant interactions among any of the main factors. For plants that were clipped, final height was negatively related to endoreduplication, suggesting that plants that were stressed the most by clipping also

endoreduplicated the most ($R^2=0.186$; $P<0.0001$) (Figure 6).

Multiple clipping experiment

Shoot biomass – Clipping Johnsongrass multiple times reduced final biomass by ~40% (Table 5, Fig. 5). There was significant variation among accessions, but no differences among plants from agricultural and non-agricultural populations.

Shoot endoreduplication – Similar to the early clipping treatment, both clipped and unclipped Johnsongrass showed endoreduplication, but multiple clipping increased endoreduplication by 35%, much more than the response to a single early clipping event (Table 5, Fig. 5). There was significant variation among accessions in the rate of endoreduplication, but there were no differences among plants from agricultural and non-agricultural populations. There were no significant interactions among any of the main factors. As for plants that experienced early clipping, plants that were clipped multiple times showed final heights that were negatively related to endoreduplication. If shorter plants reflected greater suppression by clipping, and thus greater stress, then plants that were stressed the most by clipping also endoreduplicated the most ($R^2=0.130$; $P<0.0001$) (Figure 6).

Discussion

The global invader Johnsongrass shows phenotypic and genomic plasticity in response to all biotic and abiotic stresses tested. In most cases, plant height was negatively correlated with the level of endoreduplication, suggesting that plants that were most stressed had the highest levels of endoreduplication. However, it is important to note that endoreduplication consistently occurred in all treatments, just at different magnitudes. Of primary interest, some phenotypic

and genomic responses differed between Johnsongrass accessions collected from agricultural and non-agricultural habitats. For example, plants from agricultural habitats were suppressed less by herbicides in general than plants from non-agricultural habitats, suggesting the possibility of adaptation to tolerate herbicides in populations exposed more frequently to their application. There are many such examples of plants evolving tolerance to herbicides (Heap 2015, Baucom 2016). However, we also found that this phenotypic tolerance of herbicide corresponded with greater rates of endoreduplication by plants from agricultural habitats in general, and that the specific herbicides that caused the greatest suppression also caused the highest rates of endoreduplication. In the competition experiment, the phenotypic and genomic responses to stress were less clear, but endoreduplication rates in Johnsongrass shoots from non-agricultural populations were higher than that for plants from agricultural populations across all treatments. Plants in competition that produced the longest roots also reduplicated the most. Finally, plants that were clipped, in both early and multiple clipping treatments, endoreduplicated more than those that were not clipped. The general trend across all stress treatments was that individuals experiencing more stress have higher rates of endoreduplication, which in many cases varied among Johnsongrass ecotypes. To our knowledge, this is the first evidence that endoreduplication may influence the response of an invader to multiple stresses.

The differences we found between Johnsongrass populations from agricultural and non-agricultural habitats in phenotypic and genomic responses to stress are consistent with a number of studies of plant species evolving in agricultural habitats. In a common greenhouse experiment, Bommarco et al. (2010) compared traits of the agricultural weed *Cirsium arvense* collected from populations occurring in natural environments, in semi-natural environments, on conventional farms, and on organic farms. Some of these traits were interpreted as conferring

superior competitive ability, and they found that some of these “competitive” traits differed among populations in ways that suggested the evolution of weaker competitive ability in agricultural conditions. Similarly, genetic analyses indicated some differentiation between plants collected on conventional farms versus recently established organic farms, and interpreted this as evidence for adaptation to different forms of agriculture. Leiss and Müller-Schärer (2001a) explored phenotypic variation in the ruderal weed *Senecio vulgaris* in a common garden experiment using seed collected from ruderal and agricultural populations. They found that *S. vulgaris* plants from agricultural habitats produced larger leaves and had a higher reproductive rate than plants from ruderal habitats. They also found that plants from agricultural habitats increase reproduction in response to high soil nutrient concentrations. In a second experiment with seeds of *S. vulgaris* from agricultural and ruderal populations sown reciprocally into agricultural and ruderal habitats, Leiss and Müller-Schärer (2001b) found that emergence, growth, and fecundity differed between plants from the different habitats but no indication of local adaptation.

We found that herbicides applied at a sub lethal dose generally suppressed the growth of Johnsongrass and stimulated endoreduplication. In contrast to the relatively few general comparisons of the traits and genetics of plants from agricultural and non-agricultural habitats, there is a great deal of phenotypic and genomic evidence for the rapid evolution of resistance to herbicides (see survey by Heap 2015). This is particularly true when herbicides with a single mode of action are applied repeatedly (e.g. Stankiewicz et al. 2001; Tranel and Wright 2002; Menchari et al., 2006). Genetic mutations are thought to drive much of this rapid evolution (Tranel and Wright 2002; Délye 2005), though there is a growing body of evidence for alternative mechanisms that may contribute to rapid herbicide resistance development. Menchari

et al. (2006) measured the relative frequencies of seven alleles, known to confer resistance to different herbicides in the weedy grass *Alopecurus myosuroides* collected from 243 different sites. Measurements of some alleles indicated that the evolution of herbicide resistance has occurred in this species independently in different populations and at many different times (also see Menchari et al. 2007). To experimentally select for herbicide resistance, Neve and Powles (2005) applied the herbicide diclofop-methyl to a large number of individual plants of an herbicide-susceptible biotype of *Lolium rigidum*. A low rate, 10% of the recommended field application rate, was applied that resulted in a 36% survival rate. From these they produced a “low dose-selected line”. Plants from this line showed increased tolerance of diclofop-methyl. After two other cycles of selection they produced lines that were resistant to far higher rates of herbicide application. Clearly, the evolution of herbicide resistance provides a very powerful scenario of rapid evolution to agricultural practices. In the context of our results, many cases of herbicide resistance have no known mechanism. Endoreduplication may provide a genomic mechanism for some of these cases.

In our experiments, we examined the possible role endoreduplication plays in response to common environmental stressors, including herbicides. We found that endoreduplication increased in response to herbicide and to clipping in general, but not to competition. For example, for all herbicides except sethoxydim, which had no effect on biomass, endoreduplication was negatively correlated with plant height. Furthermore, the herbicides that reduced Johnsongrass biomass the most, primisulfuron and imazethapyr, also stimulated the largest increases in endoreduplication. Similarly, clipping reduced height and also increased endoreduplication. However, competition clearly induced stress as Johnsongrass plants in competition were roughly 50% smaller than those grown alone, but there was no increase in

endoreduplication in plants experiencing competition. These results indicate that endoreduplication by Johnsongrass may be a genomic response that corresponds with some forms of stress, but not all. Both herbicide and clipping induce strong metabolic and physiological reactions, while competition is slower to progress and can manifest through more complex reactions. Perhaps if we had let the competition experiment run longer, so that competition was more intense, we may have observed a signal with endoreduplication. Further studies should consider competition intensity and duration.

To our knowledge, few other studies have compared endoreduplication in response to herbivory or simulated herbivory (Scholes and Paige 2011, 2014, 2015; Scholes et al. 2013). For example, Scholes and Paige (2011) measured endoreduplication after simulating herbivory by clipping in two varieties of *Arabidopsis thaliana*, Columbia and Landsberg erecta. They found that endoreduplication in clipped Columbia plants was greater than that of unclipped controls, and that this genomic response was positively correlated with fitness. Clipped Landsberg erecta showed a decrease in fitness with clipping and there was no difference in endoreduplication between clipped and unclipped plants. Scholes et al. (2013) reported a general relationship between endoreduplication and compensatory growth responses to damage. They established crosses between Columbia and Landsberg erecta and found that lines that undercompensated (i.e., produced the least biomass) in response to clipping had a lower level of endoreduplication. In other words, Columbia endoreduplicates and overcompensates following damage, while Landsberg erecta does not endoreduplicate or overcompensate following damage. Scholes and Paige (2014) overexpressed an endoreduplication regulator gene in Columbia and found that increasing the ability to endoreduplicate led to an even greater ability to compensate to damage mimicking herbivory.

Our results are more limited in some ways than studies of *A. thaliana*, which has a very short lifecycle. Our experiments were shorter in duration, and thus we did not measure any aspect of reproduction or fitness. Therefore, we could not assess how endoreduplication corresponded with fitness. However, our limitations may be compensated for by the discovery of correlations between endoreduplication and different stress responses by an important invasive and agricultural weed, and some that may have resulted from local adaptation.

Our results contribute to a growing body of research indicating that environmentally induced endoreduplication can be an important and common genomic response to stress in plants. In a review of endoreduplication in plants, Scholes and Paige (2015) summarized a number of stresses to which plants appeared to respond with endoreduplication and noted that endoreduplication in response to abiotic stresses was better understood than such responses to biotic stresses. Shade stress corresponds with increased endoreduplication for a number of species, and long-term exposure to UV-B can stimulate cytometric ploidy and cell growth. Moderate water stress has also been shown to increase endoreduplication in *Arabidopsis thaliana*. As noted above, salt stress can induce endoreduplication and cadmium has been reported to increase this genomic response in the roots of *Pisum sativum*.

Evidence in the literature for endoreduplication in response to biotic stresses is scant. In their recent review, Scholes and Paige (2015) pointed out that evidence for endoreduplication in response to biotic factors is limited to a few studies of simulated herbivory. They specifically noted that the effects of competition remained unexplored except in the context of stress induced by several experimentally applied allelochemicals. We found that competition appeared to induce substantial phenotypic stress, reducing Johnsongrass biomass by roughly 50%. However, this growth suppression did not correspond with an increase in endoreduplication. Interestingly

though, our competition experiments yielded a striking competitor-specific increase in endoreduplication. When Johnsongrass competed with corn, plants from non-agricultural populations significantly increased endoreduplication rates. While the exact mechanism is unknown, we speculate that endoreduplication may be higher since corn did not suppress target Johnsongrass as much as conspecifics. Perhaps through some form of signaling or other chemical process in the soil corn stimulated endoreduplication without even greater stress levels. Alternatively, corn may have competed with Johnsongrass for a different resource that increased endoreduplication, which in turn resulted in a higher biomass than otherwise would have occurred.

In sum, our results suggest that endoreduplication may play a stress-specific role in Johnsongrass, and that Johnsongrass may have evolved in both phenotypic and genomic ways to agricultural habitats in North America. This further supports our growing evidence of ecotypic divergence of agricultural and non-agricultural Johnsongrass populations that in many cases are geographically sympatric (Atwater et al. 2017; Sezen et al. 2017). While these experiments did not show that endoreduplication elicited enhanced stress responses, we did show that phenotypic performance is strongly correlated with endoreduplication. Further experiments in which genes are under or overexpressed (see Scholes and Paige 2014) are needed to make strong causal connections between stress responses and genomic responses such as endoreduplication. A better understanding of the relationship between endoreduplication and stress responses advances our understanding of how plants cope with stress in general, and may provide insight into traits that contribute to invasiveness.

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Table 1. Herbicide active ingredients, trade names, modes of action, and applied rates for low and high stress treatments.

Herbicide active ingredient	Trade name	Mode of Action	Low stress rate (0.5X)	High stress rate (1X)
glyphosate	Roundup PowerMax	EPSP Synthase inhibitor	0.150 kg/ha	0.310 kg/ha
primisulfuron	Beacon	ALS inhibitor	0.007 kg/ha	0.014 kg/ha
imazethapyr	Pursuit	ALS Inhibitor	0.057 kg/ha	0.113 kg/ha
sethoxydim	Poast Plus	ACCCase inhibitor	0.340 kg/ha	0.680 kg/ha

Table 2. ANCOVA table for aboveground biomass (cube root transformed) endoreduplication (log transformed) in response to herbicide treatments, habitat type, and accession nested within habitat type. Plant height before herbicide application and height measured at the time that endoreduplication was measured were included as covariates. Significant results are in bold type.

Response variable				
Effect type	Effect	<i>DF</i>	F ratio	<i>P</i> value
Shoot biomass				
Fixed	herbicide	4	77.722	<0.0001
Fixed	habitat	1	3.669	0.0560
Fixed	accession w/in habitat	39	1.635	0.0107
Fixed	initial height (cm)	1	61.792	<0.0001
Fixed	habitat x herbicide	4	2.850	0.0234
Endoreduplication				
Fixed	herbicide	4	11.288	<0.0001
Fixed	habitat	1	4.635	0.0194
Fixed	accession w/in habitat	39	1.066	0.368
Fixed	initial height (cm)	1	3.804	0.0038
Fixed	final height (cm)	1	14.58	0.0108

Table 3. ANCOVA table for shoot and root biomass (both cube root transformed) and shoot and root endoreduplication (both log transformed) of Johnsongrass in response to competition from corn and conspecifics, habitat type, and accession nested within habitat type. Plant height at the time of treatment and at the end of the experiment were included as covariates. There was no difference in the effects of these competitors so they are combined in this analysis. Significant results are in bold type.

Response variable				
Effect type	Effect	DF	F ratio	P value
Shoot biomass				
Fixed	competition	2	11.499	<0.0001
Fixed	habitat	1	2.045	0.1555
Fixed	accession w/in habitat	12	3.687	0.0001
Fixed	initial height (cm)	1	12.454	0.0006
Shoot endoreduplication				
Fixed	competition	2	1.537	0.2195
Fixed	habitat	1	4.138	0.0443
Fixed	accession w/in habitat	12	1.957	0.0351
Fixed	initial height (cm)	1	1.568	0.2131
Fixed	final height (cm)	1	11.163	0.0011
Root biomass				
Fixed	competition	2	14.042	<0.0001
Fixed	habitat	1	0.024	0.8759
Fixed	accession w/in habitat	12	3.840	<0.0001
Fixed	initial height (cm)	1	6.08	0.0153
Root endoreduplication				
Fixed	Competition	2	2.105	0.1268
Fixed	habitat	1	1.343	0.2490
Fixed	accession w/in habitat	12	1.092	0.3742
Fixed	initial height (cm)	1	0.025	0.8726
Fixed	final height (cm)	1	7.257	0.0082
Fixed	competition X habitat	2	2.572	0.0811

Table 4. ANCOVA table for shoot biomass (cube root transformed) and endoreduplication (log transformed) of Johnsongrass in response to early clipping (4 weeks post emergence) and multiple clippings. Factors include clipping, habitat type, and accession nested within habitat type. Plant height at the time of treatment and at the end of the experiment were included as covariates. Significant results are in bold type.

Response variable					
Effect type	Effect	DF	F ratio	P value	
Early-clip shoot biomass					
Fixed	clip	1	105.222	<.0001	
Fixed	habitat	1	0.886	0.3472	
Fixed	accession w/in habitat	27	3.813	<.0001	
Fixed	initial Height (cm)	1	5.033	0.0256	
Early-clip endoreduplication					
Fixed	clip	1	4.0695	0.0445	
Fixed	habitat	1	0.1314	0.7172	
Fixed	accession w/in habitat	27	1.5758	0.0369	
Fixed	initial height (cm)	1	1.7759	0.1836	
Multi-clip shoot biomass					
Fixed	clip	1	865.610	<0.0001	
Fixed	habitat	1	0.093	0.7602	
Fixed	accession w/in habitat	27	2.487	0.0002	
Fixed	initial Height (cm)	1	3.259	0.0726	
Multi-clip endoreduplication					
Fixed	clip	1	52.525	<0.0001	
Fixed	habitat	1	1.009	0.3161	
Fixed	accession w/in habitat	27	1.552	0.0461	
Fixed	initial Height (cm)	1	0.452	0.5021	

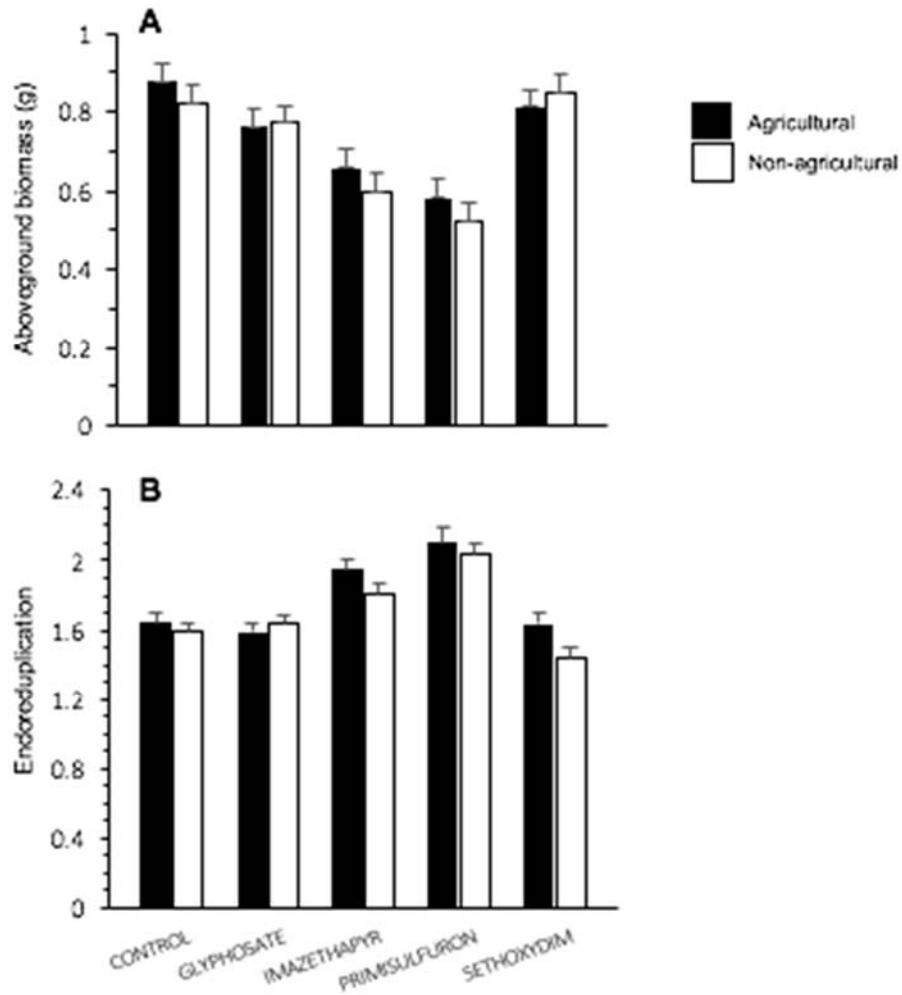


Figure 1. A) Aboveground biomass (cube root transformed) and B) endoreduplication (\log_{10} transformed) of agricultural and non-agricultural Johnsongrass populations in response to herbicide treatments. Endoreduplication measured by fluorescence events of stained DNA within suspended nuclei.

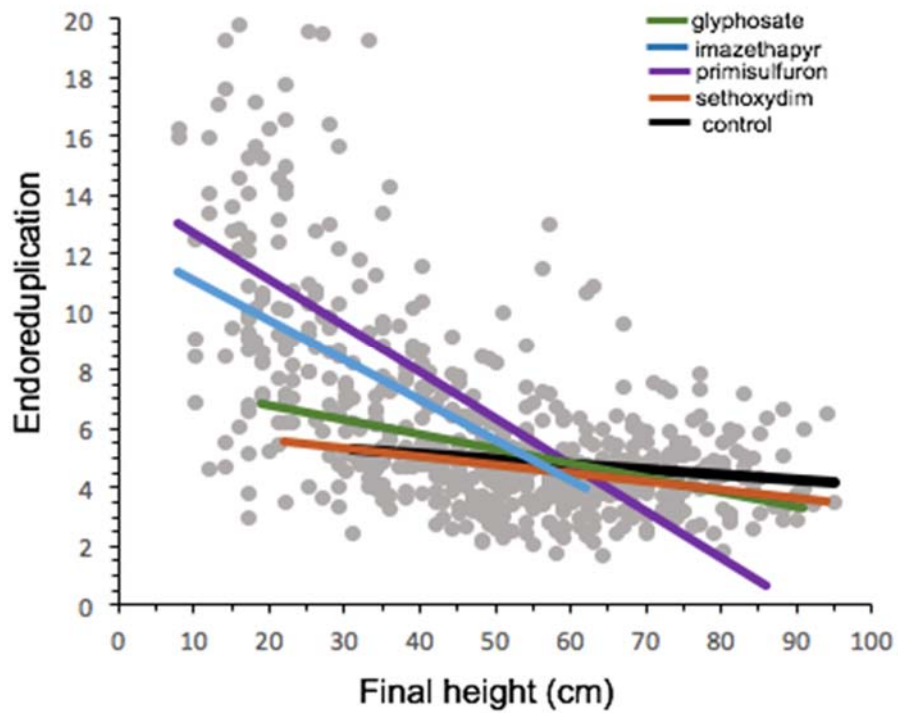


Figure 2. Correlations between Johnsongrass final height and endoreduplication when treated with herbicides. Different colored lines represent different herbicides; see Table 2 and Results for statistics. Endoreduplication measured by fluorescence events of stained DNA within suspended nuclei.

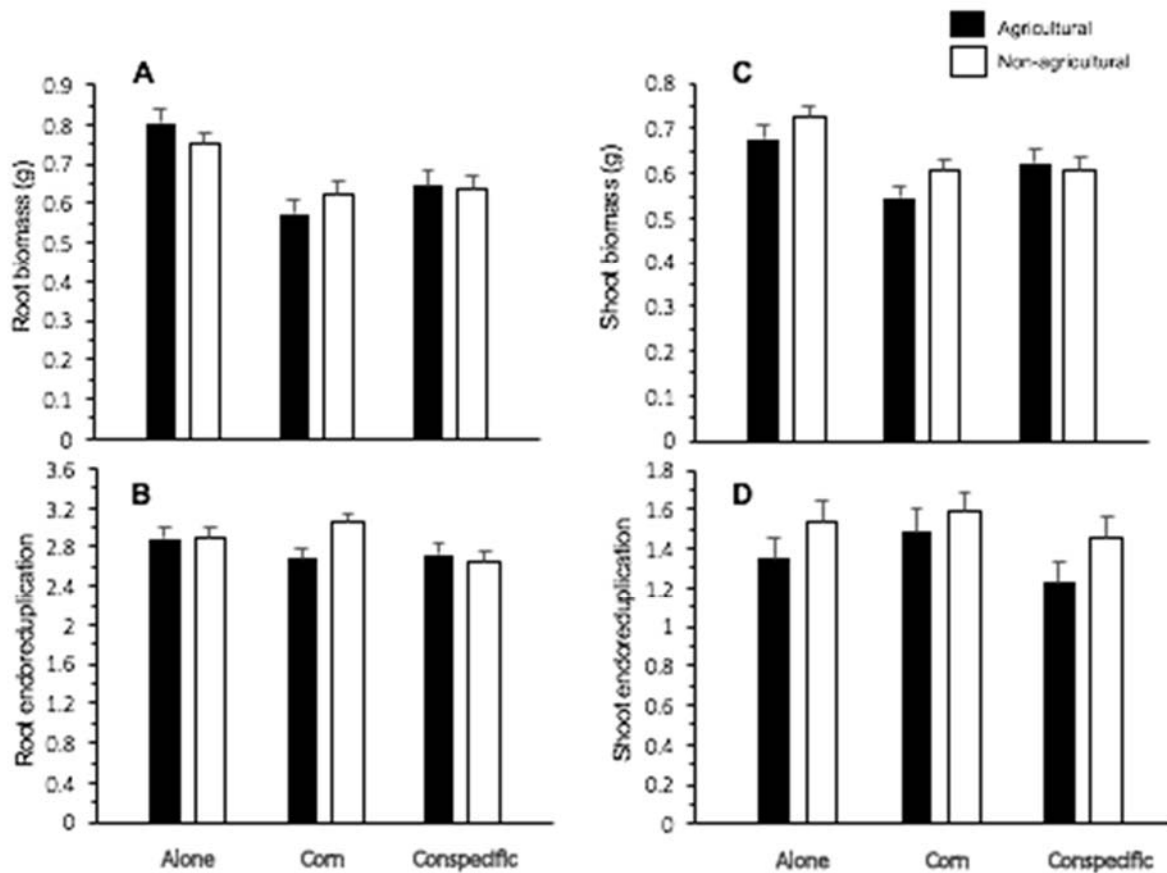


Figure 3. Root biomass (cube root transformed) A) and shoot biomass (cube root transformed) B) and the proportion of cells in Johnsongrass roots C) and shoots D) demonstrating endoreduplication when grown alone, with corn, or with a conspecific. Bars represent means and 1 SE.

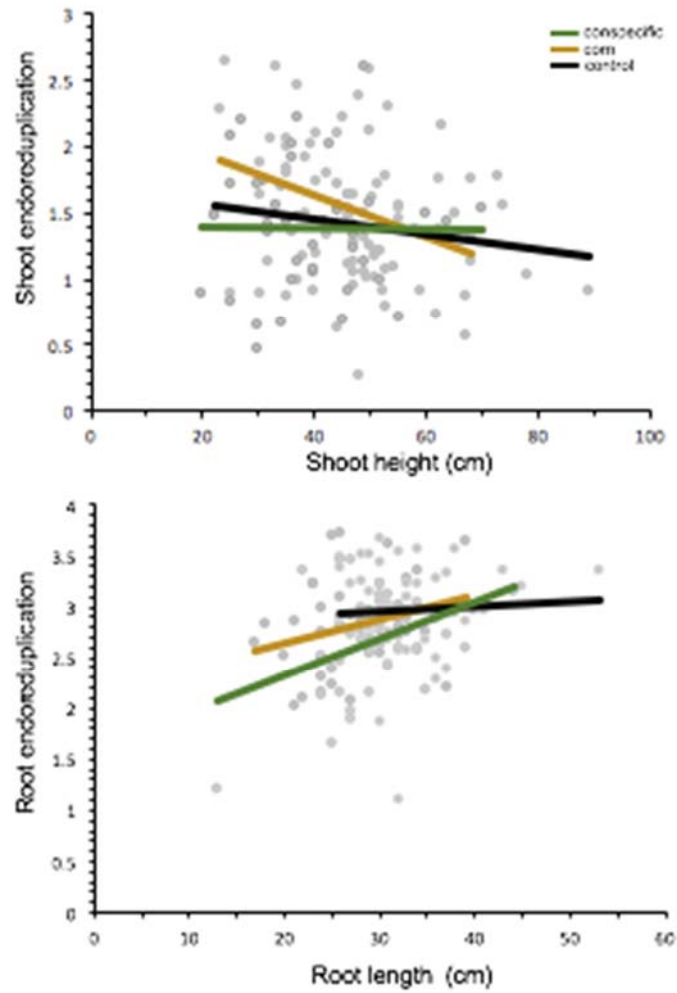


Figure 4. Correlation between shoot height A) and root length B) and endoreduplication rates for Johnsongrass plants as controls or exposed to competition with corn or conspecifics. See Table 3 and results for statistics.

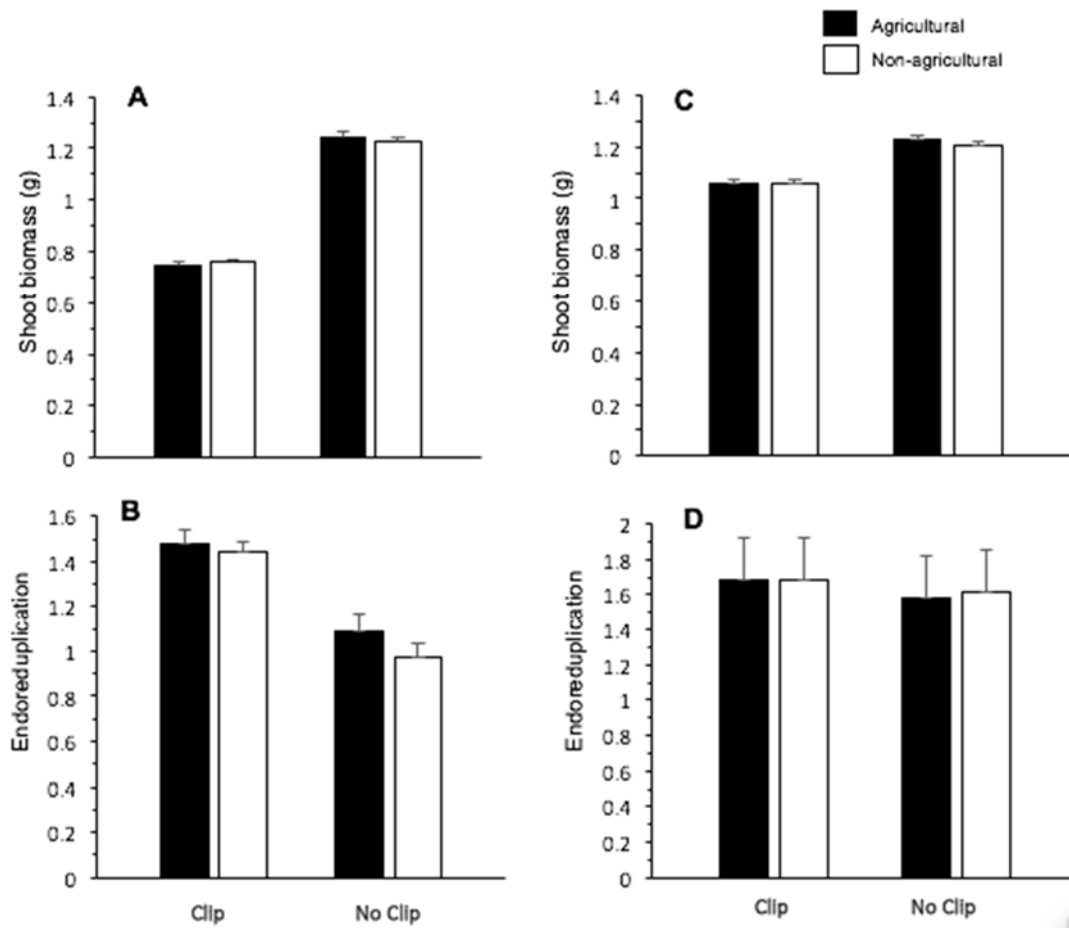


Figure 5. Shoot biomass (cube root transformed) A) and endoreduplication rates (log transformed) B) of Johnsongrass subjected to multiple rounds of clipping. Shoot biomass (cube root transformed) C) and endoreduplication rates (log transformed) D) of Johnsongrass subjected to early clipping, 21 days after transplanting seedlings. Bars represent means and 1 SE.

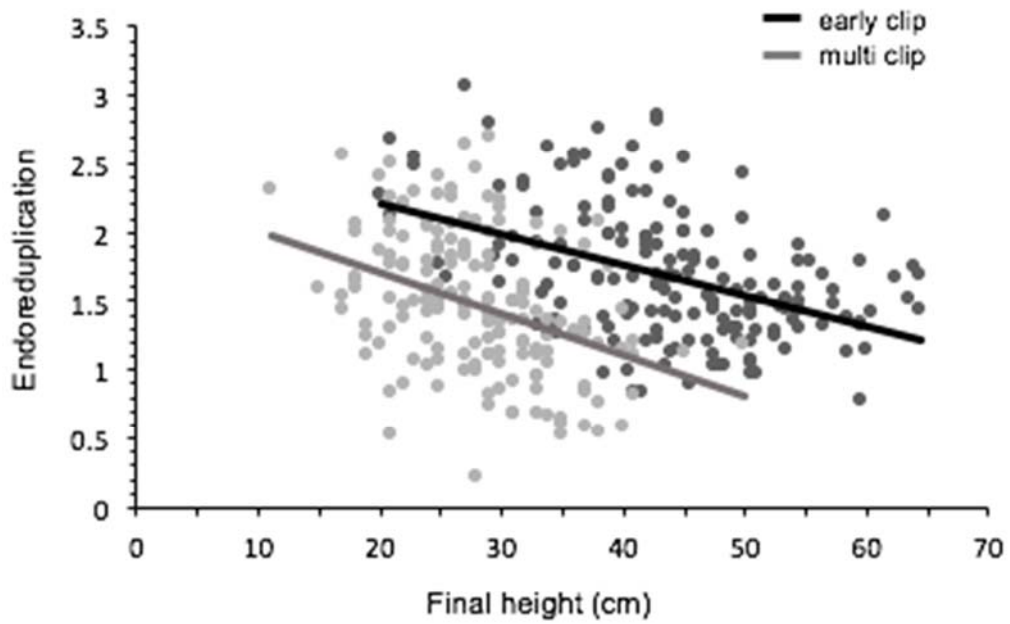


Figure 6. Correlation between final height and endoreduplication for the early clip experiment (gray symbols) and the multiple clip experiment (black symbols). Statistics are presented in Table 4 the Results.

Supplemental Materials

S1. Location and home habitat information for Johnsongrass accessions used in the three different stress experiments.

Accession ID	Pop. Name	Herb.	Clip.	Comp.	Habitat	Latitude	Longitude	Elev (m)
Al-10-1	AL-10	Y	N	N	NON-AG	34.277833	-86.889833	296
Al-11-1	AL-11	Y	N	N	NON-AG	34.648333	-87.1055	176
Al-12-1	AL-12	Y	Y	N	NON-AG	34.752167	-86.959	207
AZ-3-2	AZ-3	Y	N	N	NON-AG	33.103267	-111.97425	353
AZ-3-23	AZ-3	Y	Y	N	NON-AG	33.103267	-111.97425	353
AZ-3-25	AZ-3	Y	Y	Y	NON-AG	33.103267	-111.97425	353
AZ-3-29	AZ-3	Y	Y	Y	NON-AG	33.103267	-111.97425	353
CA-1-7	CA-1	Y	Y	N	AG	36.199022	-119.251914	92
CA-2-1	CA-2	Y	N	N	NON-AG	36.308806	-119.380639	92
CA-3-16	CA-3	Y	Y	Y	NON-AG	36.233728	-119.312081	94
CA-3-19	CA-3	Y	Y	Y	NON-AG	36.233728	-119.312081	94
GA-15-2	GA-15	Y	Y	N	NON-AG	33.96	-83.37	195
GA-3-1	GA-3	Y	N	Y	NON-AG	32.205	-82.3635	87
GA-6-1	GA-6	Y	Y	N	AG	33.525	-83.444167	195
GA-8-1	GA-8	Y	Y	N	NON-AG	34.4655	-84.6995	240
KS-1-6	KS-1	N	Y	Y	NON-AG	38.883333	-97.734444	408
KS-4-18	KS-4	Y	Y	Y	AG	38.706111	-97.427778	378
KS-4-19	KS-4	Y	N	N	AG	38.706111	-97.427778	378
KS-4-5	KS-4	Y	N	N	AG	38.706111	-97.427778	378
KS-4-6	KS-4	Y	Y	Y	AG	38.706111	-97.427778	378
KS-4-7	KS-4	Y	Y	N	AG	38.706111	-97.427778	378
NM-12-3	NM12	Y	Y	Y	AG	34.355974	-103.061773	1262
SC-3-1	SC-3	Y	Y	Y	NON-AG	34.030667	-81.588333	140
SC-5-1	SC-5	Y	Y	N	NON-AG	33.861667	-80.860167	43
SC-7-1	SC-7	Y	Y	N	NON-AG	33.290667	-81.337333	78
TX-1-15	TX-1	Y	N	N	AG	31.06	-97.3422	200
TX-1-16	TX-1	Y	Y	N	AG	31.06	-97.3422	200
TX-3-13	TX-3	Y	Y	N	AG	33.2797	-96.8927	171

TX-3-20	TX-3	N	N	Y	AG	33.2797	-96.8927	171
TX-3-5	TX-3	Y	N	N	AG	33.2797	-96.8927	171
TX-3-8	TX-3	Y	N	Y	AG	33.2797	-96.8927	171
TX-3-9	TX-3	Y	N	N	AG	33.2797	-96.8927	171
TX-4-1	TX-4	Y	Y	Y	NON-AG	34.330872	-102.976227	1240
TX-4-10	TX-4	Y	N	N	NON-AG	34.330872	-102.976227	1240
TX-4-11	TX-4	Y	N	N	NON-AG	34.330872	-102.976227	1240
TX-4-3	TX-4	Y	N	N	NON-AG	34.330872	-102.976227	1240
TX-5-10	TX-5	Y	N	N	NON-AG	34.315554	-102.773632	1196
TX-5-3	TX-5	Y	Y	Y	NON-AG	34.315554	-102.773632	1196
TX-5-5	TX-5	Y	Y	N	NON-AG	34.315554	-102.773632	1196
VA-1-1	VA-1	Y	Y	Y	AG	37.211413	-80.48768	77
VA-1-3	VA-1	Y	Y	N	AG	37.211413	-80.48768	77
VA-1-4	VA-1	Y	Y	Y	AG	37.211413	-80.48768	77
VA-1-9	VA-1	Y	N	N	AG	37.211413	-80.48768	77
