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Loss of Pleiotropic Regulatory Functions in *Tannin1*, the Sorghum Ortholog of Arabidopsis Master Regulator *TTG1*

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ABSTRACT

Transcriptional master regulators are often targeted to improve plant traits, but antagonistic pleiotropic effects of these regulators can hamper this approach. The Myb-bHLH-WDR (MBW) complex is a broadly conserved transcriptional regulator affecting pigmentation, biotic stress resistance, and abiotic stress tolerance. We investigated the function of sorghum grain pigmentation regulator *Tannin1*, the ortholog of Arabidopsis pleiotropic WD40 regulator *TTG1*, to test for conserved pleiotropic regulatory effects and to better understand the evolution of the MBW complex in Poaceae. We characterized genome-wide differential expression of leaf tissue using RNA sequencing in near-isogenic lines (NILs) that contrasted wildtype *Tan1* and loss-of-function *tan1-b* alleles, under optimal temperature and chilling stress. Notably, Gene Ontology analyses revealed no pathways with differential expression between *Tan1* and *tan1-b* NILs, suggesting that, in contrast to Arabidopsis *TTG1*, *Tannin1* has no pleiotropic regulatory role in leaves. Further, NILs had no visible difference in anthocyanin pigmentation, and no genes with known or expected function in flavonoid synthesis were differentially expressed. Genome-wide, only 18 total genes were differentially expressed between NILs, with six of these genes located inside the NIL introgression region, an observation most parsimoniously explained by *cis*-regulatory effects unrelated to *Tannin1* regulation. Comparing our findings with known function of *TTG1* orthologs in maize, rice, and Arabidopsis, we conclude that pleiotropic regulatory function in leaf tissue was likely lost in panicoid grass evolution before the sorghum-maize split. These findings inform future molecular breeding of MBW regulated traits and highlight the benefit of subfunctionalization to relieve pleiotropic constraints.

1 | Introduction

Master regulators are the primary genes in a regulatory cascade (Han et al. 2004) and underlie many key traits for plant development and environmental response, including many traits relevant for crop improvement. Master regulators of agriculturally relevant traits include Os*WRKY71* regulating biotic stress response (Liu et al. 2007), CBF regulators for cold response (Savitch et al. 2005; Zhang et al. 2020), and *SELF-PRUNING* for plant architecture (Silva et al. 2018). However, the multiple effects of master regulators may lead to antagonistic pleiotropy (Paaby and Rockman 2013). In Arabidopsis, the Myb-bHLH-WDR (MBW) complex is a canonical pleiotropic regulator, controlling multiple epidermal traits (seed and leaf flavonoid pigments, root hairs, leaf trichomes). It consists of three subunits, with the WD40-repeat transcriptional regulator (WDR) being shared across traits (*Transparent Testa Glabrata1*; *TTG1* in Arabidopsis), whereas diverse myb and bHLH transcription factors allow the complex to regulate multiple phenotypes independently (Tian and Wang 2020). MBW function is broadly conserved across plants, along with its flavonoid regulatory function (Tian and Wang 2020). MBW genes regulate key agronomic traits such as pigmentation, seed dormancy, grain tannins, bird resistance, fungal resistance, and trichome formation

in diverse crops (Carey et al. 2004; Ibraheem et al. 2010; Wu et al. 2012; Xie et al. 2019; Yang et al. 2021).

Manipulation of the MBW complex has become an important target for molecular breeding (Alahakoon et al. 2016; Zheng et al. 2021; Wang et al. 2017), but pleiotropic effects could create undesired phenotypic tradeoffs (Benech-Arnold and Rodríguez 2018; Gu et al. 2011; Huang, Yi, et al. 2022). The function of *OsTTG1* in rice is similar to Arabidopsis *TTG1*, regulating trichome formation and anthocyanins throughout the plant (Yang et al. 2021). In maize, the MBW complex has functionally diverged, and the identified WDR subunit, *Pale Aleurone Color 1* (*PAC1)*, controls seed anthocyanins but lacks regulatory function over anthocyanins in other tissues. Differing MBW regulatory functions between rice and maize suggest evolutionary divergence occurred after the separation of the Panicoideae and Oryzoideae subfamilies c. 80 million years ago (Huang, Zhang, and Columbus 2022), which leaves the role of MBW within the Panicoideae in question, including globally important crops such as sorghum, sugarcane, and several millets. Investigation of MBW function in other panicoid grasses can be used to further resolve the evolutionary history of the MBW complex in Poaceae and better understand its role in pigmentation and stress resilience in cereals.

Sorghum (*Sorghum bicolor* L. Moench) is a globally cultivated panicoid grass crop (Monk et al. 2014) in which three agronomically important MBW genes have been cloned. *Tannin1* (classically, *B1*) (Wu et al. 2012) is a WDR, *Tannin2* (classically, *B2*) (Wu et al. 2019) a bHLH, and *YELLOW SEED1* (*Y1*; classically, *Y*) (Ibraheem et al. 2010) a myb. *Tannin1* and *Tannin2* were originally identified as regulators of grain proanthocyanidins, but these genes also colocalize with quantitative trait loci (QTL) for early season chilling tolerance (germination, emergence, seedling vigor under ~0°C–10°C), particularly *Tannin1*, which colocalizes with the largest QTL *qCT04.62* (Marla et al. 2019). *Y1* was identified as a gene controlling pericarp and leaf pigmentation and has been shown to confer resistance to fungal diseases, such as anthracnose and grain mold (Boddu et al. 2005; Doggett 1970; Ibraheem et al. 2010; Nida et al. 2019). Given that the sorghum genome contains many paralogs of each of the MBW components (Morris et al. 2013), it is possible that they have redundant function, or they have neofunctionalized or subfunctionalized into contrasting roles (Birchler and Yang 2022).

We investigated the conservation of the master regulatory role of *TTG1* orthologs in panicoid grasses and possible pleiotropic effects of sorghum WDR transcriptional regulator *Tannin1*, with the main goal of testing two competing hypotheses (Platt 1964) (Figure 1). Under the first hypothesis, the pleiotropic regulatory functions of *TTG1* would be conserved in the panicoid grass lineage leading to sorghum (*Conserved ancestral pleiotropy* hypothesis; Figure 1A) and sorghum *Tannin1* would retain pleiotropic regulatory function in leaf tissue, including anthocyanin pigmentation (Figure 1B). Based on colocalization of *Tannin1* with chilling tolerance QTL (Marla et al. 2019; Schuh et al. 2024) and previous reports of *TTG1*'s role in chilling response in Arabidopsis via flavonoid pigmentation (Schulz et al. 2016) we also considered possible pleiotropic effects of *Tannin1* on chilling tolerance (Figure 1B). Alternatively, under the second hypothesis, these pleiotropic effects could have been lost during panicoid grass evolution (*Derived loss of pleiotropy* hypothesis; Figure 1C) and sorghum *Tannin1* would not have a pleiotropic regulatory role in leaves (Figure 1D). Here, we used transcriptome analyses of *Tannin1* near isogenic lines, which contrast for the *Tan1* wildtype allele versus the *tan1-b* loss-of-function allele, as well as transcriptome atlas analyses from other plants, to test these hypotheses.

2 | Results

2.1 | *Tannin1* and Several Other WDR Paralogs Are Widely Expressed Across sorghum Tissues, but the Other Co-Ortholog of *TTG1* Is Not

In this study, we used NILs contrasting for functional wildtype (*Tan1*) and nonfunctional loss-of-function (*tan1-b*) alleles under control and chilling treatments to test for a regulatory role of WDR *Tannin1* (Sobic.004G280800) in leaf tissue (Figure 1A,B). *Tan1* NILs have a chilling-sensitive BTx623 genetic background with a 2- to 10-Mb introgression from chilling-tolerant kaoliang sorghum Hong Ke Zi (HKZ), including the *Tan1* allele and part of *qCT04.62*, notated as *pCT04.62+/Tan1* (Figure 2A) (Schuh et al. 2024). Beyond the *Tannin1* region, *tan1-b* NILs are almost fully isogenic with BTx623 (Schuh et al. 2024). We first checked whether *Tannin1* is expressed in leaves, as would be expected if *Tannin1* has function in leaves (Figure 1A). For both wildtype *Tan1* (NIL+) and *tan1-b* (NIL−), *Tannin1* is expressed in leaves, under both normal and chilled conditions, with no significant difference between the lines (Figure 2B). Notably, however, *Tannin1* is significantly ($p < 10^{-4}$) downregulated under chilling conditions in both NIL+ and NIL− genotypes (Figure 2B). Next, we considered whether Sobic.004G161600, the *Tannin1* paralog with the greatest similarity to Arabidopsis *TTG1* (73%, vs. 66% for *Tannin1*), was similarly expressed in leaves. However, there is no evidence of expression for Sobic.004G161600, in either *Tan1* or *tan1-b* NILs, in either normal or chilling conditions (Figure 2C).

To further investigate the possibility of redundant or contrasting function among WDR genes in sorghum, we characterized expression across tissues for *Tannin1* and its WDR paralogs in using publicly available data from a tissue expression atlas (Figure 2D). *Tannin1* is not only expressed in seeds, as would be expected given its known role is grain pigmentation (Figure 1), and in various leaf tissues, consistent with our expression analysis above; it is also widely expressed across a diverse range of tissues, including root, stem, and inflorescence (Figure 2D). This is a similar pattern of broad expression orthologous WDR genes in maize, rice, and Arabidopsis (Figure 2E). By contrast, there is no evidence of expression for the other sorghum *TTG1* co-ortholog, Sobic.004G161600, in any tissue or time point (Figure 2D). Looking more broadly across paralogous WDR genes in sorghum, most of the *TTG1* homologs (with lower similarity to *TTG1*) were also not expressed in most tissues (Figure 2D). None of the *TTG1* homologs had a leaf-specific expression profile. Notably, however, four of the paralogs (Sobic.003G427100, Sobic.002G401500,

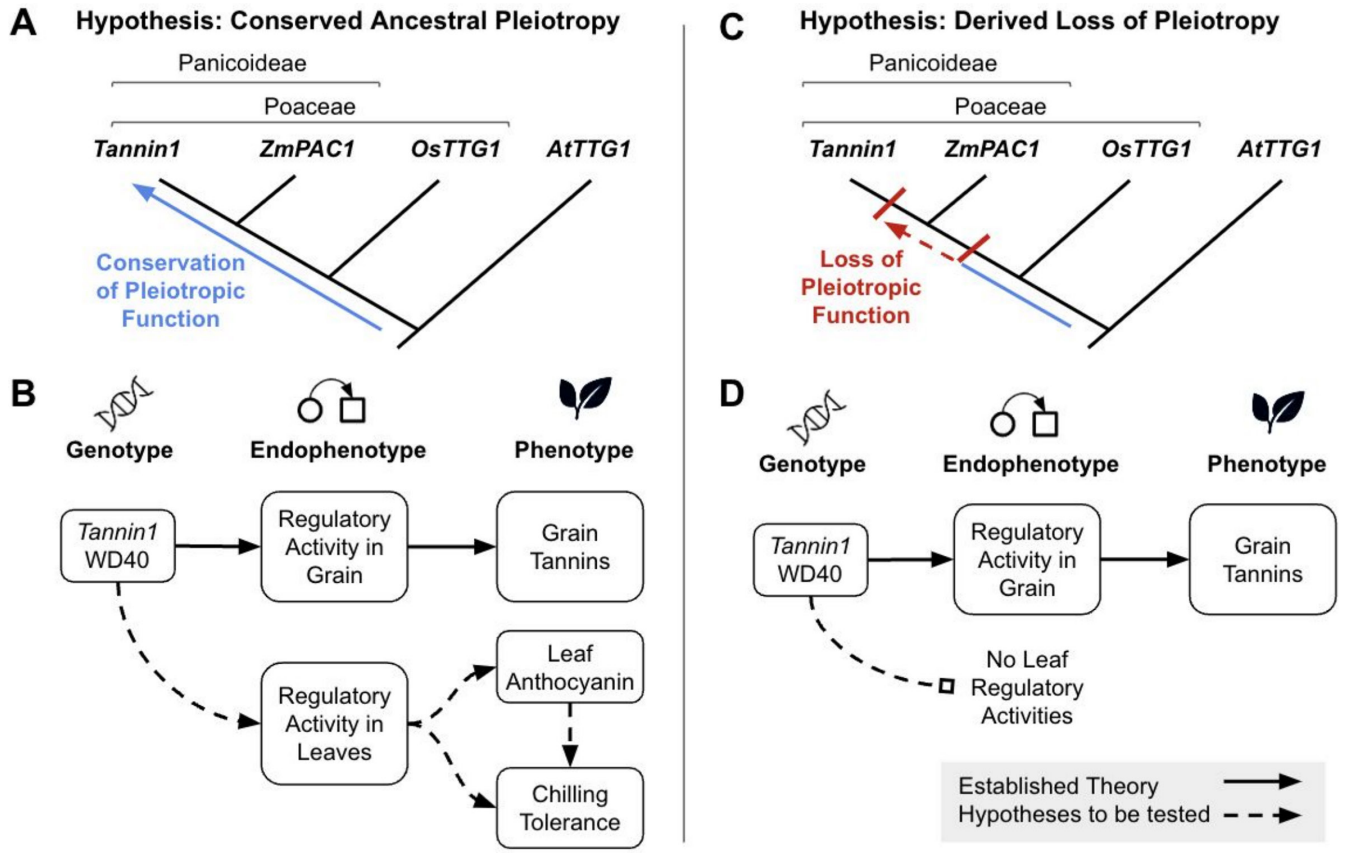


FIGURE 1 | Competing hypotheses on the evolution of *TTG1* orthologs in panicoid grasses and corresponding pleiotropic functional role of the WDR protein in sorghum leaf traits. (A) Under the *conserved ancestral pleiotropy* hypothesis, *TTG1* orthologs in cereals such as sorghum, maize, and rice would have retained regulatory functions in the leaves and seed, similar to the dicot model Arabidopsis. (B) Under this hypothesis, the pleiotropic *TTG1* regulatory function in leaf would be retained in sorghum *Tannin1*, influencing the genetics of leaf anthocyanin and possibly chilling tolerance (Yang et al. 2021; Schulz et al. 2016). (C) By contrast, under the *derived loss of pleiotropy* hypothesis, pleiotropic effects of *TTG1* orthologs in leaves would have been lost during grass evolution. (D) Under this hypothesis, genetics of leaf traits would not be influenced by *TTG1* orthologs. In panel B and D, dotted lines represent hypotheses, whereas solid lines represent established functions (Tian and Wang 2020).

Sobic.008G016100, and Sobic.003G408300) were highly and widely expressed (including in leaf tissue at various stages), suggesting they are candidate for the WDR role in MBW function in leaves.

To further characterize *TTG1* paralogs that could play a redundant or subfunctionalized WDR role in leaf MBW regulatory complexes, we conducted a phylogenetic analysis of homologous genes from sorghum, maize, rice, and Arabidopsis (Figure 3). The homology group from Phytozome, which included *TTG1*, included 71 WDR genes from the four focal species (note that this homology group does not include all WDR genes in these species). Sorghum *Tannin1*, maize *PAC1*, and rice *OsTTG1* cluster, with Arabidopsis *TTG1* as an outgroup. Another homology group, apparently sister to the *Tannin1-PAC1-OsTTG1* group, includes the putative sorghum co-ortholog of *TTG1*, Sobic.004G161600 (red highlight) and the Arabidopsis LWD genes. Notably, the four WDR genes (other than *Tannin1*) that were highly expressed in leaves (orange highlight; Sobic.003G427100, Sobic.002G401500, Sobic.008G016100, and Sobic.003G408300) are all relatively distantly related to the homology group that includes *TTG1*, *Tannin1*, *PAC1*, and *OsTTG1*. Among the four genes, only one Sobic.003G408300 is an ortholog of a characterized maize gene, *SHREK1* (*Shrunken and Embryo Defective Kernel 1*; v3 GRMZM2G081013; v5 Zm00001eb142980), but its function in kernel development (Liu et al. 2022) does not suggest a leaf WDR role for Sobic.003G408300.

2.2 | No *Trans*-Regulatory Effect on *Tannin1* Introgression on Flavonoid Biosynthesis or CBF Genes in Leaves

To test for evidence of pleiotropic leaf *trans*-regulatory functions of *Tannin1*, we looked for transcriptional changes in leaf tissue between NILs for genes and pathways that would be consistent with a role in leaf pigmentation or cold tolerance (Figure 1B). If *Tannin1* has a conserved leaf pigmentation function with *TTG1* and *OsTTG1*, we would expect *trans*-regulatory effects of the *Tannin1* introgression to cause differential expression of flavonoid pathway genes between NILs. We examined all genes with a known or hypothesized (based on homology; Morris et al. 2013) function in flavonoid biosynthesis and found none to be differentially expressed between the *Tannin1* NILs (Figure 4A). Additionally, anthocyanins visibly accumulate in leaf and stem tissue for all genotypes, with no difference observed between NIL+ and NIL− genotypes (Figure 4B).

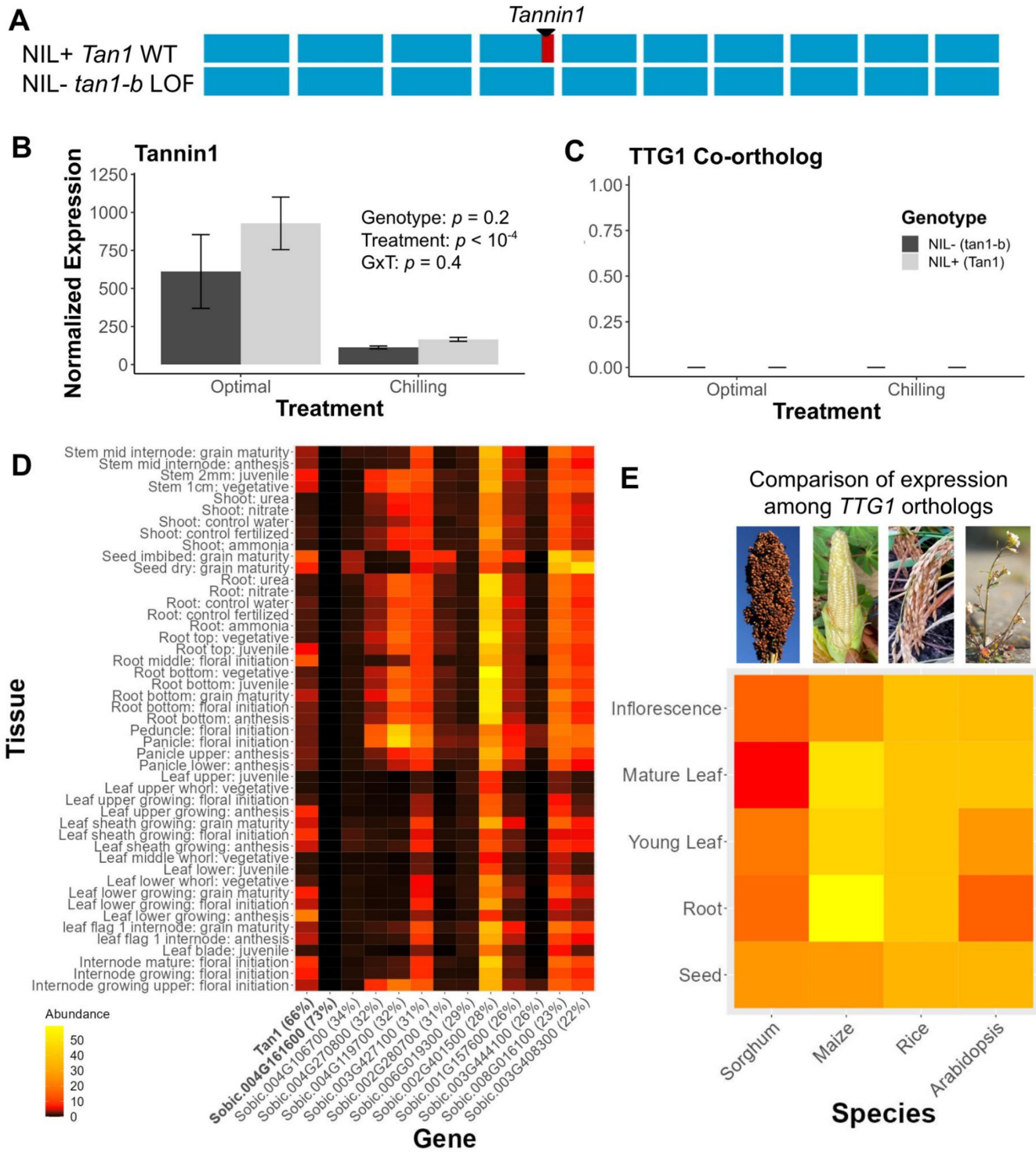


FIGURE 2 | Contrasting expression patterns of *Tannin1* and other *TTG1* homologs. (A) Sliding window analysis showing inheritance of genomic regions in NILs. Blue regions are from chilling sensitive parent (BTx623) and red from chilling tolerant (HKZ). Window size is 1 Mb. (B) Expression (± one standard error) of *Tannin1* and (C) possible *TTG1* co-ortholog, Sobic.004G161600, in leaf tissue for NILs under control and chilling treatments (one-way ANOVA *p*-values). Expression values are the mean of six genotypic replicates and normalized using DESeq2 median of ratios. (D) Expression (FPKM) of *Tan1* and other *TTG1* homologs across sorghum tissues, from Phytozome (Goodstein et al. 2012). The two *TTG1* co-orthologs are indicated in bold, with percent similarity with *TTG1* in parentheses. (E) Comparison of normalized tissue expression for WDR orthologs in sorghum (Tan1; Sobic.004G280800), maize (ZmPAC1; Zm00001eb250750), rice (OsTTG1; LOC_Os02g45810), and Arabidopsis (AtTTG1; AT5G24520). (Red: low relative expression, yellow: high relative expression). Tissues were manually curated by category. Data from Phytozome (sorghum), MaizeGDB (maize), BAR (rice), and TAIR (Arabidopsis) (Goodstein et al. 2012; Lawrence et al. 2004; Swarbreck et al. 2008; Waese and Provart 2017). Sorghum, maize image credit: G. Morris. Rice image credit: https://commons.wikimedia.org/wiki/File:20201102.Hengnan.Hybrid_rice_Sanyou-1.6.jpg. Arabidopsis image credit: https://commons.wikimedia.org/wiki/File:Arabidopsis_thaliana.jpg.

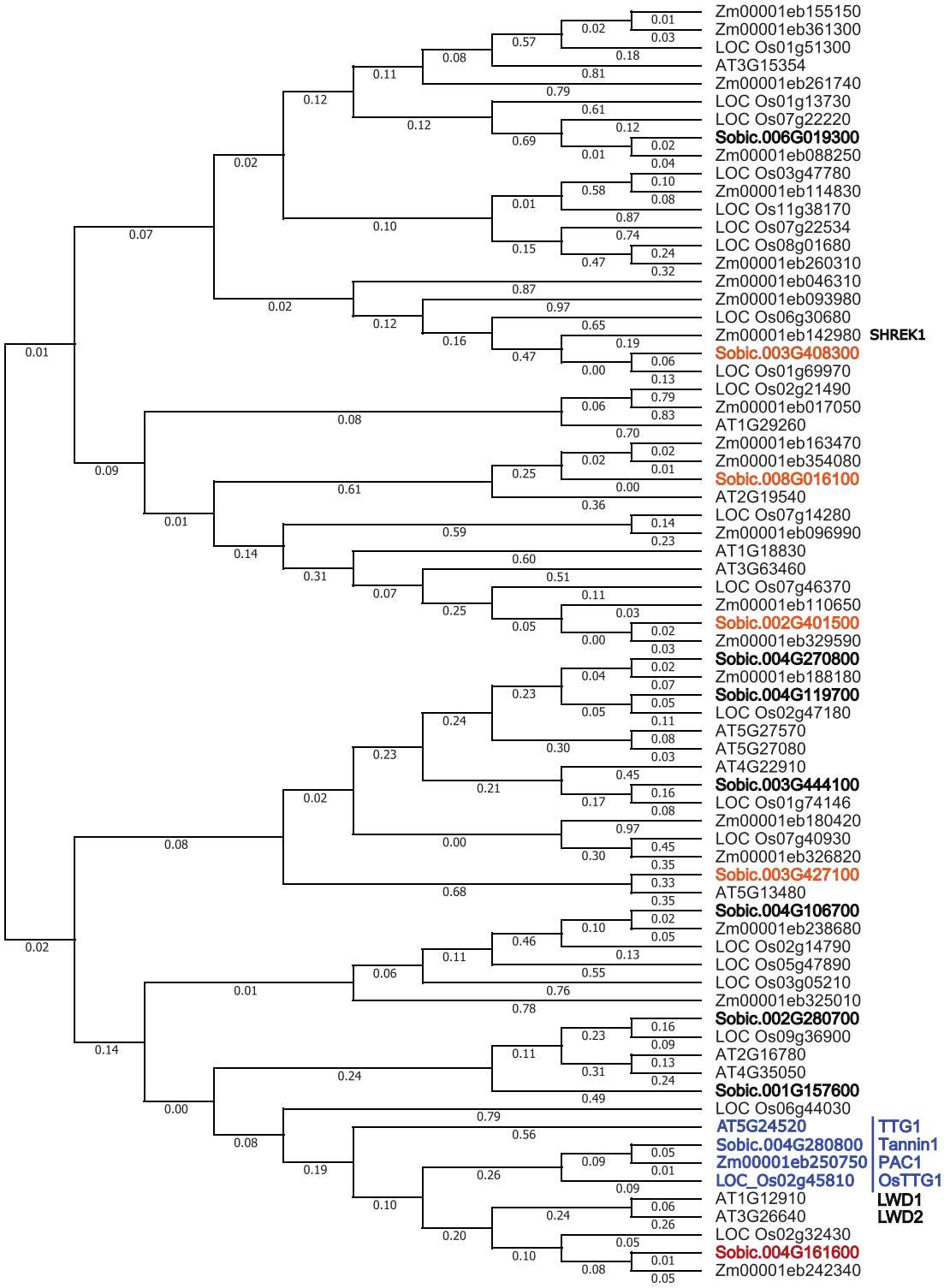


FIGURE 3 | Phylogenetic analysis indicates that leaf-expressed WDR paralogs of *Tannin1* are distantly related to *Tannin1* and *TTG1*. Maximum likelihood phylogeny of WDR genes (CDS) from sorghum (v3 "Sobic" IDs), maize (v5 "Zm" IDs), rice ("LOC_OS" IDs), and Arabidopsis ("AT" IDs). Labels are branch lengths. Arabidopsis *TTG1* (At5G24520), as well as its cereal orthologs sorghum *Tannin1* (Sobic.004G280800), maize *PAC1* (Zm00001eb250750), and rice *OsTTG1* (LOC_Os02g45810), are noted in blue. Three other cloned WDR genes, maize *Shrunken and Embryo Defective Kernel (SHREK1)* and Arabidopsis *Light-regulated WD1* (*LWD1*) and *Light-regulated WD2* (*LWD2*), are also noted. The other sorghum co-ortholog of *TTG1* (Sobic.004G161600), which has no evidence of leaf expression (see Figure 2C,D) is noted in red. Four sorghum *TTG1* homologs that were highly expressed in leaf tissue and broadly expressed overall (see Figure 2D), noted in orange, are candidates for the WDR that acts in leaves. Functional annotation of Arabidopsis genes is provided in Table S1.

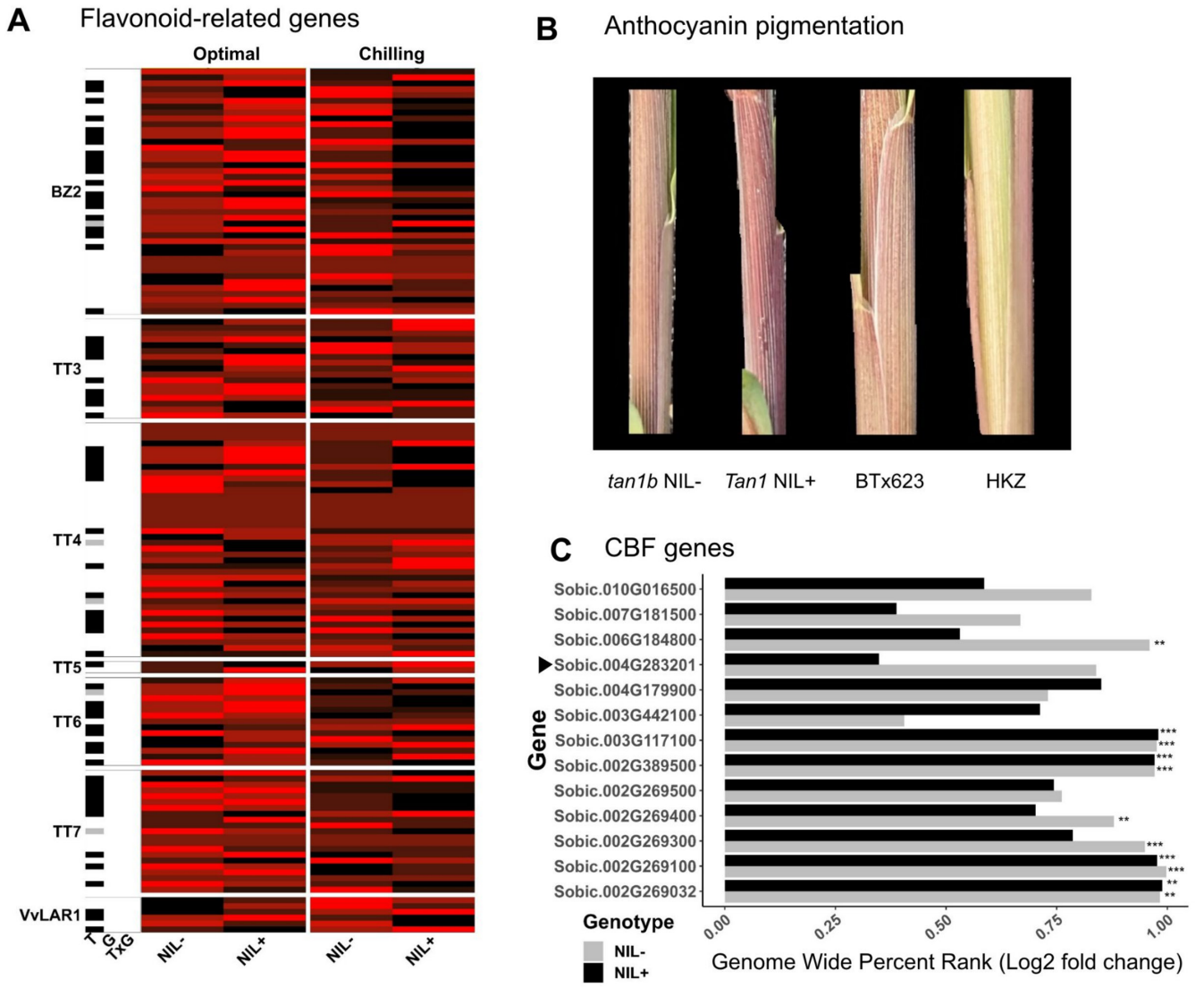
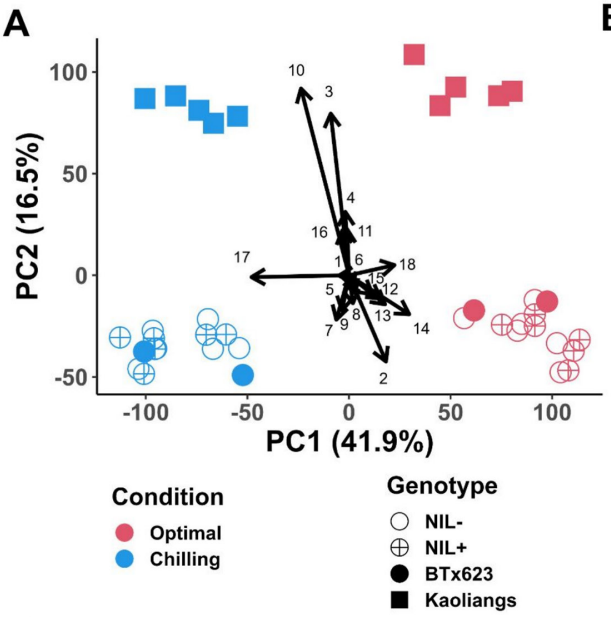


FIGURE 4 | Absence of pleiotropic *trans* regulation by *Tannin1* of anthocyanins or CBF cold response regulators in leaf tissue. (A) Expression of genes with known or predicted function in flavonoid synthesis. Data are shown for genes under optimal (orange) and chilling (blue) conditions. For a given gene, red indicates highest expression rank, whereas black represents the lowest. Median of ratios normalized mean expression from six genotypic replicates per treatment is displayed in the heatmap cell. Labels indicate the reference homolog in Arabidopsis (TT3, TT4, TT5, TT6, TT7), maize (BZ2), or grape (VvLAR) (Morris et al. 2013). The left sidebar shows significance of effect (black: $p < 0.05$; gray: $0.05 < p < 0.1$; white: $p > 0.1$) for treatment (T), genotype (G), and genotype × treatment interaction (G × T). (B) Anthocyanin accumulation in stem tissue by genotype under chilling conditions. NIL parents are included, BTx623 as negative (*tan1-b*) control and HKZ as positive (*Tannin1*) control (Composite image with background set to black for clarity). (C) Genome-wide percent rank of log2 fold change in predicted CBF orthologs. Expression is measured as the mean of six genotypic replicates and normalized using DESeq2's median of ratios method. Significance values are calculated for log2 fold change per NIL in response to treatment. No significant difference ($p > 0.05$) is observed for log2 fold change between NILs in any CBF ortholog. The triangle indicates the CBF homolog that is within the NIL+ introgressions. Significance codes are: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Since previous studies had posited *Tannin1* as a potential regulator of chilling tolerance (Figure 1B) and CBF genes are known regulators of cold tolerance in Arabidopsis and maize (Thomashow 2010), we tested whether *Tannin1* introgression had a *trans*-regulatory effect on CBF expression (Figure 4C). Though several were up or down regulated due to chilling, no CBF orthologs were significantly differentially expressed between NILs ($p = 1$) (Figure 4C). Further, there was no statistically significant upregulation ($p < 0.05$) for previously identified genes involved in other known chilling tolerance pathways (Marla et al. 2017), including lipid remodeling, NPQ, or phytohormone biosynthesis in *Tan1* versus *tan1-b* NILs (Figure S1).

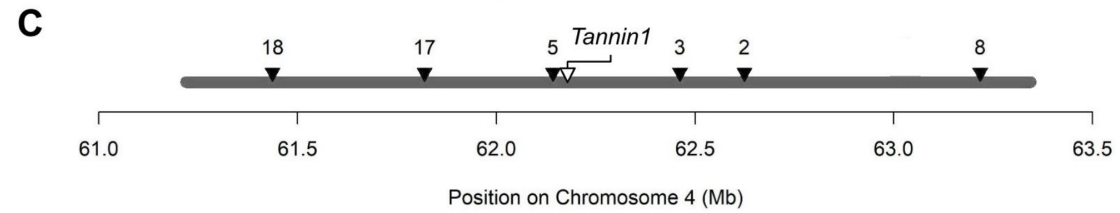
2.3 | Expression Pattern Suggests Independent Regulation Among Differentially Expressed Genes

To test for any additional *trans*-regulatory effects of the *Tannin1* introgression across the transcriptome, or *cis*-regulatory effects of other genes in the introgression, we conducted a principal component analysis (PCA; Figure 5A). The PC1 axis,



B

Ind	Gene ID	Annotation	*p*-value G	*p*-value GxT
1	Sobic.002G051300	flavin-dependent monooxygenase 1	$<10^{-4}$	$<10^{-4}$
2	**Sobic.004G284001**		$<10^{-4}$	1
3	**Sobic.004G282800**	GMC oxidoreductase	$<10^{-4}$	0.05
4	Sobic.009G248801		$<10^{-4}$	1
5	**Sobic.004G278650**	BURP domain-containing	0.001	1
6	Sobic.004G184800		0.02	0.7
7	Sobic.006G073400	F-box family protein	0.002	0.4
8	**Sobic.004G291200**		0.04	1
9	Sobic.006G249100		0.02	0.9
10	Sobic.010G208750	ribosomal protein L20	$<10^{-4}$	$<10^{-4}$
11	Sobic.010G085500		0.7	$<10^{-4}$
12	Sobic.007G092900	cytochrome P450, fam 71, subfam A	1	$<10^{-4}$
13	Sobic.008G183900	NAC domain containing protein 42	0.9	$<10^{-4}$
14	Sobic.002G323500	alpha/beta-Hydrolases superfamily	1	$<10^{-4}$
15	Sobic.002G100200		0.9	0.008
16	Sobic.002G278800	Ribonuclease T2 family protein	0.2	$<10^{-4}$
17	**Sobic.004G274700**	SNARE associated Golgi protein	0.9	$<10^{-4}$
18	**Sobic.004G270000**	clathrin adaptor complexes	$<10^{-4}$	$<10^{-4}$



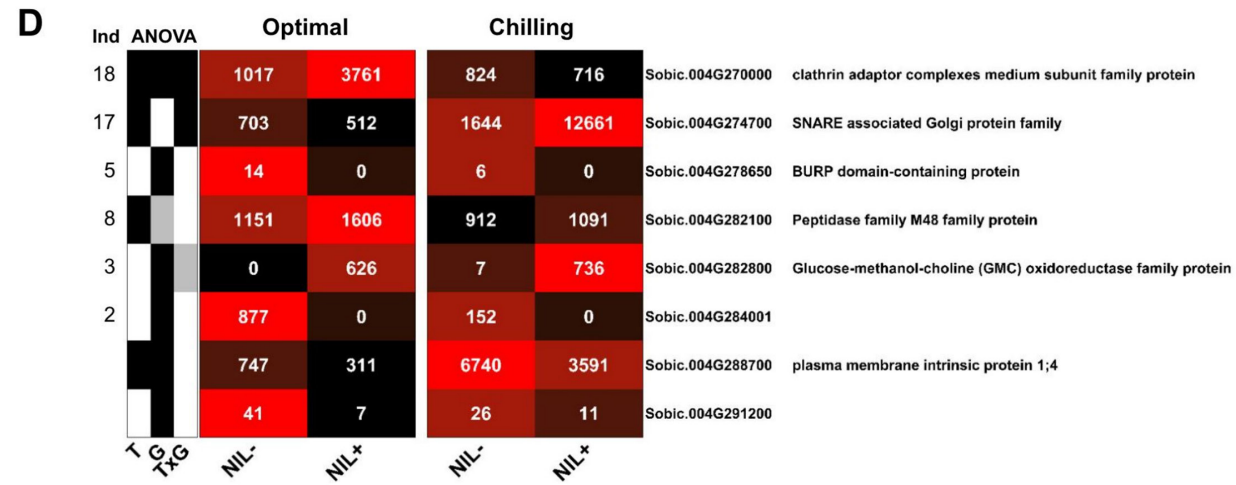


FIGURE 5 | Global gene expression analysis identifies *trans* and *cis* regulatory effects of the introgression. (A) Principal coordinates analysis of global gene expression of parent lines and NILs under optimal and chilling conditions. Circles indicate BTx623 genetic background (including NILs), squares indicate kaoliangs. Filled shapes indicate parent lines, whereas open shapes indicate NILs. PC1 axis resolves treatment, whereas PC2 genetic background. Arrows represents principal components (scaled up 3000× for clarity) for genes with significant ($p<0.05$; see panel B) differential expression. Direction of the arrow indicates upregulation. (B) List of differentially expressed genes (labeled with an Index, "Ind") along with Phytozome annotations. Six differentially expressed genes located within the introgression (putatively regulated in *cis*) are indicated with bold text, whereas 12 other genes (putatively regulated in *trans*) are in normal text (C) Locations of differentially expressed genes with the introgression, labeled as in the previous panel. (D) Expression heatmap of introgressed genes under optimal and chilling conditions, which had significant (ANOVA $p<0.05$) G or G×T interactions, with normalized mean expression ($n=6$). Significance is on the left (black: $p<0.05$; gray: $0.05<p<0.1$; white: $p>0.1$) and gene ID and annotation is on the right.

accounting for ~42% of the total variance, separates chilling versus control treatment. The PC2 axis, accounting for 16.5% of the total expression variance, separates the Kaoliangs parent genotypes from genotypes with BTx623 genetic background (BTx623 parent line along with both NIL+ and NIL−). Thus, there is a clear structure of four discrete clusters, corresponding to genetic background by treatment combinations, starting in the top left corner and moving clockwise: Kaoliang-Chilled, Kaoliang-Control, BTx623 background-Control, and BTx623 Background-Chilled. Notably, both *Tan1* NIL+ and *tan1-b* NIL−, which have

BTx623 genetic backgrounds, group with BTx623 for control and chilling treatments.

To identify genes that are differentially expressed due to the introgression (G effect) or due to introgression and chilling (G×T effects), we examined the expression patterns of top differentially expressed genes relative to NIL parents (Figure 5B) and plotted these on the PCA (Figure 5A). Overall, there is little effect of the introgression on global gene expression: among >30,000 annotated genes, just 18 genes were differentially expressed (G or G×T). Gene Ontology (GO) analysis indicated no statistical enrichment for any term (p-value >0.05) and none of the genes have an annotation that suggests a role in pigmentation or chilling tolerance (Figure 5B). Only two genes (#17: Sobic.004G274700, #1: Sobic.002G051300) have expression patterns (higher under chilling in *Tan1* NIL+) consistent with a role in induced chilling tolerance. A third of the differentially expressed genes (6/18) are in introgression itself (Figure 5C), likely represented *cis* regulatory differences rather than *trans* regulatory effects of *Tannin1*.

Finally, to identify any other genes in the introgressed region that could contribute to phenotypic differences between the NILs via *cis* regulation (i.e., independent of *Tannin1*), we reanalyzed differential expression just within the introgression. Even with this more powerful analysis of the 225 introgressed genes, just eight (the six genes previously noted and two more) had significant ($p<0.05$) G or G×T effects on expression (Figure 5D). This is an enrichment within the introgression relative to a genome-wide null model ($\chi^2=p<10^{-16}$). Only Sobic.004G270000 and Sobic.004G274700 exhibited G×T interactions. Only Sobic.004G274700, a putative SNARE-associated Golgi protein, is upregulated in both *Tan1* NIL+ under chilling treatment.

3 | Discussion

3.1 | *Tannin1* Is Likely Not a Pleiotropic Transcriptional Regulator of Leaf Pigmentation or Chilling Response

In this study, we investigated the conservation of WDR master regulation in sorghum, using genome-wide expression patterns in *Tannin1* NILs to test for *trans*-regulatory effects that could lead to pleiotropic effects on leaf pigmentation or chilling tolerance (Figure 1). Overall, expression patterns in NILs with contrasting *Tannin1* alleles overwhelmingly reflect BTx623 genetic background and show few regulatory differences (Figures 4 and 5), which differs notably from findings in rice *OsTTG1* mutants, which had widespread transcriptome effects (Yang et al. 2021). The paucity of differentially expressed genes ($n=18$; Figure 5) suggests the tighter control of genetic background effects using NILs, compared to previous expression studies of chilling tolerance using diverse accessions (Marla et al. 2017; Chopra et al. 2015), allowed stronger conclusions about the genotype–phenotype relationship. There was no signal of differential regulation for leaf pigmentation (Figure 4A,B) or chilling tolerance (Figure 4C) pathways between *Tan1* NILs, and only 12 genes showing evidence of *trans* regulation in leaves due *Tannin1* (Figure 5B), which would not be expected if *Tannin1* retained WDR pleiotropic regulation (Li et al. 2020; Yang et al. 2021). As far as the leaf pigmentation hypothesis (Figure 1B), the lack of pleiotropic effects on leaf anthocyanins in *Tannin1* NILs (Figure 4B) agrees with classical genetic studies of *Tannin1* (i.e., *B1*) and pigmentation loci (Doggett 1970; Mace and Jordan 2010), and further corroborates the hypothesis that *Tannin1* lacks pleiotropic leaf function (Figure 1D).

The lack of differential regulation in chilling-associated genes between NILs (Figure 4C) suggests that *Tannin1* has no *trans*-regulatory effect on canonical chilling tolerance pathways (Figure 1D). This is in line with a concurrent study of morphological and ecophysiological effects of *Tannin1* introgression (Schuh et al. 2024), which found no developmental or physiological effects on chilling response (Figure 4). This conclusion is surprising as both *Tannin1* and *Tannin2* are tightly co-located with chilling tolerance loci (Marla et al. 2019), which had suggested that grain tannins or other MBW effects contribute to chilling tolerance. It is also possible that growth chamber chilling conditions we used do not induce the same chilling response as in the field (Marla et al. 2023), and we cannot entirely reject *Tannin1* as underlying *qCT04.62*, because other environmental stressors, such as pathogens, may drive the association observed under field conditions (Nida et al. 2019). Alternatively, epistasis with other chilling tolerance alleles from Chinese sorghum (Marla et al. 2023; Marla et al. 2019), which were not introgressed here (Figure 2A), could also account for a lack of *Tannin1* effects. If the association between *Tannin1* and chilling tolerance is due to linkage, not pleiotropy, molecular breeders could target recombinations to break the linkage (Marla et al. 2023) and deploy *qCT04.62* in early planted cropping systems (Raymundo et al. 2021). Further, if *qCT04.62* is not a pleiotropic effect of *Tannin1* it must be due to an unidentified cold tolerance gene (Schuh et al. 2024), which could be relevant for cold tolerance in other crops such as maize and rice.

3.2 | Relief of Pleiotropic Constraints due to Duplication and Subfunctionalization of WDR Genes

The evidence that *Tannin1* does not have pleiotropic function in leaf tissues raises the question, what genes do fulfill the WDR role for other flavonoid systems in sorghum? Genetic studies (Doggett 1970; Mace and Jordan 2010; Ibraheem et al. 2010) suggest sorghum has at least four flavonoid systems, with largely independent regulatory controls: (i) proanthocyanidins (condensed tannins) in the testa (inner seed coat) (e.g., *Tannin1/B1*; WDR, *Tannin2/B2*; bHLH); (ii) anthocyanins in the seedling coleoptile (e.g., *Rs1* and *Rs2*; gene function unknown); (iii) anthocyanins in adult vegetative tissues (e.g., *P* and *Q*; gene function unknown); and (iv) phlobaphenes in the pericarp (outer seed coat) and 3-deoxyanthocyandin phytoalexins throughout the plant (e.g., *Y/Y1*; myb).

These genetic findings, along with evidence of abundant WDR duplication in the grass lineage leading to sorghum (Figure 3), leads to the hypothesis that the WDR role has subfunctionalized, or neofunctionalized, (Birchler and Yang 2022) across different pigmentation systems. Specifically, at least 12 other paralogous WDR regulators (Figure 3), beyond *Tannin1*, could be

considered as candidates for the WDR role for MBW function in the three other flavonoid systems listed above (ii–iv). However, the pattern of expression across tissues (Figure 2D) and the gene phylogeny (Figure 3), do not immediately suggest which among the WDR genes regulate other flavonoid systems (ii–iv). There is no evidence of expression of the other sorghum co-ortholog of *TTG1* (Sobic.004G161600; Figure 2; Figure 3), suggesting that it is either a pseudogene, or has subfunctionalized to a very limited extent of expression. Further studies of WDR paralogs in maize and sorghum (particularly the four sorghum paralogs with high leaf expression; Figures 2 and 3) will be needed to identify the WDR in each system.

In rice *OsTTG1* has been shown to regulate leaf trichome development and flavonoids in several tissues, and the bHLH transcription factor Rc (orthologous to sorghum *Tannin2* and Arabidopsis *Transparent Testa 8*) regulating testa proanthocyanidins (Gu et al. 2011; Yang et al. 2021). Notably, Rc has also been shown to pleiotropically regulate seed dormancy though regulation of ABA biosynthesis (Gu et al. 2011), one of the findings that motivated the Conserved Ancestral Pleiotropy hypothesis (Figure 1A,B). Taking these findings together, it is most parsimonious to infer a loss of function of leaf regulatory effects in the *PAC1*/*Tannin1* ancestor after the split from rice, but before the split between sorghum and maize (Figure 6). The evolutionary timing of this split will be further refined by studying MBW function in species phylogenetically intermediate between sorghum/maize and rice. Setaria (*Setaria italica*), a Panicoideae grass and model for C4 photosynthesis, has an identified but uncharacterized TTG1 ortholog (SiTTG1) which could offer valuable insights with further functional analysis (Liu et al. 2017; Pant et al. 2016).

Although Arabidopsis *TTG1* and rice *OsTTG1* regulate flavonoids in leaves, our experiments indicate that *Tannin1* does not, which is consistent with the lack of vegetative flavonoids regulation by *PAC1* in maize (Selinger and Chandler 1999). Thus, in maize and sorghum, it appears that multiple MBW contribute regulatory function independently in various tissues (Figure 6) (Boddu et al. 2005; Ibraheem et al. 2010; Nida et al. 2019). There are two possible explanations for this independent function. Either the MBW complex has lost the need for a WDR subunit in regulating many phenotypes, and the bHLH and mybs are able to regulate independently; or, perhaps more likely, the WDR paralogs (Figure 3) have subfunctionalized (Birchler and Yang 2022). Our data cannot fully rule out either explanation, as *Tannin1* and several other paralogs are expressed throughout the plant (Figure 2). *Tannin1* is broadly expressed, matching the broad expression seen in *TTG1*, *PAC1*, and *OsTTG1* (Figure 2) (Yang et al. 2021), so if *Tannin1* has subfunctionalized, it does not appear to be due to loss of expression in leaf tissue. Further, *Tannin1* and *PAC1* are both sufficient to rescue anthocyanin pigmentation in Arabidopsis *ttg1* mutants (Carey et al. 2004; Wu et al. 2012), so it is unlikely that the loss of functionality is due to coding sequence changes affecting the function of the Tannin1 or PAC1 protein. Therefore, the loss of pleiotropic function likely originates further downstream in the regulatory pathway.

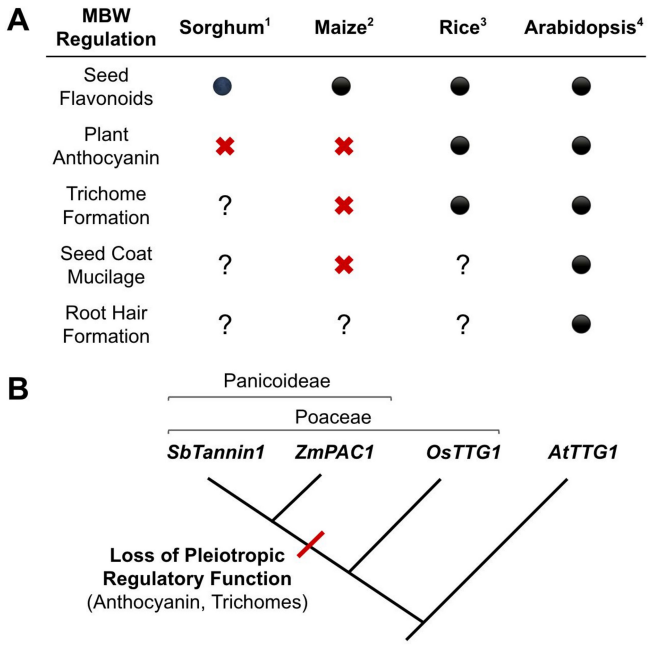


FIGURE 6 | Loss of pleiotropic functions in *TTG1* orthologs in the Panicoideae after the split from rice. (A) Evidence for loss of pleiotropic function occurring before sorghum-maize speciation, but after speciation with rice. Circles indicate when MBW function is present, question mark represents unknown, and "✖" indicates absent function. Citations: 1, (Wu et al. 2012) 2, (Selinger and Chandler 1999); 3, (Yang et al. 2021); 4, (Walker et al. 1999). (B) Hypothesis on the timing of the loss of pleiotropic functions after the panicoid grasses split from the rice lineage, but prior to the split of the maize and sorghum lineages.

3.3 | Conclusions

Overall, the hypothesis of subfunctionalization of WDRs in sorghum, and perhaps other panicoid grasses (Figures 1C,D and 6) seems most likely, in striking contrast to the WDR master regulator model from Arabidopsis, which is the dominant model of MBW function across the plant molecular biology literature (Tian and Wang 2020; Li et al. 2020; Yang et al. 2021). Unraveling conservation, subfunctionalized, and neofunctionalized of MBW roles will be useful for precise molecular breeding in sorghum and other crops as the complex regulates multiple traits interrelatedly. The sorghum findings may also suggest hypotheses on MBW function in other related crops. For instance, several millets in the Panicoideae, understudied but globally important, have active breeding programs in developing countries (Debieu et al. 2017), so elucidating MBW function in sorghum and maize can inform molecular breeding in these crops. Overall, the findings illuminate the evolutionary history of the MBW complex in the Poaceae and inform strategies to improve MBW-regulated traits in cereal crops.

4 | Material and Methods

4.1 | Plant Materials

The development of the *Tannin1* NILs was previously described (Schuh et al. 2024). Briefly, three RILs from the chilling tolerance NAM BTx623 × Hong Ke Zi (PI 567946) family were used

as starting material to reduce subsequent backcrossing (Marla et al. 2019) and crossed to BTx623. F1 progeny were selected genotypically for heterozygosity at the QTL of interest using KASP markers and phenotypically for resemblance to BTx623, the recurrent parent. Selection and backcrossing were repeated for four generations. Four suitable BC4F1 lines were then selected and selfed. From the segregating progeny, homozygotes for both alleles of the QTL of interest were selected, making eight total BC4F2 lines. Those eight lines were then advanced to the BC4F5 generation through single seed descent generating four pairs of NIL siblings (Marla et al. 2023). Only NILs1–3 were used in the current study because residual heterozygosity was detected at Tannin1 locus (Schuh et al. 2024).

4.2 | Chilling Treatment and RNA Sequencing

Experiments carried out in December 2021 using controlled environment chambers (Conviron Model CMP6050, Manitoba, Canada) at the Plant Growth Facilities at Colorado State University in Fort Collins, CO (40°34′18.6″ N 105°04′52.2″ W). Experiment designs were created and randomized using a custom R v4.1.2 script (R Core Team 2021). Following previous protocol (Marla et al. 2017), plants were potted in 1.5-in. Conetainers using Lambert LM-HP potting soil and grown using a 12-h photoperiod and 700 $\mu mol\, m^{-2} s^{-1}$ light intensity. After sowing 10 replicates of each genotype following the experimental design, all pots were allowed to germinate and grow under control temperature conditions, 28:25°C day:night, for approximately 14 days total. After the initial growth, half the plants were subjected to chilling conditions, 6:4°C day:night, beginning at the start of the dark photoperiod. Water was provided in excess using bottom watering. After a 36-h chilling treatment, 2 g of leaf tissue was collected from chilling and control plants and flash-frozen in liquid nitrogen. Frozen leaf tissue was stored at −80°C until extractions were completed. Following the manufacturer's instructions, extractions were performed using Quick-RNA Plant Miniprep Kit (ZYMO, R2024). RNA was quantified and quality tested using a Thermo Scientific NanoDrop 2000/2000c Spectrophotometer and stored at −80°C. RNA was then sent to the Kansas State University Integrated Genomics Facility (https://www.k-state.edu/igenomics/index.html) for RT-PCR, library prep, and sequencing. cDNA was sequenced using Illumina NextSeq 500, 75 cycles, and single-read chemistry (https://www.illumina.com/documents/products/appnotes/appnote-nextseq-500-wgs.pdf). Sequencing produced ~2.5 GB of data per sample, or ~30 million reads. Reads were uploaded to Illumina BaseSpace Hub by the sequencing center, and during FASTQ generation, adapter sequences were trimmed by BaseSpace.

4.3 | Differential Gene Expression Analysis

Reads were downloaded from Basespace Hub and mapped to BTx623 v3.1.1 reference genome (McCormick et al. 2018) using STAR v2.7.10a single pass mapping (Dobin et al. 2013). Subread v2.0.1 featureCounts package was then used to quantify and summarize reads (Liao et al. 2014). DE by genotype (G), treatment (T), and genotype by treatment (GxT) was calculated using DESeq2 v1.34.0 R package (Love et al. 2014). The *p*-values were obtained using the Wald test and corrected for multiple testing bias using the Benjamini–Hochberg correction. Expression was normalized across samples using DESeq2's median of ratios method. For *cis*-regulation analysis, samples were filtered by location and significant G and GxE interactions, for other analyses, DE was examined for specific genes. Heatmaps were constructed using ComplexHeatmap v2.10.0 R package (Gu et al. 2016). All other plots were constructed using the ggplot2 v3.4.2 r package (Hadley 2016). Mean expression was displayed in the heatmap cell. AgriGo: Gene Ontology Analysis Toolkit (Du et al. 2010) was used for Gene Ontology analysis. Data for expression analysis in other sorghum tissues were obtained from Phytozome (Goodstein et al. 2012). Cross-species expression comparison was performed by manually grouping tissues and assigning expression as absent, low, medium, or high for each tissue. Expression data for maize was obtained from maizegdb, rice: BAR, and Arabidopsis: TAIR (Lawrence et al. 2004; Swarbreck et al. 2008; Waese and Provart 2017).

4.4 | Phylogenetic Analyses

CDS sequences for *Tannin1* (Sobic.004G280800), *PAC1* (Zm00001eb250750), *TTG1* (AT5G24520), and *OsTTG1* (LOC_Os02g45810) were obtained from Phytozome (Goodstein et al. 2012). BLAST (Altschul 1997) was used to query the Phytozome database for paralogs of WDR in their respective species. Sequences with >25% similarity were downloaded from Phytozome and a multiple sequence alignment was created using MUSCLE (Edgar 2004). The alignment was then trimmed using ClipKIT (Steenwyk et al. 2020) to maximize the accuracy of phylogenetic inference. Evolutionary analysis and tree construction were then conducted using MEGA11 (Tamura et al. 2021). The evolutionary relationship among genes was inferred using the Maximum Likelihood method and Kimura 2-parameter model (Kimura 1980), and a discrete Gamma distribution was used to model evolutionary rate differences among sites. The rate variation model allowed for some sites to be evolutionarily invariable, and all positions with less than 95% site coverage were eliminated.

Author Contributions

TS and GPM conceived the study. TS conducted the experiments and analyzed the data. TS and GPM wrote the paper.

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Conflicts of Interest

GPM has filed a provisional patent application (WO2021189034A1) related to genetic markers at the locus of interest.

Data Availability Statement

RNA sequencing data are available at the NCBI Sequence Read Archive under Bioproject accession PRJNA1168095. The near-isogenic lines are

available from the authors by request (Geoffrey Morris; geoff.morris@colostate.edu) and will be submitted to the National Plant Germplasm System for access via the Germplasm Resources Information Network under SORGHUM GENSTOCKS.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.