

Control of Bird Feeding Behavior by *Tannin1* through Modulating the Biosynthesis of Polyphenols and Fatty Acid-Derived Volatiles in Sorghum

Peng Xie^{1,2,5}, Jiayang Shi^{1,2,5}, Sanyuan Tang^{1,5}, Chengxuan Chen^{1,2}, Aimal Khan^{1,2}, Fengxia Zhang^{1,2}, Ying Xiong³, Chao Li¹, Wei He⁴, Guodong Wang^{1,2}, Fumin Lei³, Yaorong Wu^{1,2,*} and Qi Xie^{1,2,*}

¹State Key Laboratory of Plant Genomics, Institute of Genetics and Developmental Biology, The Innovative Academy of Seed Design, Chinese Academy of Sciences, Beijing 100101, P. R. China

²University of Chinese Academy of Sciences, Beijing 100049, P. R. China

³Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, P. R. China

⁴School of Pharmaceutical Sciences, Tsinghua University, Beijing 100084, P. R. China

⁵These authors contributed equally to this article.

*Correspondence: Yaorong Wu (yrwu@genetics.ac.cn), Qi Xie (qxie@genetics.ac.cn) https://doi.org/10.1016/j.molp.2019.08.004

ABSTRACT

Bird predation during seed maturation causes great loss to agricultural production. In this study, through GWAS analysis of a large-scale sorghum germplasm diversity panel, we identified that *Tannin1*, which encodes a WD40 protein functioning in the WD40/MYB/bHLH complex, controls bird feeding behavior in sorghum. Metabolic profiling analysis showed that a group of sorghum accessions preferred by birds contain mutated *tan1-a/b* alleles and accumulate significantly lower levels of anthocyanins and condensed tannin compounds. In contrast, a variety of aromatic and fatty acid-derived volatiles accumulate at significantly higher levels in these bird-preference accessions. We subsequently conducted both sparrow feeding and sparrow volatile attractant assays, which confirmed, respectively, the antifeedant and attractant functions of these differentially accumulated metabolites. In addition, the connection between the biosynthesis pathway of anthocyanin and proanthocyanidin and the pathway of fatty acid-derived volatile biosynthesis was demonstrated by discovering that Tannin1 complex modulates fatty acid-derived volatiles. Taken together, our study identified *Tannin1* as the gene underlying the major locus controlling bird feeding behavior in sorghum, illustrating an example of the identification of an ecologically impactful molecular mechanism from field observation and providing significant insights into the chemistry of bird-plant ecological interactions.

Key words: bird damage, GWAS, metabolism, tannin, volatiles

Xie P., Shi J., Tang S., Chen C., Khan A., Zhang F., Xiong Y., Li C., He W., Wang G., Lei F., Wu Y., and Xie Q. (2019). Control of Bird Feeding Behavior by *Tannin1* through Modulating the Biosynthesis of Polyphenols and Fatty Acid-Derived Volatiles in Sorghum. Mol. Plant. **12**, 1315–1324.

INTRODUCTION

Damage by birds is one of the most severe biotic constraints on crop production worldwide (Coleman and Spurr, 2001; De Mey et al., 2012; Anderson et al., 2013). In cereal crops, birds cause damage by provoking lodging, pecking seeds, and sucking the juice of immature seeds, preventing full development of many grains and frequently encouraging mildews and other plant diseases around panicles (Tipton et al., 1970). Some of

the cereal crops most vulnerable to bird damage include wheat, barley, rice, sorghum, and millet (Dolbeer et al., 1986).

Sorghum (Sorghum bicolor ([L.]) moench) is a major global cereal crop that is a steady source of calories for more than

Published by the Molecular Plant Shanghai Editorial Office in association with Cell Press, an imprint of Elsevier Inc., on behalf of CSPB and IPPE, SIBS, CAS.

Molecular Plant

500 million people worldwide and is also an important source of forage and biofuel. Sorghum is particularly important in difficult environments because of its strong ability to resist multiple abiotic stresses (Gilbert, 2009; Xie and Xu, 2019). Bird species known to consume sorghum include sparrow (Passer domesticus), parrot, pigeon (Columba livia), and Quelea quelea, among others (De Melo and Cheschini, 2012), and yield losses in sorghum caused by birds have been reported to reach as high as 52% (Kale et al., 2014). Moreover, there are few efficient control measures to protect field-grown crops from bird predation: bagging panicles and/or building anti-bird nets can require immense manpower and material investment. It was reported that birds exhibit sorghum germplasm-dependent taste preferences, and it has been proposed that bird preferences for sorghum are correlated with phenotypes such as compact panicle shape, the extent of glume coverage, seed coat color, and tannin content (Perumal and Subramaniam, 1973). However, the molecular and/or chemical basis underlying bird feeding behavior remains unknown.

Tannins, a general term for a class of polyphenolic compounds, are very common in the plant kingdom (Hillis, 1958). The condensed tannins, also widely known as proanthocyanidins (PAs), are polymeric derivatives of flavan-3-ols (Dixon et al., 2005). The anthocyanin content in sunflower is known to be associated with resistance to bird depredation (Mason et al., 1989), while tannic acid (known as one of the hydrolyzable tannins) and PAs have astringent properties and act as deterrents that reduce food digestibility by binding dietary proteins and digestive enzymes (da Costa et al., 2008; Espinosa Gomez et al., 2018). Anthocyanin and PAs are derived from a branch of the flavonoid pathway, which is well documented in Arabidopsis (Stracke et al., 2007, 2009). The first rate-limiting step, from which chalcone is produced, is controlled by CHS (chalcone synthase) (Ferrer et al., 1999; Austin and Noel, 2003; Grotewold, 2006), after which DFR (dihydroflavonol 4-reductase) catalyzes another critical step in the biosynthesis of anthocyanin and PA, resulting in the formation of the related compound leucoanthocyanidin. Subsequently, anthocyanidin is directly biosynthesized from leucoanthocyanidin by ANS (anthocyanidin synthase) (Grotewold, 2006). Leucoanthocyanidin and anthocyanidin are directly converted into flavan-3-ols including catechin and epicatechin by LAR (leucoanthocyanidin reductase) and ANR (anthocyanidin reductase), respectively. Flavan-3-ols can subsequently polymerize into proanthocyanidin via an as yet unsolved mechanism (Xie and Dixon, 2005). Using genetic linkage mapping, Wu et al. (2012) cloned the Tannin1 (Tan1) gene, a homolog of Arabidopsis TTG1 that encodes a WD40 repeat protein functioning in the WD40-MYB-bHLH complex and controls the synthesis of anthocyanin and PA, and found that Tannin1 may have a regulatory function similar to that of Arabidopsis TTG1.

It is well known that fatty acid (FA) biosynthesis is involved in the development of major seed-storage compounds in plants (Baud et al., 2002). Acetyl-coenzyme A (CoA), which is derived from glycolysis, can be further converted into malonyl-CoA. Both acetyl-CoA and malonyl-CoA are common building blocks for

Tannin1 Modulates Bird Feeding Behavior in Sorghum

FA biosynthesis in the embryo, while malonyl-CoA also functions as a major precursor for flavonoid biosynthesis (Lepiniec et al., 2006). A large amount of plant short-chain volatile organic compounds (VOCs) originate from C₁₈ unsaturated FAs (linoleic acid or linolenic acid) via branches of the lipoxygenase (LOX) pathway (Feussner and Wasternack, 2002; Dudareva et al., 2013). Among them, C₆ and C₉ aldehydes are mainly produced from hydroperoxide FA derivatives in reactions catalyzed by hydroperoxide lyase. Short-chain aldehydes commonly are converted to alcohols by alcohol dehydrogenases (Gigot et al., 2010). Most short-chain aldehydes and alcohols naturally carry their own characteristic "fresh green" aroma in plants, and are produced as a strategy for either pollinator attraction or plant defense (Raguso, 2008; Unsicker et al., 2009; Huang et al., 2012).

There are few control measures to combat damage by birds, and crops such as sorghum and millet, which are grown in marginal agroecosystems, are known to be particularly vulnerable to yield loss from bird predation. In this study, we identified a single major locus, Tannin1, which controls both polyand bird-attractant volatiles in phenols sorahum simultaneously. Metabolic and gene expression analyses revealed that the Tannin1 complex potentially regulates the expression of SbGL2 and thus controls FA-derived volatile accumulation by affecting the FA biogenesis pathway. Our study provides new insights about the chemistry of birdplant ecological interactions and suggests multiple strategies for developing new control measures to prevent the catastrophic yield losses caused by birds each year.

RESULTS

Identification of the *Tannin1* Locus Linked to Bird Feeding Behavior by GWAS Analysis

In the course of large-scale germplasm diversity panel studies, we made the serendipitous observation of apparently extreme differences in the preferences of birds for consuming particular germplasm accessions (Figure 1A). Specifically, we analyzed two large germplasm diversity panels-one comprising 352 US Sorghum Association Panel (SAP) lines (Casa et al., 2008; Morris et al., 2013) and the other comprising 219 sweet sorghum lines (Burks et al., 2015), which were grown for two consecutive years at three Chinese sites in field experiments with no control measures to prevent bird feeding-and observed major and apparently binary differences in bird preferences for particular accessions. We phenotyped each of the accessions as being either a "bird-avoidance" or "birdpreference" accession, which revealed that 42.4% of the SAP population and 53.8% of the sweet sorghum population were "bird-avoidance" accessions (Supplemental Data 1).

Using these phenotypic data, as well as a single-nucleotide polymorphism (SNP) dataset generated for each of the accessions in the diversity panels, we conducted genome-wide association studies (GWAS; mixed linear model [MLM]) seeking to identify loci putatively associated with bird feeding behavior. A single major locus that apparently contributes to bird feeding behavior was detected in both of the populations. The most significant SNP, S4_61667908 (P = 3.41e-08), was located in the sole exon of the Tannin1 gene



Figure 1. Bird Preferences in the Open Field, and GWAS Analysis of Bird Feeding Behavior and Condensed Tannin Content in Natural Sorghum Populations.

(A) Representative lines from the SAP population illustrating the extent of bird feeding under no protection in the field. Left shows a bird-preference sorghum line and right shows a bird-avoidance sorghum line. Scale bar, 1 cm.

(B–D) Genome-wide Manhattan plots of bird feeding behavior in the natural populations. (B) Bird feeding behavior in the SAP sorghum population. (C) Bird feeding behavior in the sweet sorghum population. (D) Condensed tannin content in the SAP population. Arrows indicate the SNPs most strongly associated with bird feeding behavior and condensed tannin content. The gray dashed line indicates a region associated with both bird feeding behavior and tannin content.

(E) Natural variantions of Tannin1 detected in sorghum germplasms.

on chromosome 4 in the SAP population (Figure 1B and Supplemental Table 1), while the SNP most strongly associated with bird feeding behavior in the sweet sorghum population was $W_4_58856219$ (P = 4.02e-07), which is located very close to Tannin1 (Figure 1C and Supplemental Table 1). Also, the major locus Tannin1 was detected by GWAS of condensed tannin content in the SAP population (Figure 1D and Supplemental Table 1). The overlap between the genomic regions associated with bird feeding behavior and tannin content indicates a definite connection between these traits in sorghum (Figure 1B-1D). Tannin1 is a homolog of Arabidopsis TTG1 and encodes a WD40 repeat protein that is assumed to affect the stability of basic helixloop-helix (bHLH) and MYB transcriptional factors that control the biosynthesis of anthocyanins and PAs in sorghum (Wu et al., 2012). Wild-type Tannin1 and two mutated alleles, tan1-a/b, were found in the SAP population (Figure 1E and Supplemental Data 1). In addition, we observed no significant differences when we performed a binary logistic regression analysis of the bird-avoidance/bird-preference data versus panicle shape, glume coverage, the presence/ absence of awns, or seed coat color. These results correct the previously reported findings (Perumal and Subramaniam, 1973) (Supplemental Figure 1).

Metabolites of the Anthocyanin and PA Biosynthesis Pathway Are Responsible for Bird Avoidance

Previous work has demonstrated that Tannin1 regulates the expression of anthocyanin and PA biosynthesis genes including DFR, LDOX, and ANR (Grotewold, 2006; Wu et al., 2012); therefore, we used liquid chromatography-mass spectrometry (LC-MS)-based targeted metabolic profiling analysis to examine whether metabolites of this pathway may contribute to the observed bird feeding behavior. Specifically, we measured 15 metabolites from the anthocyanin and PA biosynthesis pathway in filling-stage seeds that were harvested from six bird-avoidance (Tan1) and 12 bird-preference sorghum accessions (tan1-a/b) (Supplemental Table 2). We found that the bird-preference accessions had significantly reduced accumulation of pathway metabolites, including quercetin, rutin, and kaempferol, involved in flavonoid synthesis; and leucocyanidin, catechin/epicatechin, anthocyanin (peonidin, petunidin, and malvidin), and PA involved in anthocyanin and PA biosynthesis (Figure 2A, Supplemental



Figure 2. Identification of Metabolites for Anthocyanin and PA Biosynthesis Pathways and Sparrow Feeding Experiments. (A) Fifteen metabolites of the anthocyanin and PA biosynthesis pathways were examined in the six bird-avoidance sorghum accessions and 12 birdpreference sorghum accessions; note that different forms of some metabolites were also detected. For comparison of the relative amounts of metabolites from bird-avoidance and bird-preference sorghum samples, the mean values of each metabolite, calculated from six bird-avoidance accessions, were set as 1.0. Student's *t*-test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

(B and C) Inferential statistical evaluation of the weight of remaining seeds upon treatment with different secondary metabolites. Student's t-test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

(D and E) Sparrow feeding experiments were performed with seeds treated using various concentrations (high versus low, see concentrations in Supplemental Table 3) of malvidin and PA. Upper and lower panels show seed amount before and after these feeding assay experiments. Scale bars, 10 cm.

Figure 2A, and Supplemental Data 2). An MBW complex consisting of TT2 (MYB), TT8 (bHLH), and TTG1, a WD-repeat (WDR) protein, is necessary for PA biosynthesis in Arabidopsis (Baudry et al., 2004; Gonzalez et al., 2008). This suggests that genetic polymorphism(s) at the Tannin1 (ortholog of TTG1) locus of the bird-preference accessions may downregulate anthocyanin and PA biosynthesis pathway by decreasing transcriptional regulation of the MBW complex.

It is known that tannins can reach levels of more than 5% in sorghum seeds, most of which is stored in the seed coat (Mehansho et al., 1983). We therefore dissected caryopses and quantified the PA content as a percentage of the total weight of the seed coat material. Simple ammonium ferric citrate-staining-based analysis showed that the condensed tannin content of seed coats was more than 4-fold greater than that of whole seeds (Supplemental Figure 2B). Next, we conducted a series of standard feeding experiments whereby sparrows were offered seeds of the BTx623 accession (a bird-preference accession) that were untreated or had been soaked in aqueous solutions of quercetin, kaempferol, catechin, malvidin, or PA. Whereas we observed no significant difference in the amount of seeds eaten for the quercetin, kaempferol, and catechin samples (Supplemental Figure 3), treatment with malvidin or PA (condensed tannins) significantly reduced the amount of seeds eaten by sparrows (Figure 2B-2E; Supplemental Videos 1 and 2). In addition, we found that treatment with tannic acid (a hydrolyzable tannin compound) also significantly reduced the preference of the sparrows for the seed (Supplemental Figure 4 and Supplemental Video 3). Moreover, we noted that the extent of the reduction increased with increasing concentration of these compounds (Figure 2B-2E and

Tannin1 Modulates Bird Feeding Behavior in Sorghum

Supplemental Figure 4). Given that these three compounds are polyphenols and that the flavonoid monomers tested apparently had no effect, we conclude that condensed and hydrolyzable tannins are at least partially responsible for the observed differences in bird feeding behavior.

Bird-Preference Sorghum Accessions Accumulate VOCs to Attract Bird Predation

It is known that some plant secondary metabolic products such as VOCs facilitate the attraction of pollinators and seed dispersers (Knudsen et al., 2004, 2006). On the other hand, plants can also divert substantial resources to the production of aposematic volatiles, which are also produced by aggregating animals, to advertise their toxicity to their predators (Gittleman and Harvey, 1980; Ruxton and Sherratt, 2006; Barlow et al., 2017; Wei et al., 2019). To explore whether the volatile components are related to bird attraction in sorghum, we next used gas chromatography (GC)-MS to measure short-chain volatiles in filling-stage seeds. Nine of the 23 detected VOCs were present at significantly higher levels in the bird-preference accessions than in the birdavoidance accessions, with the increase in some FA-derived volatiles reaching as high as 30-fold (Figure 3A and Supplemental Data 3). This finding led us to speculate that the substantially increased volatile content of the bird-preference sorghum accessions may function as an attractant semiochemical for birds.

To test our hypothesis that these volatiles are birdattractant compounds, we conducted sparrow-attracting exper-

Molecular Plant

Figure 3. Identification of Volatile Components Related to Bird Feeding and Sparrow Behavioral Assays.

(A) Twenty-three different kinds of volatiles were measured in six bird-avoidance and 12 bird-preference sorghum accessions, the same as used for anthocyanin and PA metabolic analysis. For comparison of the relative VOC content from bird-avoidance and bird-preference sorghum samples, the mean values of each VOC, calculated from six bird-avoidance accessions, were set as 1.0. Student's *t*-test: *P < 0.05, **P < 0.01, ***P < 0.001.

(**B** and **C**) Average residence time for a bird per minute was evaluated using birds placed in experimental cages with seeds treated with 1-octen-3-ol or hexanal (or ethanol control). Student's *t*-test: *P < 0.05, ***P < 0.001.

(D) Comparison of linolenic acid measurements between *Tan1* and *tan1-a/b* accessions. For comparison of the relative linolenic acid content from bird-avoidance and bird-preference sorghum samples, the mean value calculated from bird-avoidance accessions was set as 1.0. Student's *t*-test: *P < 0.05.

iments using two volatiles that exhibited statistically significant differences in accumulation between the bird-avoidance and bird-preference sorghum accession samples: 1-octen-3-ol and hexanal. Because the physiological concentration of

1-octen-3-ol was found to range from ${\sim}0.002$ to ${\sim}1~\mu\text{g/g}$ fresh weight (FW) while that of hexanal ranged from \sim 0.01 to \sim 5 μ g/ g FW in the seeds of soybean, barley, and wheat (Cramer et al., 2005; Matsui et al., 2018), we soaked the filling seeds in sorghum panicles with 1 µg/g 1-octen-3-ol or 5 µg/g hexanal to perform bird feeding experiments. Values from these tests were scored as the average residence time of a bird for the control versus volatile treatments, and we found that the presence of both 1-octen-3-ol and hexanal in sorohum seeds caused a significant increase in the duration of sparrow residence (Figure 3B and 3C; Supplemental Videos 4 and 5). Thus, compared with the bird-avoidance accessions, bird-preference sorghum accessions produce increased levels of volatiles that specifically attract birds to approach, thereby at least partially explaining the observed behavioral differences from our large-scale field-based germplasm diversity quantitative genetics studies.

Loss of *Tannin1* Function Enhances Fatty Acid-Derived Volatile Production through Downregulation of *SbGL2* Expression

Previous metabolic flux analysis in *Arabidopsis* revealed that loss of function of *TTG1*, *TT2*, or *TT8* could lead to accumulation of long-chain FAs in the embryo by indirectly inhibiting the genes involved in FA biosynthesis (Chen et al., 2014, 2015; Wang et al., 2014) (Supplemental Figure 5). We subsequently measured the amount of long-chain linolenic acid in filling seeds of different sorghum accessions. Strikingly, higher accumulation

Molecular Plant

Figure 4. Analysis of Gene Expression in PA and VOC Biogenesis Pathways.

(A-E) Relative expression of genes involved in the fatty acid (FA)-derived volatile pathways in randomly selected (three accessions each) Tan1, tan1-a, and tan1-b accessions at the seed-filling stage. (A) SbGL2 is involved in regulation of the FA pathway. (B) SbMOD1.1. (C) SbCDS2.1. (D) SbFAD3.2 are involved in synthesis of FAs. (E) SbLOX3 is involved in decomposition of FAs. Student's *t*-test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. (F-J) Relative expression of genes involved in the anthocyanin and PA biosynthesis pathways in the same Tan1, tan1-a, and tan1-b accessions at the seed-filling stage. (F) SbCHS and (G) SbF3H are involved in synthesis of flavonoids. (H) SbDFR, (I) SbANS (J), and SbLAR are involved in synthesis of anthocyanins and specific PAs. Student's *t*-test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

tor that functions as a key negative regulator of FA biosynthesis in Arabidopsis (Shi et al., 2012; Li et al., 2018). To further investigate how Tannin1 regulates FA-derived volatile accumulation in bird-preference sorghum accessions, we examined the expression of SbGL2, the only homolog of Arabidopsis GL2in sorghum, and found that it was significantly less expressed in tan1-a/b accessions (Figure 4A). Moreover, a potential MYB binding element. "ACCAAAC." was found in the promoter region of the SbGL2 gene. This indicates that the MBW complex might directly regulate the expression of SbGL2. To analyze whether the expression of FA metabolic pathway genes is altered in tan1 accessions, we compared the FA metabolic pathway gene expression in Tan1, tan1-a, and tan1-b accessions (three accessions per genotype) (Supplemental Table 2). We observed significantly upregulated expression of genes in the FA biosynthesis pathway, including SbMOD1.1 (enoyl-[acyl-carrierprotein] reductase [NADH]), SbCDS2.1 (cvtidine diphosphate diacylalycerol synthase 2), and SbFAD3.2 (fatty acid desaturase 3), in the tan1-a/b accessions as compared with the Tan1 accessions

of linolenic acid was observed in three bird-preference (*tan1-a/b*) accessions than in three bird-avoidance (*Tan1*) accessions (Figure 3D). Decomposition of FAs in various FA-derived volatile components via the LOX pathway is also well known in plants (Dudareva et al., 2013) (Supplemental Figure 5). This result demonstrates that *Tannin1* indeed negatively controls FA levels and then leads to the reduction of the downstream FA-derived volatiles in sorghum.

In *Arabidopsis*, studies have shown that phosphorylated TTG1 can promote the accumulation of FAs by down-regulating *GL2*, which encodes a homeodomain transcription fac-

(Figure 4B–4D). *SbLOX3*, which is involved in FA decomposition, was also significantly upregulated in the *tan1-a/b* accessions (Figure 4E).

We also measured the relative expression levels of several key genes involved in the biosynthesis of anthocyanins and PAs. Expression of *SbCHS* and *SbF3H*, which are involved in flavonoid synthesis, *SbDFR* and *SbANS*, which are involved in anthocyanin synthesis, and *SbLAR*, which is involved in PA synthesis, were significantly downregulated in *tan1-a/b* sorghum accessions, consistent with previous reports (Wu et al., 2012) (Figure 4F–4J). These results suggest a possibility that genetic

Molecular Plant

Figure 5. A Working Model for the Ecological Impact of the *Tannin1* Locus.

A proposed working model for bird avoidance and bird preference associated with *Tannin1*. The intact WD40/MYB/bHLH complex promotes anthocyanin and PA accumulation in sorghum seeds to prevent bird attacks. Mutations in the WD40 protein Tannin1 lead to low amounts of anthocyanins and PAs but high levels of volatile organic compounds (VOCs) to attract feeding birds.

polymorphisms at a single locus, *Tannin1*, may have extensive metabolic regulatory impacts on multiple anabolic and catabolic pathways of plant phenylpropanoid and FA metabolism, which ultimately determine whether a given sorghum accession will be deemed an attractive food source by birds.

DISCUSSION

In this study, we identified *Tannin1* underlying a major locus controls bird feeding behavior in sorghum. *Tannin1* encodes a WD40 protein functioning in the WD40/MYB/bHLH complex. Our observations about the increased accumulation of FA-derived volatiles in multiple tan1 accessions are consistent with our observations from the same accessions regarding the decrease of metabolites from the anthocyanin and PA biosynthesis pathways: the accessions with high volatile content have low accumulation of anthocyanin and PA biosynthesis pathway metabolites, and vice versa (Figure 5). Thus, beyond accumulating volatiles that function as semiochemicals that attract feeding by birds, the birdpreference accessions also have the ostensibly feedingpromoting feature of decreased condensed tannin content, which may reduce astringency in the oral cavity of birds and thereby improve the perceived taste and/or digestibility of seeds from these accessions.

High-tannin sorghum may gradually lose its competitiveness when compared with non-tannin sorghum for feeding by mammals, such as monkeys and rats, due to the presence of indigestible chemical compounds (Mehansho et al., 1983; Shimada, 2006; Espinosa Gomez et al., 2018). In this study, we showed experimentally that sparrow feeding behavior was also affected by increased levels of anthocyanins and condensed tannin compounds in sorghum grains. It is known that plants and animals emit VOCs to attract stakeholders to pursue mutual benefit or protect themselves from predators. We also found that FA-derived volatiles could attract sparrows to seek and stay. Instances of massive numbers of harmful birds being guided from elsewhere in the field to fly toward, feed on, and destroy filling and mature crop seeds may be explained by this strong attraction to aromatic volatiles. Although we have demonstrated the role of gene expression in the control of FA-derived volatiles by Tannin1 complex, all intermediates and enzyme activities involved in the biogenesis pathway require further investigations in the future.

The particular effect of sorghum *Tannin1* on the transcriptional regulatory function of bHLH and MYB transcriptional factors, as well as characterization of how this WD40 protein finely regulates the expression of downstream genes, awaits confirmatory studies. Nevertheless, our discovery that *Tannin1* has an enormous impact on the feeding preferences of birds would guide molecular markerassisted selection efforts in crop improvement programs seeking to limit or prevent bird damage to agricultural production systems. Moreover, our insights about which compounds attract birds and which compounds are distasteful to birds suggest a variety of novel control measures that could be undertaken to prevent yield losses, for example, by chemically or genetically blocking the activities of enzymes in attractant volatile biosynthesis and/or programming of condensed tannin content.

METHODS

Materials

A diverse sorghum panel consisting of 571 accessions was collected. The sorghum natural population comprising 352 lines was derived from the SAP, which has been previously reported (Morris et al., 2013). The sweet sorghum natural population comprising 219 lines was collected from the United States Department of Agriculture (USDA). All seeds were ordered from the USDA Agricultural Research Service.

Phenotype Evaluation

The diverse sorghum panels were planted under a complete randomized block design in three different areas of China (Beijing [$39.5^{\circ}N$, $116.4^{\circ}E$], Sanya [$18.3^{\circ}N$, $108.8^{\circ}E$], and Yinchuan [$38.5^{\circ}N$, $106.3^{\circ}E$] of Ningxia province), with duplicates per site in years 2016 and 2017. Each line was planted in a single 4-m-long plot with 1-m row spacing. A reference line, BTx623, which was used as a host plant to attract bird predation, was randomly planted every 20 lines. The morphological trait panicle shape was scored as three levels: 0 (Disperse type), 1 (Medium type), and 2 (Tight type). Glume coverage was scored as six levels: 0 (Very low glume coverage), 1 (Low glume coverage), 2 (Moderate glume coverage), 3

Molecular Plant

(High glume coverage), 4 (Very high glume coverage), and 5 (All enveloped). Awn was scored as a qualitative trait: 0 (Awn) and 1 (Awnless). Seed coat color was evaluated at the mature stage and scored as five levels: 0 (White), 1 (Wax), 2 (Yellow), 3 (Yellowish-brown), and 4 (Reddish-brown). For evaluation of bird avoidance, we scored the bird- avoidance trait as "0" (bird-avoidance) and "1" (bird-preference) due to a certain appearance of quality character. Phenotype data that were recorded in at least 8 out of 12 repeats were included in subsequent analysis, and otherwise were evaluated as missing data.

Statistical Contribution and Significance Tests

Panicle shape, awn, and seed coat color were scored as unordered variables, glume coverage was scored as an ordered variable, and the condensed tannin content was scored as a continuous variable. The statistical contribution of the five bird avoidance-related traits was calculated by binary logistic regression analysis. Statistical analysis was performed using IBM SPSS statistics for Windows, version 19.0 (SPSS, Chicago, IL, USA). Other statistical significance tests were calculated using a general Student's *t*-test.

Genome-wide Association Analysis

The annotation information for the sorghum reference genome BTx623 was downloaded from the Phytozome website (https://phytozome.jgi. doe.gov). The genotyping by sequencing (GBS) SNP markers for the SAP population were obtained from a community resource as previously described (Morris et al., 2013), and are publicly available at http://www. morrislab.org/data. The GBS SNP markers for the sweet sorghum natural population were obtained from a previous sweet sorghum association study (Burks et al., 2015). Heterozygous SNP loci were changed to unknown. SNPs with more than 20% missing data and minor allele frequencies below 0.05 were removed. In total, 82 430 SNP markers for the SAP population and 41 997 SNP markers for the sweet sorghum population were used for GWAS analysis. The compressed MLM was used to perform GWAS analysis by means of the Genome Association and Prediction Integrated Tool (GAPIT) (Lipka et al., 2012). Kinship (K) was calculated using the default VanRaden method in GAPIT (VanRaden, 2008). The significant P-value threshold was determined by Bonferroni correction (Sedgwick, 2012).

Metabolic Analysis of Anthocyanin, PA Biosynthesis, and Volatiles

Eighteen accessions were grown in the field as two randomized replicates. We selected three panicles 15 days after the start of the filling stage for each accession and mixed all samples for grinding in liquid nitrogen. Samples were used for comprehensive metabolic profiling analysis.

For metabolic profiling analysis of 15 metabolites in the PA pathway, the sample was prepared with sonication by adding 2 ml of 80% methanol solution (containing lidocaine as an internal standard, 0.08 µM), and the resulting extract was separated on a ZORBAX Extend C18 column (100 mm × 2.1 mm internal diameter, 1.8 μm; Agilent, USA). The column temperature was maintained at 35°C and the autosampler was conditioned at 4°C. The mobile phase consisted of water (solution A, 0.1% formic acid) and acetonitrile (solution B, 0.1% formic acid) at a flow rate of 0.40 ml/min. The gradient conditions for the mobile phase were as follows: 1% \rightarrow 99.5% B at 0.1–15.5 min; 99.5% \rightarrow 99.5% B at 15.5–17 min. The system was then equilibrated using the initial condition for 3 min. The injection volume was 2 µl. Mass spectra were acquired in positive mode. The operating parameters were set as follows: capillary voltage, 4000 V (+); nebulizer pressure, 20 pounds per square inch gauge; drying gas flow rate, 13 l/min; gas temperature, 225°C; sheath gas flow rate, 12 I/min; sheath gas temperature, 350°C; and fragmentor, -380 V.

The sample preparation and analysis of volatile metabolites were performed as previously described with minor modifications (Wei et al., 2016). In brief, 200 mg of fresh sample power was weighed, and 50 ng

Tannin1 Modulates Bird Feeding Behavior in Sorghum

of 2-heptone, as an internal standard, was added during sample preparation. Next, a 50/30-µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS)-coated solid-phase microextraction (SPME; Supelco) technique was used to measure the volatile metabolites released from the sorghum sample. The headspace was sampled for 0.5 h at 30°C with DVB/CAR/PDMS-coated SPME and analyzed by GC-MS. Injection was performed by thermal desorption of the SPME in the injector at 250°C for 0.5 min in the splitness mode. GC-MS analysis was performed using an Agilent 7890A GC/5975C MSD system. Metabolites were separated on a DB-5 MS column (30 m \times 0.25 mm, 0.25 μm film thickness) with He as the carrier gas (1.5 ml/min). The initial oven temperature was held at 40°C for 1.5 min, then ramped by 5°C/min to 150°C, then ramped by 15°C/min to 260°C, and kept at 260°C for 7 min. The inlet temperature was set at 250°C. The ionization was processed in El positive mode. The temperatures of the ion source and quadrupole were set at 230°C and 150°C, respectively. The mass spectra were acquired at a scanning range of 50-400 m/z. The metabolites were identified according to the retention time and mass spectra of authentic standards, and some of the metabolites were identified by comparison with the mass spectra in the National Institute of Standards and Technology and Wiley libraries (Agilent Technologies, Palo Alto, CA).

The extraction and analysis of FAs in the filling seeds were carried out as described previously (Poirier et al., 1999; Chen et al., 2012). The quantitative chemical trait condensed tannin content was measured in fresh matured seeds of 352 SAP lines by a determination (ISO 9648: 1988, MOD). The relative concentrations of metabolites and volatiles were calculated using an internal standard method, and normalized to the unit FW of sample. We selected a characteristic ion for each metabolite, and quantified the metabolites according to the following formula: $C = A/A_{IS} \times C_{IS}$, where C is the concentration of metabolite, A is the peak area of the characteristic ion for each metabolite, and IS is the abbreviation for the internal standard (lidocaine for LC-MS analysis and 2-heptone for GC-MS analysis); W represents sample weight in grams. For comparison of the relative amount of metabolite from birdavoidance and bird-preference sorghum samples, the mean values of each metabolite, calculated from six bird-avoidance accessions, were set as 1.0.

qPCR

Expression of the *Tannin1* gene was confirmed by qPCR. Total RNA was extracted from sorghum seeds at the filling and mature stage using a pure RNA extraction kit (Huayueyang, Beijing) for polyphenol samples, then washed off and digested by RNase-free DNase I (Invitrogen). A cDNA synthesis kit (TransGen) was used to reverse transcribe 2 μ g of RNA of each sample. qPCR from 1 μ I of cDNA diluted 10 times was performed using SYBR Green qPCR mix (TransGen) on an Applied Biosystems 7900HT Fast Real-Time PCR System. Data were calculated using the Δ Ct method and each sample was replicated three times. The sorghum actin *EIF* was used as a reference gene (Reddy et al., 2016). Relevant information for all primer sequences is given in Supplemental Table 4. The flanking sequences of the primers for all the accessions are given in Supplemental Figure 6.

Sparrow Feeding Experiments

We used 15–20 sparrows to perform the three biological repeats of the feeding experiments in cages for each metabolite of the anthocyanin and PA biosynthesis pathway (Malvidin: CAS 643-84-5; PA: CAS 4852-22-6; tannic acid: CAS 1401-55-4; quercetin: CAS 117-39-5; kaempferol: CAS 520-18-3; catechin: CAS 154-23-4) and two selected volatile components 1-octen-3-ol (CAS 3391-86-4) and hexanal (CAS 66-25-1). Each standard cage was a unified model measuring 75 × 45 × 45 cm. All sparrows were kept in four phytotrons under 26°C and 60% humidity with 16 h of daylight and 8 h of darkness. Sparrows were fed millet or other crop seeds for 3 days before starting treatment experiments due to the selective memory for food intake. Each metabolite or volatile feeding

Molecular Plant

experiment was carried out in an independent phytotron under good ventilation to avoid cross-contamination of aromatic flavors. All sparrow feeding experiments followed the animal experiment guidelines of the Institute of Zoology, the Chinese Academy of Sciences.

To verify the effect of metabolites of the anthocyanin and PA pathway on sparrow feeding behavior, we used a line, BTx623, carrying a *tan1-b* allele (no tannin content) as a control. Thirty grams of BTx623 dry seeds soaked with water was referred to as the control, and the same amount of seeds were soaked for 30 min with different concentrations of each metabolite. The weight of seeds left by sparrows for each experiment was evaluated at day 3 and day 5. For bird-behavior experiments with FA-derived volatiles, four panicles at the filling stage of a line, P260 (very low volatile production), were put into the cages. Two panicles were soaked for 30 min in 1 μ g/g 1-octen-3-ol or 5 μ g/g hexanal while the other two panicles were soaked with ethanol, which was referred to as the control. For evaluation of the attraction of sparrows by volatiles, the average residence time for a bird in the supplement-treatment side or control side per randomly selected minute was analyzed (Supplemental Table 3).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at Molecular Plant Online.

FUNDING

This research was supported by grants from the Agricultural Breeding Program in NingXia Province (2014NYYZ0401) and the National Key R&D Program of China (2018YFD1000704), Key Deployment Projects of Chinese Academy of Sciences (KFZD-SW-112-01), and S.T. is supported by the Youth Innovation Promotion Association of Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

Q.X., Y.W., and P.X. designed the research strategy and guided the project. P.X. and J.S. performed most of the experiments. P.X., S.T., A.K., and C.L. performed phenotyping of traits in the field. P.X. and C.C. performed the GWAS and binary logistic regression analyses. F.Z. performed the determination of metabolites of volatiles and ultraperformance LC–MS analysis. J.S. performed the qPCR experiments. P.X., J.S., and Y.X. performed the feeding experiments. P.X., J.S., C.C., Y.W., and Q.X. analyzed the data. P.X., J.S., Y.W., and Q.X. wrote the manuscript. All authors have read, discussed, and contributed to the manuscript.

ACKNOWLEDGMENTS

We thank the US Sorghum Association Panel for providing seeds of sorghum accessions. The authors declare no competing interests.

Received: June 15, 2019 Revised: August 22, 2019 Accepted: August 22, 2019 Published: September 23, 2019

REFERENCES

- Anderson, A., Lindell, C.A., Moxcey, K.M., Siemer, W.F., Linz, G.M., Curtis, P.D., Carroll, J.E., Burrows, C.L., Boulanger, J.R., Steensma, K.M.M., et al. (2013). Bird damage to select fruit crops: the cost of damage and the benefits of control in five states. Crop Prot. 52:103–109.
- Austin, M.B., and Noel, A.J.P. (2003). The chalcone synthase superfamily of type III polyketide synthases. Nat. Prod. Rep. 20:79–110.
- Barlow, S.E., Wright, G.A., Ma, C., Barberis, M., Farrell, I.W., Marr, E.C., Brankin, A., Pavlik, B.M., and Stevenson, P.C. (2017). Distasteful nectar deters floral robbery. Curr. Biol. 27:2552–2558.e3.
- Baud, S., Boutin, J.P., Miquel, M., Lepiniec, L., and Rochat, C. (2002). An integrated overview of seed development in *Arabidopsis thaliana* ecotype WS. Plant Physiol. Biochem. 40:151–160.

- Baudry, A., Heim, M.A., Dubreucq, B., Caboche, M., Weisshaar, B., and Lepiniec, L. (2004). TT2, TT8, and TTG1 synergistically specify the expression of BANYULS and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. Plant J. **39**:366–380.
- Burks, P.S., Kaiser, C.M., Hawkins, E.M., and Brown, P.J. (2015). Genomewide association for sugar yield in sweet sorghum. Crop Sci. 55:2138–2148.
- Casa, A.M., Pressoir, G., Brown, P.J., Mitchell, S.E., Rooney, W.L., Tuinstra, M.R., Franks, C.D., and Kresovich, S. (2008). Community resources and strategies for association mapping in sorghum. Crop Sci. 48:30–40.
- Chen, M.X., Wang, Z., Zhu, Y.N., Li, Z.L., Hussain, N., Xuan, L.J., Guo, W.L., Zhang, G.P., and Jiang, L.X. (2012). The effect of TRANSPARENT TESTA2 on seed fatty acid biosynthesis and tolerance to environmental stresses during young seedling establishment in arabidopsis. Plant Physiol. 160:1023–1036.
- Chen, M.X., Xuan, L.J., Wang, Z., Zhou, L.H., Li, Z.L., Du, X., Ali, E., Zhang, G.P., and Jiang, L.X. (2014). TRANSPARENT TESTA8 inhibits seed fatty acid accumulation by targeting several seed development regulators in *Arabidopsis*. Plant Physiol. **165**:905–916.
- Chen, M.X., Zhang, B., Li, C.X., Kulaveerasingam, H., Chew, F.T., and Yu, H. (2015). TRANSPARENT TESTA GLABRA1 regulates the accumulation of seed storage reserves in *Arabidopsis*. Plant Physiol. 169:391–402.
- Coleman, J.D., and Spurr, E.B. (2001). Farmer perceptions of bird damage and control in arable crops. N. Z. Plant Prot. 54:184–187.
- Cramer, A.C.J., Mattinson, D.S., Fellman, J.K., and Baik, B.K. (2005). Analysis of volatile compounds from various types of barley cultivars. J. Agric. Food Chem. **53**:7526–7531.
- da Costa, G., Lamy, E., Silva, F.C.E., Andersen, J., Baptista, E.S., and Coelho, A.V. (2008). Salivary amylase induction by tannin-enriched diets as a possible countermeasure against tannins. J. Chem. Ecol. 34:376–387.
- De Melo, C., and Cheschini, J. (2012). Damage caused by birds in sorghum (Sorghum bicolor) crops in central Brazil. Bioagro 24:33–38.
- De Mey, Y., Demont, M., and Diagne, M. (2012). Estimating bird damage to rice in Africa: evidence from the Senegal river valley. J. Agric. Econ. 63:175–200.
- Dixon, R.A., Xie, D.Y., and Sharma, S.B. (2005). Proanthocyanidins—a final frontier in flavonoid research? New Phytol. **165**:9–28.
- Dolbeer, R.A., Woronecki, P.P., and Bruggers, R.L. (1986). Reflecting tapes repel blackbirds from millet, sunflowers, and sweet corn. Wildlife Soc. B 14:418–425.
- Dudareva, N., Klempien, A., Muhlemann, J.K., and Kaplan, I. (2013). Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytol. **198**:16–32.
- Espinosa Gomez, F.C., Serio Silva, J.C., Santiago Garcia, J.D., Sandoval Castro, C.A., Hernandez Salazar, L.T., Mejia Varas, F., Ojeda Chavez, J., and Chapman, C.A. (2018). Salivary tanninbinding proteins are a pervasive strategy used by the folivorous/ frugivorous black howler monkey. Am. J. Primatol. 80:e22737.
- Ferrer, J.L., Jez, J.M., Bowman, M.E., Dixon, R.A., and Noel, J.P. (1999). Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. Nat. Struct. Biol. 6:775–784.
- Feussner, I., and Wasternack, C. (2002). The lipoxygenase pathway. Annu. Rev. Plant Biol. 53:275–297.
- Gigot, C., Ongena, M., Fauconnier, M.L., Wathelet, J.P., Du Jardin, P., and Thonart, P. (2010). The lipoxygenase metabolic pathway in plants: potential for industrial production of natural green leaf volatiles. Biotechnol. Agron. Soc. **14**:451–460.

Molecular Plant

- Gilbert, N. (2009). Averting a climate-led food crisis in Africa. Nature https://doi.org/10.1038/news.2009.585.
- Gittleman, J.L., and Harvey, P.H. (1980). Why are distasteful prey not cryptic? Nature 286:149–150.
- Gonzalez, A., Zhao, M., Leavitt, J.M., and Lloyd, A.M. (2008). Regulation of the anthocyanin biosynthetic pathway by the TTG1/ bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. Plant J. 53:814–827.
- Grotewold, E. (2006). The genetics and biochemistry of floral pigments. Annu. Rev. Plant Biol. 57:761–780.
- Hillis, W.E. (1958). Formation of condensed tannins in plants. Nature 182:1371.
- Huang, M., Sanchez-Moreiras, A.M., Abel, C., Sohrabi, R., Lee, S., Gershenzon, J., and Tholl, D. (2012). The major volatile organic compound emitted from *Arabidopsis thaliana* flowers, the sesquiterpene (E)-beta-caryophyllene, is a defense against a bacterial pathogen. New Phytol. **193**:997–1008.
- Kale, M.A., Dudhe, N., Kasambe, R., and Bhattacharya, P. (2014). Crop depredation by birds in Deccan plateau, India. Int. J. Biodivers. 2014:8.
- Knudsen, J.T., Eriksson, R., Gershenzon, J., and Stahl, B. (2006). Diversity and distribution of floral scent. Bot. Rev. **72**:1–120.
- Knudsen, J.T., Tollsten, L., Groth, I., Bergstrom, G., and Raguso, R.A. (2004). Trends in floral scent chemistry in pollination syndromes: floral scent composition in hummingbird-pollinated taxa. Bot. J. Linn. Soc. 146:191–199.
- Lepiniec, L., Debeaujon, I., Routaboul, J.M., Baudry, A., Pourcel, L., Nesi, N., and Caboche, M. (2006). Genetics and biochemistry of seed flavonoids. Annu. Rev. 57:405–430.
- Li, C.X., Zhang, B., Chen, B., Ji, L.H., and Yu, H. (2018). Site-specific phosphorylation of TRANSPARENT TESTA GLABRA1 mediates carbon partitioning in *Arabidopsis* seeds. Nat. Commun. 9:571.
- Lipka, A.E., Tian, F., Wang, Q.S., Peiffer, J., Li, M., Bradbury, P.J., Gore, M.A., Buckler, E.S., and Zhang, Z.W. (2012). GAPIT: genome association and prediction integrated tool. Bioinformatics 28:2397– 2399.
- Mason, J.R., Bullard, R.W., Dolbeer, R.A., and Woronecki, P.P. (1989). Red-winged blackbird (*Agelaius phoeniceus* L) feeding response to oil and anthocyanin levels in sunflower meal. Crop Prot. 8:455–460.
- Matsui, K., Takemoto, H., Koeduka, T., and Ohnishi, T. (2018). 1-Octen-3-ol is formed from its glycoside during processing of soybean [*Glycine max* (L.) Merr.] seeds. J. Agric. Food Chem. 66:7409–7416.
- Mehansho, H., Hagerman, A., Clements, S., Butler, L., Rogler, J., and Carlson, D.M. (1983). Modulation of proline-rich protein-biosynthesis in rat parotid-glands by sorghums with high tannin levels. Proc. Natl. Acad. Sci. U S A 80:3948–3952.
- Morris, G.P., Ramu, P., Deshpande, S.P., Hash, C.T., Shah, T., Upadhyaya, H.D., Riera-Lizarazu, O., Brown, P.J., Acharya, C.B., Mitchell, S.E., et al. (2013). Population genomic and genome-wide association studies of agroclimatic traits in sorghum. Proc. Natl. Acad. Sci. U S A 110:453–458.
- Perumal, R.S., and Subramaniam, T.R. (1973). Studies on panicle characters associated with bird resistance in sorghum. Madras Agric. J. 60:256–258.
- Poirier, Y., Ventre, G., and Caldelari, D. (1999). Increased flow of fatty acids toward beta-oxidation in developing seeds of *Arabidopsis* deficient in diacylglycerol acyltransferase activity or synthesizing medium-chain-length fatty acids. Plant Physiol. **121**:1359–1366.

Tannin1 Modulates Bird Feeding Behavior in Sorghum

- Raguso, R.A. (2008). Wake up and smell the roses: the ecology and evolution of floral scent. Annu. Rev. **39**:549–569.
- Reddy, P.S., Reddy, D.S., Sivasakthi, K., Bhatnagar-Mathur, P., Vadez, V., and Sharma, K.K. (2016). Evaluation of sorghum [Sorghum bicolor (L.)] reference genes in various tissues and under abiotic stress conditions for quantitative real-time PCR data normalization. Front. Plant Sci. 7:529.
- Ruxton, G.D., and Sherratt, T.N. (2006). Aggregation, defence and warning signals: the evolutionary relationship. Proc. Biol. Sci. 273:2417–2424.
- Sedgwick, P. (2012). Multiple significance tests: the Bonferroni correction. Br. Med. J. 344. https://doi.org/10.1136/bmj.e509.
- Shi, L., Katavic, V., Yu, Y.Y., Kunst, L., and Haughn, G. (2012). Arabidopsis glabra2 mutant seeds deficient in mucilage biosynthesis produce more oil. Plant J. 69:37–46.
- Shimada, T. (2006). Salivary proteins as a defense against dietary tannins. J. Chem. Ecol. 32:1149–1163.
- Stracke, R., De Vos, R.C.H., Bartelniewoehner, L., Ishihara, H., Sagasser, M., Martens, S., and Weisshaar, B. (2009). Metabolomic and genetic analyses of flavonol synthesis in *Arabidopsis thaliana* support the in vivo involvement of leucoanthocyanidin dioxygenase. Planta 229:427–445.
- Stracke, R., Ishihara, H., Barsch, G.H.A., Mehrtens, F., Niehaus, K., and Weisshaar, B. (2007). Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. Plant J. 50:660–677.
- Tipton, K.W., Floyd, E.H., Marshall, J.G., and Mcdevitt, J.B. (1970). Resistance of certain grain sorghum hybrids to bird damage in Louisiana. Agron. J. 62:211–213.
- Unsicker, S.B., Kunert, G., and Gershenzon, J. (2009). Protective perfumes: the role of vegetative volatiles in plant defense against herbivores. Curr. Opin. Plant Biol. 12:479–485.
- VanRaden, P.M. (2008). Efficient methods to compute genomic predictions. J. Dairy Sci. 91:4414–4423.
- Wang, Z., Chen, M.X., Chen, T.L., Xuan, L.J., Li, Z.L., Du, X., Zhou, L.H., Zhang, G.P., and Jiang, L.X. (2014). TRANSPARENT TESTA2 regulates embryonic fatty acid biosynthesis by targeting FUSCA3 during the early developmental stage of *Arabidopsis* seeds. Plant J. 77:757–769.
- Wei, G., Tian, P., Zhang, F., Qin, H., Miao, H., Chen, Q., Hu, Z., Cao, L., Wang, M., Gu, X., et al. (2016). Integrative analyses of nontargeted volatile profiling and transcriptome data provide molecular insight into VOC diversity in cucumber plants (*Cucumis sativus*). Plant Physiol. 172:603–618.
- Wei, J.N., Shao, W.B., Cao, M.M., Ge, J., Yang, P.C., Chen, L., Wang, X.H., and Kang, L. (2019). Phenylacetonitrile in locusts facilitates an antipredator defense by acting as an olfactory aposematic signal and cyanide precursor. Sci. Adv. 5:eaav5495.
- Wu, Y.Y., Li, X.R., Xiang, W.W., Zhu, C.S., Lin, Z.W., Wu, Y., Li, J.R., Pandravada, S., Ridder, D.D., Bai, G.H., et al. (2012). Presence of tannins in sorghum grains is conditioned by different natural alleles of Tannin1. Proc. Natl. Acad. Sci. U S A 109:10281–10286.
- Xie, D.Y., and Dixon, R.A. (2005). Proanthocyanidin biosynthesis-still more questions than answers? Phytochemistry 66:2127–2144.
- Xie, Q., and Xu, Z.H. (2019). Sustainable agriculture: from sweet sorghum planting and ensiling to ruminant feeding. Mol. Plant 12:603–606.