THESIS

UNLOCKING SORGHUM ADAPTIVE POTENTIAL THROUGH INVESTIGATIONS INTO PLEIOTROPIC CONTROL OF CHILLING TOLERANCE BY *TANNIN1*

Submitted by

Anthony Schuh

Department of Soil and Crop Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2023

Master's Committee:

Advisor: Geoffrey Morris

Cristiana Argueso
Kelly Wrighton

ABSTRACT

UNLOCKING SORGHUM ADAPTIVE POTENTIAL THROUGH INVESTIGATIONS INTO PLEIOTROPIC CONTROL OF CHILLING TOLERANCE BY *TANNIN1*

Chilling tolerant crops can positively impact agricultural sustainability through lengthened growing seasons and improved water and nitrogen use efficiency. In sorghum (*Sorghum bicolor* [L.] Moench), the fourth most grown grain, coinheritance of *qSbCT04.62*, the largest effect chilling tolerance locus, with *Tannin1*, the major gene underlying undesirable grain proanthocyanidins, has stymied breeding for chilling tolerance. To investigate the genetic basis of *qSbCT04.62* including its coinheritance with *Tan1*, we developed near isogenic lines (NILs) with chilling tolerant haplotypes around *qCT04.62*.

In the first study we genotype the NILs and investigate the introgressions physiological control over the cold stress response. Genome sequencing revealed that the *CT04.62*+ NILs introgressions on chr04 include *Tannin1*, a homolog of Arabidopsis cold regulator CBF, peak SNPs for *qCT04.62* from multi-family NAM, and 61.2-62 Mb of HKZ × BTx623 NAM family *qCT04.62* confidence interval. Grain tannins were correlated with *Tan1* genotype, revealing heterogeneity in one NIL at *Tannin1*. Controlled environment chilling assays found no genotype by environment interaction on growth by chilling *per se* in parents or NILs. Cold germination was reduced at 15°C and superior at 20 and 25°C in the chilling tolerant parent compared to chilling sensitive, but unchanged between NILs. The introgression also did not regulate a chilling induced increase in non-photochemical quenching.

In the second study we investigated *Tan1* function with a transcriptome analysis of the NIL's response to chilling stress. *Tannin1* was widely expressed in sorghum tissues but did not promote a transcriptional response in chilling tolerance related molecular pathways including lipid remodeling, phytohormone signaling, CBF upregulation, photoprotection, and ROS mitigation. GO analysis also found no significant term enrichments at the $p < 0.1$ threshold. Only 17 genes had expression patterns regulated by polymorphisms in the introgressions, seven cis, and ten trans, with little evidence of co-regulation. Further, *Tannin1* was functionally divergent from its Arabidopsis ortholog *TTG1* and other WD40 orthologs in regulating leaf anthocyanin biosynthesis.

Overall, these findings suggest that linkage, not pleiotropy, underpins the coinheritance of *Tan1* and *CT04.62+*, unlocking the use of *CT04.62+* for sorghum improvement. Further, these results imply a lack of deleterious fitness effects of *tan1* alleles in commercial grain sorghum varieties and suggest the possibility of an unknown cold tolerance regulator which, if identified, could have implications for crop improvement of chilling tolerance outside sorghum.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER II:

CHAPTER I: MAPPING GENOTYPE TO PHENOTYPE FOR CHILLING TOLERANCE IN SORGHUM TO FACILITATE MOLECULAR BREEDING

The Importance of Crop Adaptation: Historical and Modern

Crop adaptation ensures crop resilience in the face of external instability. Likely beginning more than 12000 years ago, crop adaptation remains the foundation of agriculture (Zeder, 2011), responding to a myriad of factors, including human preference, pest pressure, variable markets, and changes in agricultural practice (Evans, 1997). In its simplest form, crop adaptation takes place over dozens to hundreds of generations, driven by both artificial and natural selection and genetic drift (Robertson, 1960).

In the 21st century, increasing the rate of crop adaptation has become more and more necessary. This century brings the difficulties of climatic uncertainty, water shortages, soil degradation, and a rising global population (FAO, 2019; Lobell et al., 2008; Qaim, 2020). Fortunately, molecular advances of the 20th and 21st centuries have enabled faster, genetically targeted methods known as molecular breeding. Molecular breeding employs genetic markers to assist in selecting difficult-to-phenotype, complex, and quantitative traits, reducing linkage drag and making it possible to breed plants with minimized cost and time (Moose & Mumm, 2008).

Adapting Crops for Chilling Tolerance: Why it is Desirable and Important

Chilling tolerance refers to a plant's ability to survive and grow in cool, above-freezing temperatures (Lyons, 1973). It is a beneficial trait for crops grown in temperate regions and is generally lacking in those of lowland tropical origins. Subsequently, it is of significant interest in staple crops such as maize and rice, and its incorporation into germplasm has allowed their range to expand significantly into cooler areas with short growing seasons (Frei, 2000; Khush, 1997). For agronomic purposes, chilling tolerance is generally considered two traits, early and late

season chilling tolerance. For many crops, including maize, sorghum, and rice, chilling tolerance is relevant during the early season as it increases vigor and emergence for earlier plantings (Miedema et al., 1987; Sthapit, 1992; Yu & Tuinstra, 2001) and can have a substantial positive effect on yield (Ellis, 1992; Liu et al., 2019; Raymundo et al., 2021).

Several environmental factors contribute to a cropping system benefiting from more chilling tolerant crops: a short growing season (Frei, 2000) or frequent late-season droughts paired with cold winters (Raymundo et al., 2021). In environments with these conditions, early season chilling tolerance can extend a growing season (Long & Spence, 2013), which adds flexibility and makes it easier to avoid unfavorable conditions. In addition, extended growing seasons also increase choices for crop rotations (Lin, 2011), can increase yields, and reduce the need for herbicide and fertilizer (McDonald & Gill, 2009; Mosier et al., 1998; Vida et al., 2006). Chilling tolerance also decreases the chance of poor or failed harvests by making crops more resilient to unpredictable weather conditions (Raymundo et al., 2021).

Physiological Basis for Chilling Sensitivity/Damage in Plants

In plants, there are two main mechanisms underlying chilling stress, reduction in enzymatic function and increased membrane rigidity (Lyons, 1973; Raison & Orr, 1990). Symptoms manifest on all scales and include poor germination and field emergence, retarded growth, physical damage such as surface pitting, necrosis, discoloration, and physiological symptoms such as photoinhibition and respiratory acceleration with decreased ATP production. Phase changes in membrane lipids at chilling temperatures result in decreased membrane fluidity leading to membrane leakage and interference with the function of membrane-bound enzymes (Raison, Lyons, & Thomson, 1971; Raison, Lyons, Mehlhorn, et al., 1971). Chilling-resistant plants often have membrane compositions containing a smaller proportion of high melting point

fatty acids (N. Murata et al., 1992; Nishida & Murata, 1996, p. 5; Roughan, 1985), reducing the temperature at which a phase change occurs to below freezing (Lyons & Asmundson, 1965).

Chilling stress affects metabolism by interfering with membrane-bound enzymes in the mitochondria. The loss of membrane fluidity raises the activation energy of the membrane-bound enzymes in the TCA cycle with less effect on the unbound enzymes (Lyons & Raison, 1970). This leads to metabolic imbalance and the accumulation of toxic intermediate products such as pyruvate, acetaldehyde, and ethanol (T. Murata, 1969). The plant's inability to withstand the build-up of these intermediate products is thought to cause many physical symptoms of chilling (Lyons, 1973).

A similar phenomenon is also responsible for photoinhibition under chilling stress (Lyons, 1973). The activation energy for reactions in the chloroplast dramatically increases under chilling temperatures, significantly reducing the photosystem's efficiency of sunlight absorption (Shneyour et al., 1973). The unabsorbed sunlight is then free to react with O_2 and form reactive oxygen species (ROS), which can overwhelm the plant's repair mechanisms and cause damage to the plant's thylakoid membranes (Nishiyama et al., 2011). Depending on the severity, this damage can be reversible or irreversible (Ort, 2001; Taylor & Rowley, 1971). Photoinhibition and ROS build-up may also be partially responsible for leaf damage and death (Demmig-Adams & Adams, 2006).

Membrane lipid composition within seeds also correlates with low-temperature germination (Dogras et al., 1977). Therefore, it is hypothesized that interferences with respiration and the subsequent decrease in ATP are responsible for the reduced rate of germination among chilling-sensitive plants (Lyons, 1973).

Genetic Regulation in Plant Chilling Response

The CBF regulon is induced by jasmonate and is thought to be the primary regulator of cold response. Jasmonate is a stress response hormone (Santino et al., 2013) and a known upstream regulator of many biological processes, including root inhibition, anthocyanin accumulation, trichome initiation, male fertility, and leaf senescence (Hu et al., 2017). Jasmonate is also a key player in cold response signaling and is shown to be involved with cold tolerance in Arabidopsis and cold-sensitive species, including rice and tomato (Du et al., 2013; Hu et al., 2013; F. Wang et al., 2016). Cold induces JA biosynthesis genes, increasing JA-Ile levels. JA-Ile upregulates CBF/DREB1 dependent or independent signaling to increase the cold stress response (Hu et al., 2013). JA also inhibits growth by repressing gibberellic acid, which is thought to play a role in the cold response (Yang et al., 2012).

The CBF Regulon is a group of about 100 genes regulated by CBF (C-repeat binding factor) transcription factors (Fowler & Thomashow, 2002) and is well-known as a cold response pathway. In Arabidopsis, cold-induced biosynthesis of JA activates the ICE (Inducer of CBF Expression) pathway (Hu et al., 2013), which in turn induces CBF1, CBF2, and CBF3 transcription factors (Medina et al., 1999). These then activate the transcription of many COR (Cold-Regulated) genes through a C-repeat/dehydration responsive regulatory element (CRT/DRE) present in their promoters, which in turn mobilize cold acclimation (Stockinger et al., 1997).

Lipid Remodeling Involved in Chilling Response in Plants

Lipid remodeling is the inducible change in membrane lipid composition, affecting fluidity and phase change temperature (Upchurch, 2008). It is a well-known response to abiotic stresses (Liu et al., 2019) and is well-studied as an adaptation that mitigates cell damage during

cold acclimation in Arabidopsis (Degenkolbe et al., 2012; Li et al., 2008; Moellering et al., 2010; X. Wang et al., 2006). Generally during cold stress, plants will increase the unsaturation of their membranes to raise the melting point, which increases membrane stability to counteract the chilling-induced loss in membrane fluidity. This is accomplished through shifts in the ratio of saturated to unsaturated lipids composing the membrane. However, the specifics can vary by species and are strongly influenced by biosynthetic pathways present in the plant (Liu et al., 2019). Studies have identified several lipid shifts that suggest this phenomenon (Barnes et al., 2016; Welti et al., 2002; M. Zhang et al., 2005). To mobilize these shifts, lipid signaling molecules, phosphatidic acid (PA), lysophospholipids, oxylipins, and sphingolipids are created by the action of phospholipase on glycoglycerolipids and phospholipids (Arisz et al., 2013; Hou et al., 2016; Ruelland et al., 2002). Further, these signaling molecules can also influence phytohormone signaling, growth, development, and protein function, potentially playing a broader role in cold stress response (Hou et al., 2016).

Importance of Chilling Tolerance in Sorghum

Sorghum is an important crop in dryland agriculture and food security. Because of its relative tolerance among cereals to drought, heat, and low soil fertility, it is widely grown in regions limited by these abiotic stresses (Prasad & Staggenborg, 2010). In the U.S., sorghum fits a similar niche to maize but performs better in dry areas (Staggenborg et al., 2008). It is also a model system for several orphan crops, including pearl millet, proso millet, and foxtail millet, all of which are drought resistant. Chilling tolerance in sorghum is an important trait for dryland agricultural improvement, potentially affecting more efficient water and land use in the great plains. Currently, sorghum must be planted relatively late in the U.S. sorghum belt because it grows poorly at temperatures under 15°C (Taylor & Rowley, 1971). However, earlier planted

sorghum could take advantage of early-season rainfall and is predicted to increase yields, reduce the risk of "terminal droughts," extend the growing season and increase rotation options (Raymundo et al., 2021).

Physiology of Chilling Tolerance in Sorghum

Sorghum is considered extremely sensitive to chilling, especially when compared with maize. This is quantified by a significantly reduced photosynthetic rate during chilling and a relatively low capacity for post-chill recovery (Taylor & Rowley, 1971). Consistent with the hypothesis put forward by Lyons in 1973, in sorghum, several factors have been found to modulate the levels of photodamage during chilling, namely temperature, length of chilling exposure, light intensity, and time of chilling during the photoperiod. Colder temperatures and longer exposure increase photodamage, as well as increasing intensities of light. It was also found that sorghum has higher levels of photodamage when chilling occurs at the beginning of a photoperiod vs. mid-photoperiod (Taylor & Rowley, 1971).

In sorghum breeding lines, there is little standing variation for early season chilling tolerance (Salas Fernandez et al., 2014), though this is not universal among sorghum. A group of Northern Chinese landraces known as Kaoliangs have long been noted for their remarkable chilling tolerance. The Kaoliangs consistently perform better in early season emergence, early season vigor, and cool temperature germination when compared with elite inbred lines and commercial hybrids (Franks et al., 2006; Stickler et al., 1962).

From a mechanistic standpoint, little is known about the underlying physiology of the Kaoliangs' chilling tolerance. There is limited evidence that membrane composition may play a role as well as the CBF regulon (Marla et al., 2017). A joint transcriptome-lipidome analysis compared chilling tolerant NSZ and chilling susceptible BTx623. It was found that

phosphatidylcholine (PC), a glycerolipid associated with increased membrane stability, was less abundant in chilling tolerant NSZ under chilling conditions. The expression of a PLDα1 gene that encodes an enzyme that breaks down PC followed the same pattern (Marla et al., 2017). This is consistent with findings in Arabidopsis, where PC levels decrease more dramatically in cold-tolerant lines (Welti et al., 2002). The same transcriptome study also noted the up-regulation of a single *CBF*-associated gene in NSZ during chilling.

Genetics and Genomics of Chilling Tolerance in Sorghum

In field trials, variability for chilling tolerance is expressed as germination, emergence, and seedling vigor and is considered a heritable trait (Yu & Tuinstra, 2001). Furthermore, multiple association mapping studies have linked these traits with various QTL located across all ten sorghum chromosomes (Burow et al., 2011; Fiedler et al., 2016; Knoll et al., 2008; Marla et al., 2019). For early season emergence and cold germination, several notable QTL clusters are located across chromosomes 8 and 9 and at the end of chromosomes 2, 4, and 7. For early season vigor, QTL are also located on nearly every chromosome, but notable regions have appeared in multiple studies on chromosomes 1, 2, and 4 (Casto et al., 2021). Furthermore, agronomically important dwarfing and tannin genes are closely linked to several of these QTL: *Tan1* on chromosome 4, *Tan2* on chromosome 2, *Dw1* on chromosome 9, and *Dw3* on chromosome 7, suggesting potential significance for these particular genes through pleiotropic interactions. Moreover, as height and lack of tannins are highly selectively targeted traits, antagonistic interactions between chilling tolerance, tannins, and plant height may underlie the lack of variance for chilling tolerance within American sorghum germplasm (Marla et al., 2019).

Evolutionary evidence also supports *Tan1* and *Tan2* as potential pleiotropic regulators. In plants, flavonoids, including grain tannins, are produced by a highly conserved pathway and are

generally considered a metabolic endpoint (Shahin Hassanpour et al., 2011). The proanthocyanidin biosynthesis pathway is known to be regulated by the canonical regulatory MBW complex, which involves three subunits: MYB, bHLH (basic-helix-loop-helix), and WDR (WD40-repeat). In sorghum, *Tan1* is the W subunit, and *Tan2* is the B subunit. In Arabidopsis, other traits are also known to be pleiotropically regulated by the MBW complex, including leaf trichome initiation, root hair formation, anthocyanin production, and the presence of seed coat mucilage (B. Zhang & Hülskamp, 2019). Genetic mutants in this complex can have several phenotypes depending on the mutated subunit. This is attributable to the multiple homologous proteins with functional interchangeability for the M and B subunits, each specific for one or several MBW-associated phenotypes. The W subunit is shared across all five traits (B. Zhang & Hülskamp, 2019). It is unknown to what extent this function is conserved in sorghum, but major pleiotropic regulation of chilling tolerance signaling may be possible.

Little work has been done in sorghum to link JA or CBF with chilling tolerance directly. Still, comparative transcriptome analysis between BTx623 (chilling sensitive) and Kaoliangs (Chilling Tolerant) provides preliminary evidence that these are involved in the chilling response. In addition, under chilling conditions, two sorghum orthologs of *CBF3* were differentially upregulated in NSZ, and one, *SbCBF6*, was highly upregulated in chilling tolerant vs. chilling susceptible sorghum lines (Chopra et al., 2015; Marla et al., 2017). Also, under chilling conditions, JA biosynthesis genes (coding orthologs of OPR, 12-oxyphytodienoic acid reductase, and AOS, allene oxide synthase) were found to be differentially upregulated in chilling tolerant sorghum lines (Marla et al., 2017). Taken together, these two pieces of evidence provide support for the involvement of JA and CBF regulon signaling in sorghum chilling tolerance.

Mechanistic Hypothesis of Chilling Tolerance in Sorghum

From the above-cited research, at least three hypotheses could explain chilling tolerance mechanisms in sorghum, including Jasmonate/CBF signaling, lipid remodeling, and ROS mitigation. Jasmonate/CBF signaling has been extensively studied in Arabidopsis and is upstream for many of the organisms' cold and freezing tolerance responses. Furthermore, it has a conserved function in many grains such as rice, barley, wheat, and maize (Guo et al., 2019; Ito et al., 2006; Marozsán-Tóth et al., 2015; Qin et al., 2004), which makes it a strong candidate for investigation as an integral player in the sorghum chilling tolerance response. In sorghum, there are 12 CBF homologs, two of which were differentially upregulated during transcriptomic analysis (Marla et al., 2017). Though QTL analysis did not associate these two genes with chilling tolerance (Marla et al., 2019), this is not unexpected and suggests that these two genes are likely not causal in the differential chilling response. Moreover, the differential upregulation of JA biosynthesis genes in chilled Kaoliangs (Marla et al., 2017) further strengthens this interpretation, as JA is an upstream regulator of the CBF regulon, and its synthesis would contribute to the induction of the CBF pathway leading to a heightened chilling response. These findings give us a glimpse into potential mechanisms of chilling tolerance in sorghum and is a starting point for future investigation into the sorghum chilling response.

Lipid remodeling and ROS mitigation also have precedence in Arabidopsis and chilling-sensitive crops and have transcriptomic and lipidomic support in sorghum. In Marla et al., 2017, many lipids were differentially present under chilling in the tolerant plants, including the membrane stabilizing lipid PC. Furthermore, multiple lipid metabolism genes were differentially regulated under chilling, as were genes associated with ROS mitigation.

It is also reasonable to suggest the involvement of several independent mechanisms contributing to chilling tolerance in sorghum. This can be seen by the oligogenic nature of the trait and the varied genotypic expression. We also must consider a novel, uncharacterized adaptation. Finally, Marla et al., 2017 propose a unified model that combines all three hypotheses. They postulate that chilling-induced lipid remodeling promotes JA synthesis, which upregulates CBF and activates ROS mitigation (Marla et al., 2017).

Next Steps Towards Breeding for Chilling Tolerant Sorghum

Currently, breeding for more chilling tolerant sorghum can follow several avenues. Multiple QTL are known, many of which are free from negative associations. Because of the divergent genetic background of the Chinese sorghum, prebreeding is necessary to mitigate linkage drag and facilitate the transfer of alleles into breeding programs. Further, as chilling tolerance is a relatively complex trait, we will need to understand better the molecular and physiological basis of chilling tolerance variation in the kaoliangs and how these map to individual QTL and genes. Further, at this time, the associations between *Tan1*, *Tan2*, *Dw1,* and *Dw3* and major effect chilling QTL are locking up a significant portion of the chilling tolerance variation, rendering a comprehensive understanding of how genotype maps to phenotype even more vital. This research will require controlled genetic studies using NILs and mutants to fine-map and clone the unfavorably associated QTL. If undertaken, these measures will accelerate sorghum chilling tolerance adaptation, aid in food security, and help to improve agriculture for future generations.

REFERENCES: CHAPTER I

Arisz, S., van Wijk, R., Roels, W., Zhu, J.-K., Haring, M., & Munnik, T. (2013). Rapid phosphatidic acid accumulation in response to low temperature stress in Arabidopsis is generated through diacylglycerol kinase. Frontiers in Plant Science, 4. https://www.frontiersin.org/articles/10.3389/fpls.2013.00001

Barnes, A. C., Benning, C., & Roston, R. L. (2016). Chloroplast Membrane Remodeling during Freezing Stress Is Accompanied by Cytoplasmic Acidification Activating SENSITIVE TO FREEZING2. *Plant Physiology*, *171*(3), 2140–2149. https://doi.org/10.1104/pp.16.00286

Burow, G., Burke, J. J., Xin, Z., & Franks, C. D. (2011). Genetic dissection of early-season cold tolerance in sorghum (Sorghum bicolor (L.) Moench). *Molecular Breeding*, *28*(3), 391–402. http://dx.doi.org.ezproxy2.library.colostate.edu/10.1007/s11032-010-9491-4

Casto, A. L., Murphy, K. M., & Gehan, M. A. (2021). Coping with cold: Sorghum cold stress from germination to maturity. *Crop Science*, *61*(6), 3894–3907. https://doi.org/10.1002/csc2.20609

Chopra, R., Burow, G., Hayes, C., Emendack, Y., Xin, Z., & Burke, J. (2015). Transcriptome profiling and validation of gene based single nucleotide polymorphisms (SNPs) in sorghum genotypes with contrasting responses to cold stress. *BMC Genomics*, *16*(1), 1040. https://doi.org/10.1186/s12864-015-2268-8

Degenkolbe, T., Giavalisco, P., Zuther, E., Seiwert, B., Hincha, D. K., & Willmitzer, L. (2012). Differential remodeling of the lipidome during cold acclimation in natural accessions of Arabidopsis thaliana. *The Plant Journal*, *72*(6), 972–982. https://doi.org/10.1111/tpj.12007

Demmig-Adams, B., & Adams, W. W. (2006). Photoprotection in an ecological context: The remarkable complexity of thermal energy dissipation. *New Phytologist*, *172*(1), 11–21. https://doi.org/10.1111/j.1469-8137.2006.01835.x

Dogras, C. C., Dilley, D. R., & Herner, R. C. (1977). Phospholipid Biosynthesis and Fatty Acid Content in Relation to Chilling Injury during Germination of Seeds 12. *Plant Physiology*, *60*(6), 897–902.

Du, H., Liu, H., & Xiong, L. (2013). Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. *Frontiers in Plant Science*, *0*. https://doi.org/10.3389/fpls.2013.00397

Ellis, R. H. (1992). Seed and seedling vigour in relation to crop growth and yield. *Plant Growth Regulation*, *11*(3), 249–255. https://doi.org/10.1007/BF00024563

Evans, L. T. (1997). Adapting and improving crops: The endless task. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *352*(1356), 901–906. https://doi.org/10.1098/rstb.1997.0069

FAO. (2019). Safeguarding against economic slowdowns and downturns.

Fiedler, K., Bekele, W. A., Matschegewski, C., Snowdon, R., Wieckhorst, S., Zacharias, A., & Uptmoor, R. (2016). Cold tolerance during juvenile development in sorghum: A comparative analysis by genomewide association and linkage mapping. *Plant Breeding*, *135*(5), 598–606. https://doi.org/10.1111/pbr.12394

Fowler, S., & Thomashow, M. F. (2002). Arabidopsis Transcriptome Profiling Indicates That Multiple Regulatory Pathways Are Activated during Cold Acclimation in Addition to the CBF Cold Response Pathway[W]. *The Plant Cell*, *14*(8), 1675–1690. https://doi.org/10.1105/tpc.003483

Franks, C. D., Burow, G. B., & Burke, J. J. (2006). A Comparison of U.S. and Chinese Sorghum Germplasm for Early Season Cold Tolerance. *Crop Science*, *46*(3), 1371–1376. https://doi.org/10.2135/cropsci2005.08-0279

Frei, O. M. (2000). Changes in yield physiology of corn as a result of breeding in northern Europe. *Maydica*, *45*(3), 173–183.

Guo, J., Ren, Y., Tang, Z., Shi, W., & Zhou, M. (2019). Characterization and expression profiling of the ICE-CBF-COR genes in wheat. *PeerJ*, *7*, e8190. https://doi.org/10.7717/peerj.8190

Hou, Q., Ufer, G., & Bartels, D. (2016). Lipid signalling in plant responses to abiotic stress. *Plant, Cell & Environment*, *39*(5), 1029–1048. https://doi.org/10.1111/pce.12666

Hu, Y., Jiang, L., Wang, F., & Yu, D. (2013). Jasmonate Regulates the INDUCER OF CBF EXPRESSION–C-REPEAT BINDING FACTOR/DRE BINDING FACTOR1 Cascade and Freezing Tolerance in *Arabidopsis*. *The Plant Cell*, *25*(8), 2907–2924. https://doi.org/10.1105/tpc.113.112631

Hu, Y., Jiang, Y., Han, X., Wang, H., Pan, J., & Yu, D. (2017). Jasmonate regulates leaf senescence and tolerance to cold stress: Crosstalk with other phytohormones. *Journal of Experimental Botany*, *68*(6), 1361–1369. https://doi.org/10.1093/jxb/erx004

Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2006). Functional Analysis of Rice DREB1/CBF-type Transcription Factors Involved in Cold-responsive Gene Expression in Transgenic Rice. *Plant and Cell Physiology*, *47*(1), 141–153. https://doi.org/10.1093/pcp/pci230

Khush, G. S. (1997). Origin, dispersal, cultivation and variation of rice. *Plant Molecular Biology*, *35*(1), 25–34. https://doi.org/10.1023/A:1005810616885

Knoll, J., Gunaratna, N., & Ejeta, G. (2008). QTL analysis of early-season cold tolerance in sorghum. *Theoretical and Applied Genetics*, *116*(4), 577–587. https://doi.org/10.1007/s00122-007-0692-0

Li, W., Wang, R., Li, M., Li, L., Wang, C., Welti, R., & Wang, X. (2008). Differential Degradation of Extraplastidic and Plastidic Lipids during Freezing and Post-freezing Recovery in Arabidopsis thaliana*. *Journal of Biological Chemistry*, *283*(1), 461–468. https://doi.org/10.1074/jbc.M706692200

Lin, B. B. (2011). Resilience in Agriculture through Crop Diversification: Adaptive Management for Environmental Change. *BioScience*, *61*(3), 183–193. https://doi.org/10.1525/bio.2011.61.3.4

Liu, X., Ma, D., Zhang, Z., Wang, S., Du, S., Deng, X., & Yin, L. (2019). Plant lipid remodeling in response to abiotic stresses. *Environmental and Experimental Botany*, *165*, 174–184. https://doi.org/10.1016/j.envexpbot.2019.06.005

Lobell, D. B., Burke, M. B., Tebaldi, C., Mastrandrea, M. D., Falcon, W. P., & Naylor, R. L. (2008). Prioritizing Climate Change Adaptation Needs for Food Security in 2030. *Science*, *319*(5863), 607–610. https://doi.org/10.1126/science.1152339

Long, S. P., & Spence, A. K. (2013). Toward Cool C4 Crops. *Annual Review of Plant Biology*, *64*(1), 701–722. https://doi.org/10.1146/annurev-arplant-050312-120033

Lyons, J. M. (1973). Chilling Injury in Plants. *Annual Review of Plant Physiology*, *24*(1), 445–466. https://doi.org/10.1146/annurev.pp.24.060173.002305

Lyons, J. M., & Asmundson, C. M. (1965). Solidification of unsaturated/saturated fatty acid mixtures and its relationship to chilling sensitivity in plants. *Journal of the American Oil Chemists' Society*, *42*(12Part2), 1056–1058. https://doi.org/10.1007/BF02636905

Lyons, J. M., & Raison, J. K. (1970). Oxidative Activity of Mitochondria Isolated from Plant Tissues Sensitive and Resistant to Chilling Injury 1. *Plant Physiology*, *45*(4), 386–389.

Marla, S. R., Burow, G., Chopra, R., Hayes, C., Olatoye, M. O., Felderhoff, T., Hu, Z., Raymundo, R., Perumal, R., & Morris, G. P. (2019). Genetic Architecture of Chilling Tolerance in Sorghum Dissected with a Nested Association Mapping Population. *G3 Genes|Genomes|Genetics*, *9*(12), 4045–4057. https://doi.org/10.1534/g3.119.400353

Marla, S. R., Shiva, S., Welti, R., Liu, S., Burke, J. J., & Morris, G. P. (2017). Comparative Transcriptome and Lipidome Analyses Reveal Molecular Chilling Responses in Chilling-Tolerant Sorghums. *The Plant Genome*, *10*(3), plantgenome2017.03.0025. https://doi.org/10.3835/plantgenome2017.03.0025

Marozsán-Tóth, Z., Vashegyi, I., Galiba, G., & Tóth, B. (2015). The cold response of CBF genes in barley is regulated by distinct signaling mechanisms. *Journal of Plant Physiology*, *181*, 42–49. https://doi.org/10.1016/j.jplph.2015.04.002

McDonald, G. K., & Gill, G. S. (2009). Improving crop competitiveness with weeds: Adaptations and trade-offs. In *Crop Physiology: Applications for Genetic Improvement and Agronomy* (pp. 449–488). Academic Press.

Medina, J., Bargues, M., Terol, J., Pérez-Alonso, M., & Salinas, J. (1999). The Arabidopsis CBF Gene Family Is Composed of Three Genes Encoding AP2 Domain-Containing Proteins Whose Expression Is Regulated by Low Temperature but Not by Abscisic Acid or Dehydration1. *Plant Physiology*, *119*(2), 463–470. https://doi.org/10.1104/pp.119.2.463

Miedema, P., Post, J., & Groot, P. J. (1987). The effects of low temperature on seedling growth of maize genotypes. Pudoc.

Moellering, E. R., Muthan, B., & Benning, C. (2010). Freezing Tolerance in Plants Requires Lipid Remodeling at the Outer Chloroplast Membrane. *Science*, *330*(6001), 226–228. https://doi.org/10.1126/science.1191803

Moose, S. P., & Mumm, R. H. (2008). Molecular Plant Breeding as the Foundation for 21st Century Crop Improvement. *Plant Physiology*, *147*(3), 969–977. https://doi.org/10.1104/pp.108.118232

Mosier, A., Kroeze, C., Nevison, C., Oenema, O., Seitzinger, S., & van Cleemput, O. (1998). Closing the global N2O budget: Nitrous oxide emissions through the agricultural nitrogen cycle. *Nutrient Cycling in Agroecosystems*, *52*(2), 225–248. https://doi.org/10.1023/A:1009740530221

Murata, N., Ishizaki-Nishizawa, O., Higashi, S., Hayashi, H., Tasaka, Y., & Nishida, I. (1992). Genetically engineered alteration in the chilling sensitivity of plants. *Nature*, *356*(6371), 710–713. https://doi.org/10.1038/356710a0

Murata, T. (1969). Physiological and Biochemical Studies of Chilling Injury in Bananas. *Physiologia Plantarum*, *22*(2), 401–411. https://doi.org/10.1111/j.1399-3054.1969.tb07392.x

Nishida, I., & Murata, N. (1996). CHILLING SENSITIVITY IN PLANTS AND CYANOBACTERIA: The Crucial Contribution of Membrane Lipids. *Annual Review of Plant Physiology and Plant Molecular Biology*, *47*(1), 541–568. https://doi.org/10.1146/annurev.arplant.47.1.541

Nishiyama, Y., Allakhverdiev, S. I., & Murata, N. (2011). Protein synthesis is the primary target of reactive oxygen species in the photoinhibition of photosystem II. *Physiologia Plantarum*, *142*(1), 35–46. https://doi.org/10.1111/j.1399-3054.2011.01457.x

Ort, D. R. (2001). When There Is Too Much Light. *Plant Physiology*, *125*(1), 29–32. https://doi.org/10.1104/pp.125.1.29

Prasad, P. V. V., & Staggenborg, S. (2010). *Growth and Production of Sorghum and Millets*.

Qaim, M. (2020). Role of New Plant Breeding Technologies for Food Security and Sustainable Agricultural Development. *Applied Economic Perspectives and Policy*, *42*(2), 129–150. https://doi.org/10.1002/aepp.13044

Qin, F., Sakuma, Y., Li, J., Liu, Q., Li, Y.-Q., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2004). Cloning and Functional Analysis of a Novel DREB1/CBF Transcription Factor Involved in Cold-Responsive Gene Expression in Zea mays L. *Plant and Cell Physiology*, *45*(8), 1042–1052. https://doi.org/10.1093/pcp/pch118

Raison, J. K., Lyons, J. M., Mehlhorn, R. J., & Keith, A. D. (1971). Temperature-induced Phase Changes in Mitochondrial Membranes Detected by Spin Labeling. *Journal of Biological Chemistry*, *246*(12), 4036–4040. https://doi.org/10.1016/S0021-9258(18)62136-2

Raison, J. K., Lyons, J. M., & Thomson, W. W. (1971). The influence of membranes on the temperature-induced changes in the kinetics of some respiratory enzymes of mitochondria. *Archives of Biochemistry and Biophysics*, *142*(1), 83–90. https://doi.org/10.1016/0003-9861(71)90261-X

Raison, J. K., & Orr, G. R. (1990). Proposal for a better understanding of the molecular basis of chilling injury. In *Chilling Injury of Horticultural Crops* (pp. 146–161). CRC Press.

Raymundo, R., Sexton-Bowser, S., Ciampitti, I. A., & Morris, G. P. (2021). Crop modeling defines opportunities and challenges for drought escape, water capture, and yield increase using chilling-tolerant sorghum. *Plant Direct*, *5*(9), e349. https://doi.org/10.1002/pld3.349

Robertson, A. (1960). A theory of limits in artificial selection. *Proceedings of the Royal Society of London. Series B. Biological Sciences*, *153*(951), 234–249. https://doi.org/10.1098/rspb.1960.0099

Roughan, P. G. (1985). Phosphatidylglycerol and Chilling Sensitivity in Plants. *Plant Physiology*, *77*(3), 740–746.

Ruelland, E., Cantrel, C., Gawer, M., Kader, J.-C., & Zachowski, A. (2002). Activation of Phospholipases C and D Is an Early Response to a Cold Exposure in Arabidopsis Suspension Cells. *Plant Physiology*, *130*(2), 999–1007. https://doi.org/10.1104/pp.006080

Salas Fernandez, M. G., Schoenbaum, G. R., & Goggi, A. S. (2014). Novel Germplasm and Screening Methods for Early Cold Tolerance in Sorghum. *Crop Science*, *54*(6), 2631–2638. https://doi.org/10.2135/cropsci2014.01.0025

Santino, A., Taurino, M., De Domenico, S., Bonsegna, S., Poltronieri, P., Pastor, V., & Flors, V. (2013). Jasmonate signaling in plant development and defense response to multiple (a)biotic stresses. *Plant Cell Reports*, *32*(7), 1085–1098. https://doi.org/10.1007/s00299-013-1441-2

Shahin Hassanpour, Naser Maheri-Sis, Behrad Eshratkhah, & Farhad Baghbani Mehmandar. (2011). Plants and secondary metabolites (Tannins): A Review. *International Journal of Forest, Soil and Erosion*, *1*(1), 47–53.

Shneyour, A., Raison, J. K., & Smillie, R. M. (1973). The effect of temperature of the rate of photosynthetic electron transfer in chloroplasts of chilling-sensitive and chilling-resistant plants. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *292*(1), 152–161. https://doi.org/10.1016/0005-2728(73)90259-4

Staggenborg, S. A., Dhuyvetter, K. C., & Gordon, W. B. (2008). Grain Sorghum and Corn Comparisons: Yield, Economic, and Environmental Responses. *Agronomy Journal*, *100*(6), 1600–1604. https://doi.org/10.2134/agronj2008.0129

Sthapit, B. R. (1992). CHILLING INJURY OF RICE CROP IN NEPAL: A REVIEW. *J. Inst. Agric. Anim. Sci.*, *13*, 1–32.

Stickler, F. C., Pauli, A. W., & Casady, A. J. (1962). Comparative Responses of Kaoliang and Other Grain Sorghum Types to Temperature1. *Crop Science*, *2*(2), cropsci1962.0011183X000200020015x. https://doi.org/10.2135/cropsci1962.0011183X000200020015x

Stockinger, E. J., Gilmour, S. J., & Thomashow, M. F. (1997). Arabidopsis thaliana CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat$\overline{\text{D}}$RE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl. Acad. Sci. USA*, 6.

Taylor, A. O., & Rowley, J. A. (1971). Plants under Climatic Stress: I. Low Temperature, High Light Effects on Photosynthesis. *Plant Physiology*, *47*(5), 713–718. https://doi.org/10.1104/pp.47.5.713

Upchurch, R. G. (2008). Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnology Letters*, *30*(6), 967–977. https://doi.org/10.1007/s10529-008-9639-z

Vida, F. B. P. de, Laca, E. A., Mackill, D. J., Fernández, G. M., & Fischer, A. J. (2006). Relating rice traits to weed competitiveness and yield: A path analysis. *Weed Science*, *54*(6), 1122–1131. https://doi.org/10.1614/WS-06-042R.1

Wang, F., Guo, Z., Li, H., Wang, M., Onac, E., Zhou, J., Xia, X., Shi, K., Yu, J., & Zhou, Y. (2016). Phytochrome A and B Function Antagonistically to Regulate Cold Tolerance via Abscisic Acid-Dependent Jasmonate Signaling. *Plant Physiology*, *170*(1), 459–471. https://doi.org/10.1104/pp.15.01171

Wang, X., Li, W., Li, M., & Welti, R. (2006). Profiling lipid changes in plant response to low temperatures. *Physiologia Plantarum*, *126*(1), 90–96. https://doi.org/10.1111/j.1399-3054.2006.00622.x

Welti, R., Li, W., Li, M., Sang, Y., Biesiada, H., Zhou, H.-E., Rajashekar, C. B., Williams, T. D., & Wang, X. (2002). Profiling Membrane Lipids in Plant Stress Responses: ROLE OF PHOSPHOLIPASE Dα IN FREEZING-INDUCED LIPID CHANGES IN ARABIDOPSIS*. *Journal of Biological Chemistry*, *277*(35), 31994–32002. https://doi.org/10.1074/jbc.M205375200

Yang, D.-L., Yao, J., Mei, C.-S., Tong, X.-H., Zeng, L.-J., Li, Q., Xiao, L.-T., Sun, T. -p., Li, J., Deng, X.-W., Lee, C. M., Thomashow, M. F., Yang, Y., He, Z., & He, S. Y. (2012). Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. *Proceedings of the National Academy of Sciences*, *109*(19), E1192–E1200. https://doi.org/10.1073/pnas.1201616109

Yu, J., & Tuinstra, M. R. (2001). Genetic Analysis of Seedling Growth under Cold Temperature Stress in Grain Sorghum. *Crop Science*, *41*(5), 1438–1443. https://doi.org/10.2135/cropsci2001.4151438x

Zeder, M. A. (2011). The Origins of Agriculture in the Near East. *Current Anthropology*, *52*(S4), S221–S235. https://doi.org/10.1086/659307

Zhang, B., & Hülskamp, M. (2019). Evolutionary Analysis of MBW Function by Phenotypic Rescue in Arabidopsis thaliana. *Frontiers in Plant Science*, *10*, 375. https://doi.org/10.3389/fpls.2019.00375

Zhang, M., Barg, R., Yin, M., Gueta-Dahan, Y., Leikin-Frenkel, A., Salts, Y., Shabtai, S., & Ben-Hayyim, G. (2005). Modulated fatty acid desaturation via overexpression of two distinct ω-3 desaturases differentially alters tolerance to various abiotic stresses in transgenic tobacco cells and plants. *The Plant Journal*, *44*(3), 361–371. https://doi.org/10.1111/j.1365-313X.2005.02536.x

CHAPTER II: THE MAJOR CHILLING TOLERANCE LOCUS IN SORGHUM q*SbCT04.62* IS NOT A PLEIOTROPIC EFFECT OF *TANNIN1*

Introduction

As the developed world faces emerging challenges that threaten agricultural productivity and food security, including climatic instability, degradation of soils, and water shortages (Berry et al., 2015; Wheeler & von Braun, 2013), crop adaptation is an important tool for developing solutions (Maggio et al., 2015). Chilling tolerance is a trait with the potential to make an impact on food security. Breeding more chilling tolerant crops can improve agricultural sustainability by increasing crop yields under abiotically limiting conditions such as cold, drought, and poor soil. Chilling tolerant crops are planted earlier in the spring, increasing yield through a lengthened growing season and better-aligning crops' evapotranspirative needs with environmental patterns (Long & Spence, 2013; Raymundo et al., 2021). Early planting of chilling tolerant crops can also minimize nitrogen loss by limiting runoff and N_20 emissions through reduced fallow periods (Mosier et al., 1998).

Sorghum is a tropical-origin crop that is important for agricultural sustainability. It is the fourth most produced cereal crop and is an economically important commodity that is widely cultivated, particularly in semi-arid environments (Monk et al., 2014). Historically, breeding for chilling tolerance in sorghum has been unsuccessful. There is little chilling tolerance variation in commercial breeding populations which requires breeders and geneticists to introgress chilling tolerance from exotic germplasm, which has genetic backgrounds incompatible with elite breeding populations (Franks et al., 2006).

Molecular genetics has provided new technologies to acquire useful alleles from exotic genetic backgrounds (Moose & Mumm, 2008). Linkage and association mapping can identify

loci associated with a particular trait (QTL). Positive alleles of the QTL can then be selected using molecular markers. Phenotyping is only required in the initial trait mapping, which reduces the time and cost of bringing the trait to fixation in a population. Further, it allows breeders to select for the trait in isolation and minimize the introgression of unfavorable background genetics (i.e. linkage drag) (Knoll & Ejeta, 2008).

Multiple chilling tolerance mapping studies in sorghum have shown a complex genetic architecture (Burow et al., 2011; Knoll et al., 2008; Moghimi et al., 2019; Ortiz et al., 2017). A more recent study using joint linkage mapping (JLM) in large nested association mapping (NAM) families further identified several co-localizations between QTL and genes regulating grain tannins (*Tannin1* and *Tannin2)* and height (*Dw1* and *Dw3*) (Marla et al., 2019). In particular, *qSbCT04.62*, a major effect QTL, co-localizes precisely with *Tannin1*, a canonical grain tannin regulator (Wu et al., 2012), with the peak SNP from JLM less than 50 kb from the *Tannin1* gene (Sobic.004G280800; Marla et al., 2019). Unfortunately, the colocalization is antagonistic, causing grain tannins, a commercially unacceptable trait, to be co-inherited with the chilling tolerance allele. In contrast, tannin-free grain is inherited with the chilling susceptible allele. Furthermore, this association between *Tan1* and cold tolerance is unlikely to be due to

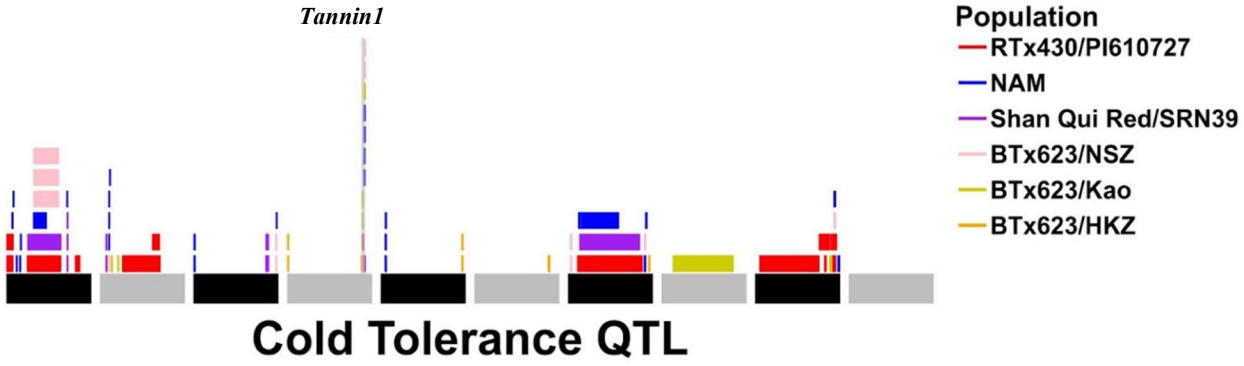

Figure 2.1. QTL from NAM and biparental mapping studies for cold tolerance in sorghum (Burow et al 2011, Marla et al 2019, Knoll et al 2008). *Tannin1* location on chromosome 4 is shaded. Data is from the Sorghum QTL Atlas (Mace et al 2019).

chance as it is seen across mapping studies spanning multiple years and environments (Fig. 2.1; Burow et al., 2011, Marla et al., 2019, Knoll., et al 2008). Notably, *Tannin1* is a sorghum ortholog of Arabidopsis *TTG1* (Wu et al., 2012), the WD40 subunit of the MBW regulatory complex, and a master regulator of epidermal traits (Tian & Wang, 2020). *TTG1* is known to be a major pleiotropic regulator of flavonoid biosynthesis, root hair and trichome development, and the presence of seed coat mucilage. It is unknown if *Tan1* shares any of these functions beyond the regulation of seed proanthocyanidins and what pleiotropic effects *Tan1* might have on commercial sorghum cultivars besides promoting grain tannins.

The association between chilling tolerance and grain tannins in sorghum could be explained by either of two competing hypotheses, each with different consequences for breeding. If the association is caused by linkage between *Tannin1* and the *qCT04.62* causal variant (quantitative trait nucleotide; QTN), then it is possible through recombination to break the linkage and use the trait for breeding. Alternatively, if the association is based on pleiotropic control of both traits by *Tan1,* the allele will be unusable by breeders, and the path forward is more complex. To test these hypotheses, NILs were created using marker assisted selection (MAS) to introgress *Tan1* and part of *CT04.62+* into a chilling sensitive sorghum background. RILs from the HKZ-BTx623 NAM family were used as starting material and then further backcrossed to BTx623 (Marla et al., 2023). In this study, we used the NILs to run genetically controlled experiments to elucidate the genotype to phenotype relationship between *Tan1* and chilling tolerance (Fig. 2.2). Though the NIL+ vs NIL- were segregating for grain tannins and functional/non-functional *Tan1* alleles, we found this to have no measurable effect on chilling tolerance. For this reason, we suspect the *qCT04.62* QTN was not introgressed during the NIL creation. This finding supports the linkage hypothesis and provides breeders with a proof of

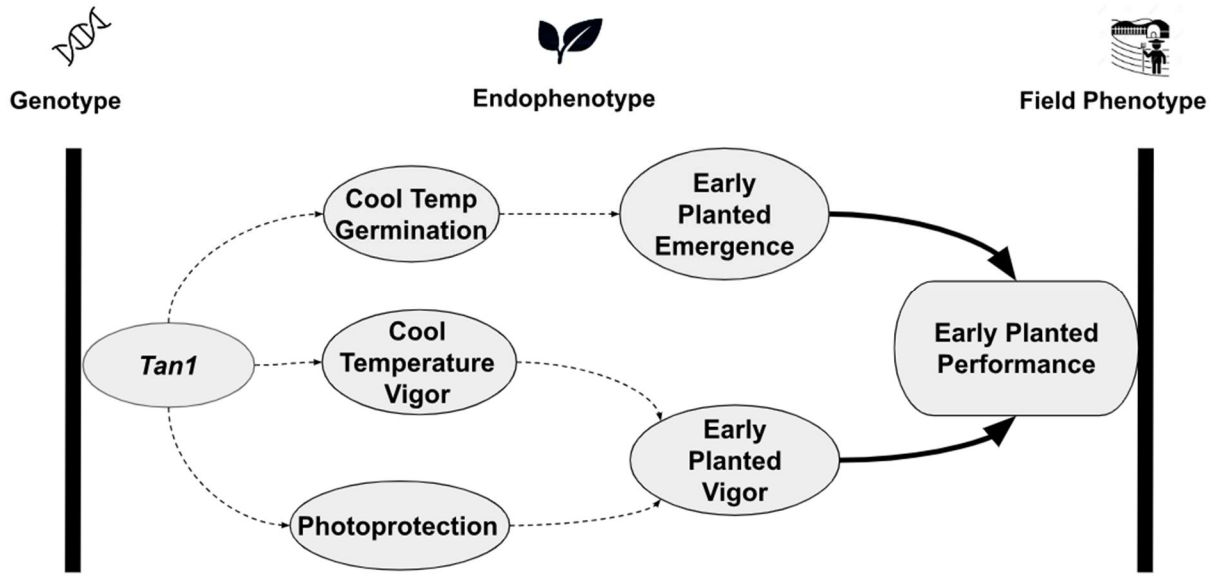

Figure 2.2. Genotype to phenotype map showing hypothetical associations between *Tan1* and early planted performance in sorghum.

concept for breaking the *Tan1* chilling tolerance linkage. Though field validation is still needed, these results allow scientists to unlock *CT04.62+* for breeding, develop more chilling tolerant sorghum varieties, and take a step towards creating more resilient agricultural systems.

Results

NILs are heterogeneous at Tannin1 and part of qCT04.62 but homogenous at other loci.

To characterize the positive and negative NIL haplotypes for each NIL family, the introgression of HKZ DNA into the BTx623 background was assessed using low-coverage genome resequencing. After filtering for high-quality biallelic SNPs, a genome-wide average of 3500 SNPs per Mb were used in genotyping across NILs and parental lines (Fig. S1). In NIL 1, 2, and 3 families, there is under 5% genomic segregation between biological replicates, and also, under 5% of the total genome originates from HKZ outside the introgressions. Conversely, in the NIL4 family, one NIL4+ individual appears heterozygous for most of chromosome 10 (Fig. 2.3).

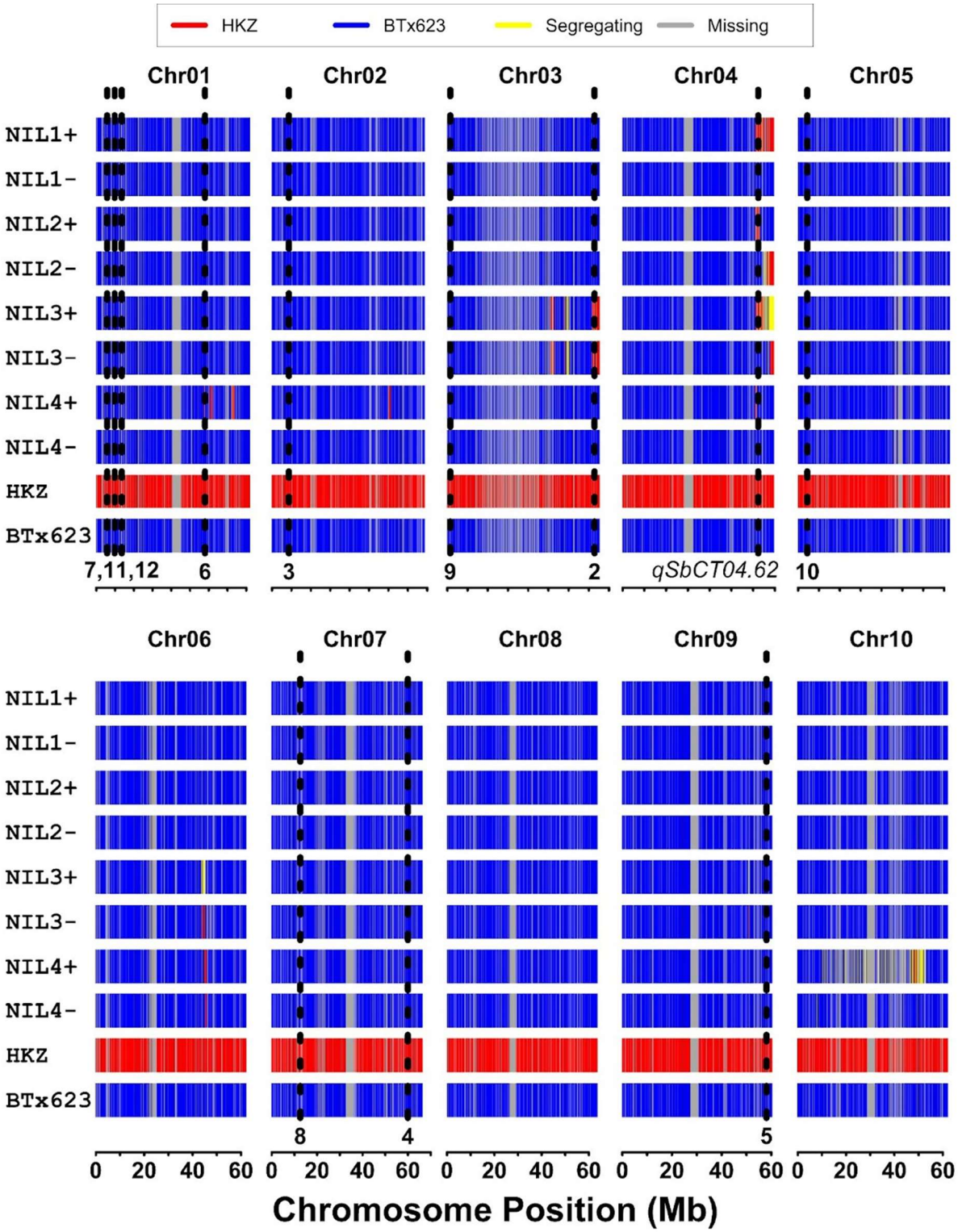

Figure 2.3. Whole genome haplotype analysis of NILs selected for and against HKZ haplotype at *qCT.4-62*. Sliding window scan of low coverage genotype data at 10 kb resolution. Visualization is of alternate allele number relative to HKZ for two individuals per genotype. Red is $alternate^{x}/alternate^{HKZ} >= 0.5$, purple is $0.2 < alternate^{x}/alternate^{HKZ} < 0.5$, yellow is when color call differs between genotype replicates; grey is missing data. Dotted lines are chilling tolerance QTL (Marla et al, 2019; Table 2.1).

In NILs, 1+, 2+, and 3+ genomic regions ranging in size from 3-15 Mb were introgressed from the recurrent parent and encompassed *Tannin1* (Chr04: 62,315,396), a sorghum CBF ortholog (Chr04: 62,486,634) of rice chilling tolerance regulator *OsDREB1G* (Moon et al., 2019), peak

Table 2.1. Chilling tolerance QTL from Marla et al., 2019. Visualized in Fig. 2.3.

Index	QTL	Chrom	Position (bp)
1	qSbCT04.62	Chr04	62,368,531
2	qSbEPEC_3-72	Chr03	72,791,601
3	qSbCT02.08	Chr02	8,672,301
4	qSbCT07.59	Chr07	59,915,577
5	qSbCT09.57	Chr09	58,070,153
6	qSbCT01.57	Chr01	57,941,435
7	qSbCT01.06	Chr01	5,730,743
8	qSbCT07.10	Chr07	12,580,350
9	qSbEPSV1_3-01	Chr03	1,447,612
10	qSbCT05.04	Chr05	4,403,613
11	qSbCT01.13	Chr01	13,526,795

SNPs from chilling tolerance JLM (S4_62368531, S4_62455479), and ~25% of *qCT04.62* confidence interval from single linkage mapping in the HKZ × BTx623 NAM family. NIL 4+ has a smaller introgression of about 1 Mb, which also includes ~25% of *qCT04.62* CI but lacks *Tannin1*, JLM peak SNPs, or the *CBF* ortholog (Fig. 2.4 A&B).

To test whether any known chilling tolerance QTL besides *qCT04.62* are also segregating between the NIL pairs, we plotted chilling tolerance QTL identified by Marla et al., 2019 over the introgression data (Fig. 2.3). Besides chromosome 10 in NIL4+, there appear to be few HKZ introgressions in the NILs, and none overlapping with CT QTL, with the exception of the NIL3 family, where one small introgression on chromosome 3 spans the early season emergence QTL *qEPEC.3-72* in both NIL+ and NIL- individuals (Fig. 2.3, Table 2.1). Further, NIL families 1-3 segregate for functional/non-functional alleles of *Tan1* between the positive and negative lines,

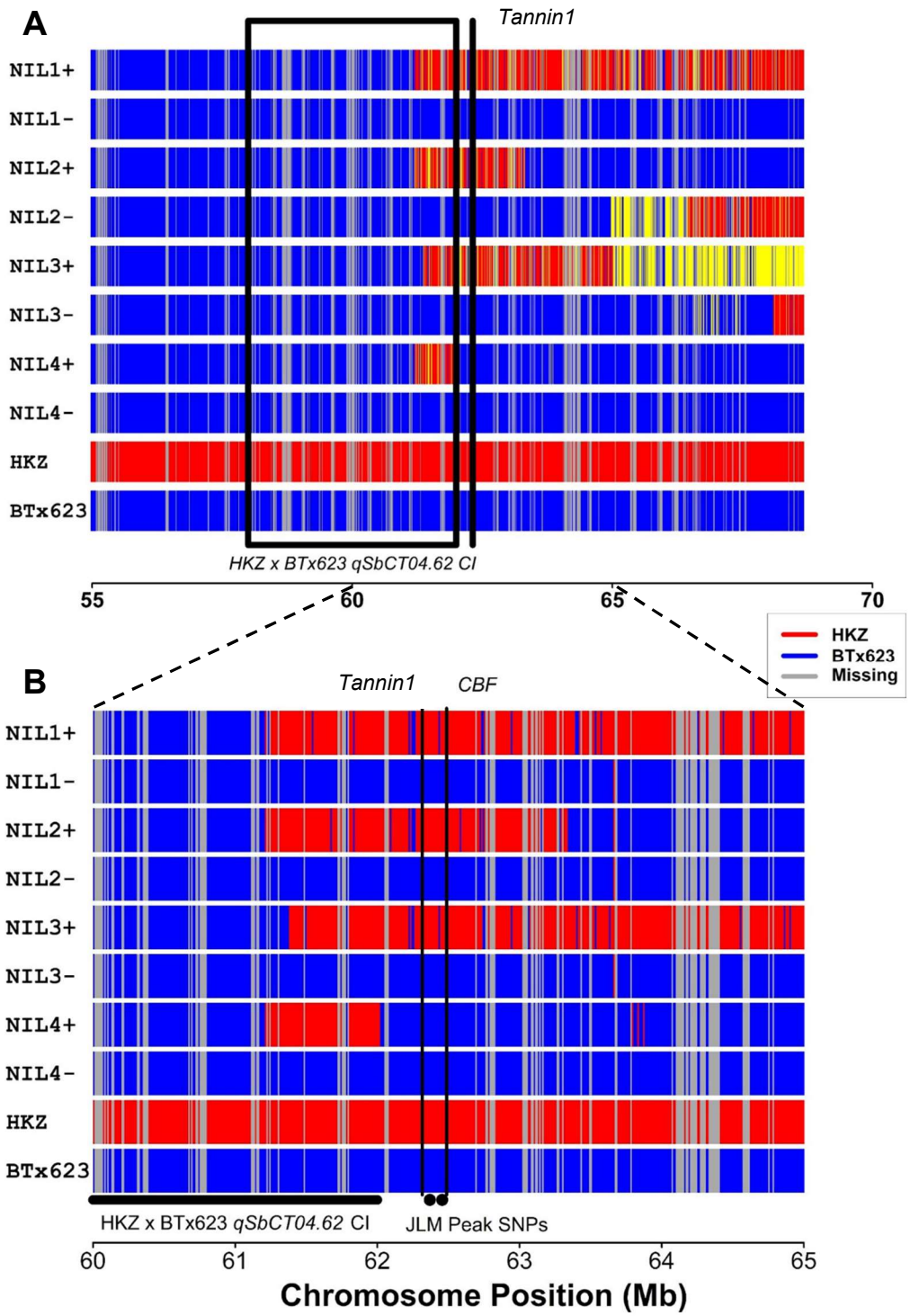

Figure 2.4. Targeted haplotype analysis of NILs selected for and against HKZ haplotype at *qCT.4-62*. (**A**) High resolution view of *qCT4.64* and introgression site on chromosome 4, 55–65Mb. *Tannin1* location is denoted by black line. Confidence interval for *qCT04.62* in HKZ NAM family is denoted by a black box. (**B**) High resolution view of region surrounding *Tannin1*. Black lines are genes (*CBF* and *Tannin1*) and dots are peak SNPS from JLM as identified by Marla et. al., 2019. *qCT04.62* confidence interval is denoted by horizontal black bar.

while the NIL4 family appears fixed for *tan1b*. Overall, the segregation patterns of all NIL families establish their usefulness for testing the *Tan1* linkage-pleiotropy hypothesis.

The presence of tannins reveals heterozygosity at Tannin1 in NIL4+ progenitor

To validate the genotyping and test for homozygosity at *Tan1,* a bleach test was used to test for the presence of grain tannins (Fig. 2.5). As expected, all NIL- lines were tannin deficient and fixed for the *tan1b* allele. In NIL1-3+, tannins were present in all seeds, consistent with genotypic predictions. In NIL4+, we expected fixation for the *tan1b* allele but observed segregation for tannin+/tannin- seeds, suggesting heterozygosity at *Tannin1* in NIL4+'s direct progenitor (Fig. 2.5). This finding prompted us to exclude the NIL4 family from further experimentation to avoid confounding effects from segregating *Tannin1* alleles.

Tan1 does not regulate low-temperature germination.

Previous mapping studies in the field showed that *qCT04.62* regulates early season emergence in the field (Burow et al., 2011; Knoll et al., 2008; Marla et al., 2019). To test this hypothesis in controlled laboratory conditions, we conducted low-temperature germination tests in BTx623, HKZ, and NIL 1 and 2 families. The NIL3 family was excluded from this test to avoid confounding effects from the introgression of *qEPEC.3-72*. At 15°C there was no germination on day one and no significant genotypic differences on days two and three, but a highly significant genotypic effect (25%; $p < 10^{-4}$) on day 4 with HKZ and DKS38-16 having lower overall germination after four days than BTx623 or either NIL + or - which all grouped (Fig. 2.6A). At 20°C, there is a significant genotypic effect on day one ($p = 0.01$), with HKZ having a higher germination rate than BTx623, NILs, or DKS38-16 (BTx623: 16%, NILs: 8%, HKZ: 25%). Again, the NILs group together. Further, no genotypic differences between lines during days 2-4 (Fig. 2.6B; $p = 0.5$-0.8). Again,

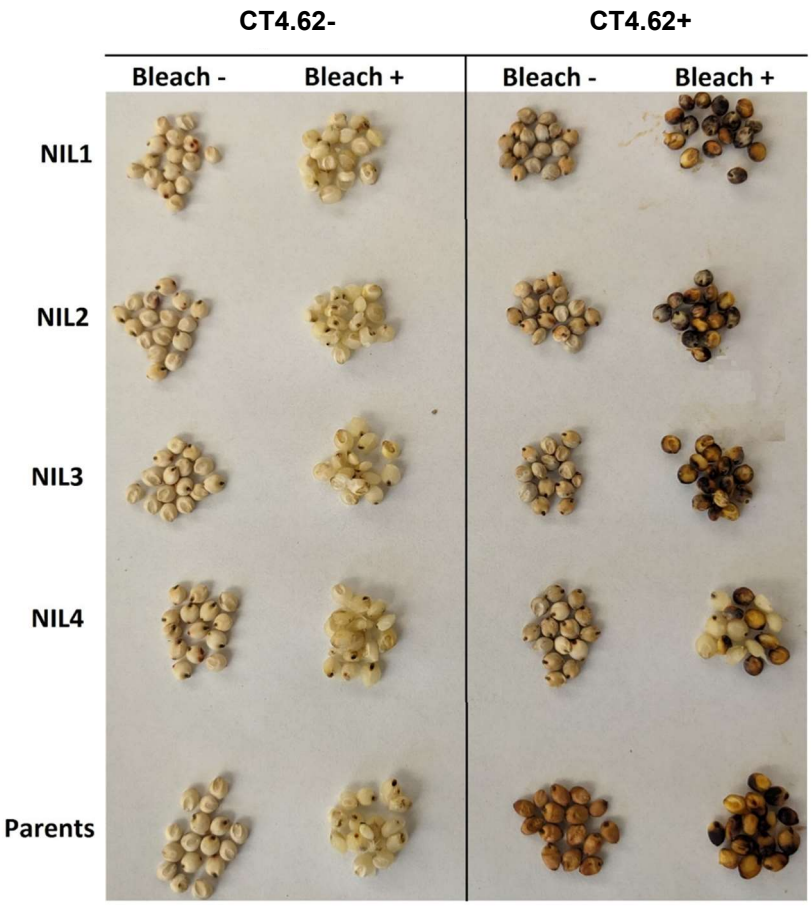

Figure 2.5. Bleach test for presence of grain tannins in NILs. NILs are organized vertically by family with parents as controls at bottom. QTL +/- denotes selection for CT4.64+ or CT4.64- in NIL families and the allele itself in parents. Tannin containing seeds darken when soaked in NaOH/bleach solution, while non-tannin seeds become white/yellow.

at 25° there is a significant genotype effect on days one and two ($p < 10^{-4}$), with HKZ having 25% greater germination (Fig. 2.6C). Overall, slower germination of HKZ at 15°C combined with faster germination at higher temps suggests genotypic control of temperature-dependent germination in HKZ and BTx623. Additionally, the lack of significant differences between NILs and the persistent grouping of both NILs with BTx623 further suggests that these effects are not regulated by *pCT04.62+/Tan1*.

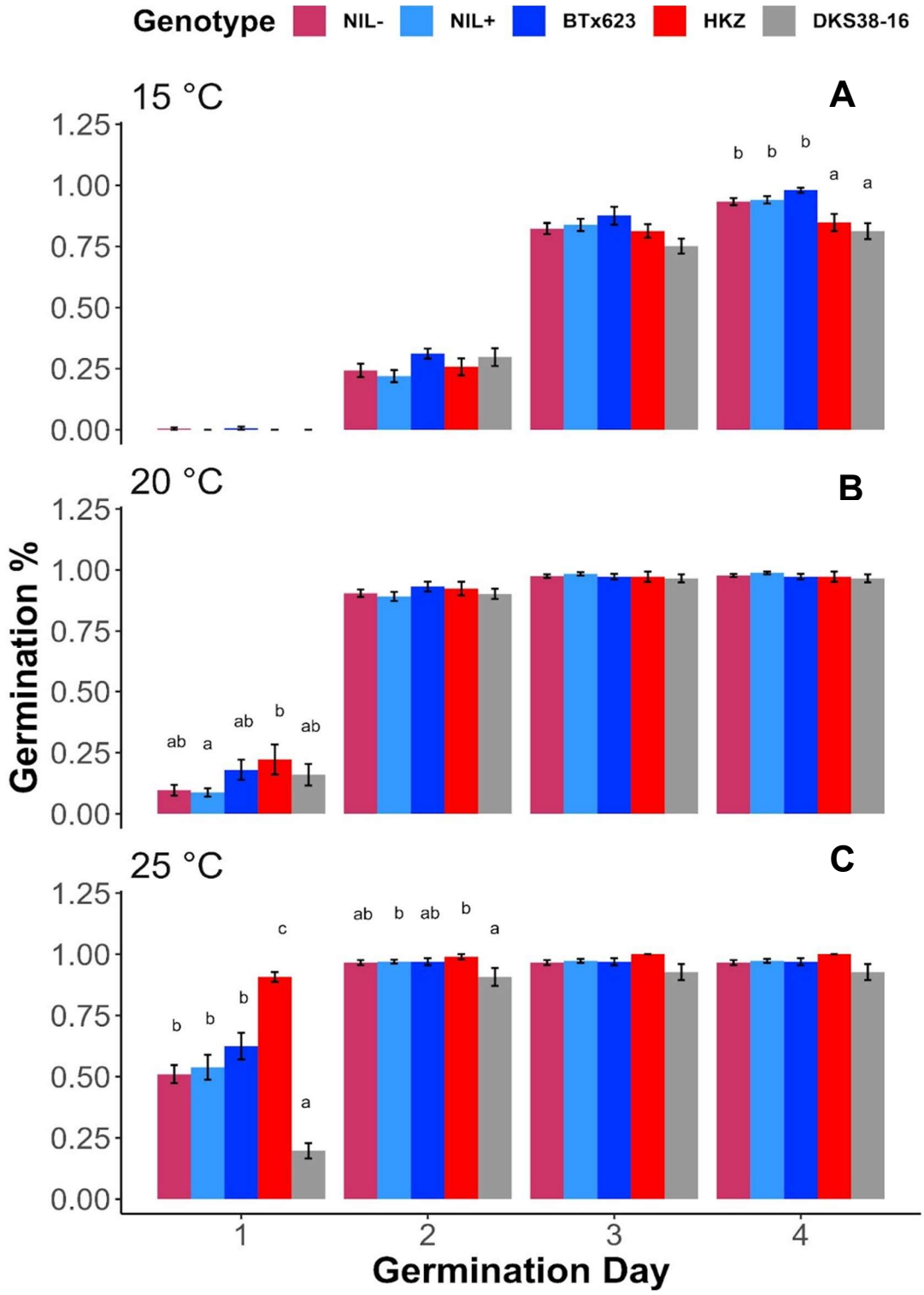

Figure 2.6. The effect of chilling on germination in NILs and parent lines. NIL+ and NIL- are means from NIL 1 and 2 families. DKS38-16 is a commercial hybrid. Error bars span +/- one standard error (**A**) Germination rate at 15°C. Day 3 *** (**B**) Germination rate at 20°C. Day 1 * (**C**) Germination rate at 25°C. Day 1 ***, Day 2 *. Significance codes: $p < 0.001$ = ***, $p < 0.01$ = **, $p < 0.05$ = *. Pairwise comparisons were made using Tukey HSD. DKS38-16 is a commercial hybrid used as a positive control for germination.

No genotype by environment interaction on growth under chilling per se

To better understand the relationship between early planted vigor and chilling tolerance per se and to test the hypothesis that we introgressed the causal factor underlying the regulation of chilling tolerance by *qCT.04-62*, we subjected NILs and parents to testing under controlled chilling conditions. During month-long temperature treatments, all lines exhibit significant treatment effects ($p < 10^{-4}$) but lack significant genotype or genotype by treatment (GxT) effect for dry weight ($p = 0.3$, $p = 0.5$). However, HKZ has a slightly higher mean weight (1.5 g) than other lines, though this is non-significant (Fig. 2.7A; $p = 0.1$–0.3). Conversely, a three-day cold shock treatment with a week-long recovery resulted in significant treatment and genotype effects ($p < 10^{-4}$, $p < 10^{-4}$), but not GxT (Fig. 2.7B; $p = 0.07$). Compared to other tested genotypes, the genotype effects are driven primarily by vigorous HKZ size gain during warm temperature phases for both chilled and control HKZ plants. Overall, HKZ has a faster growth rate when

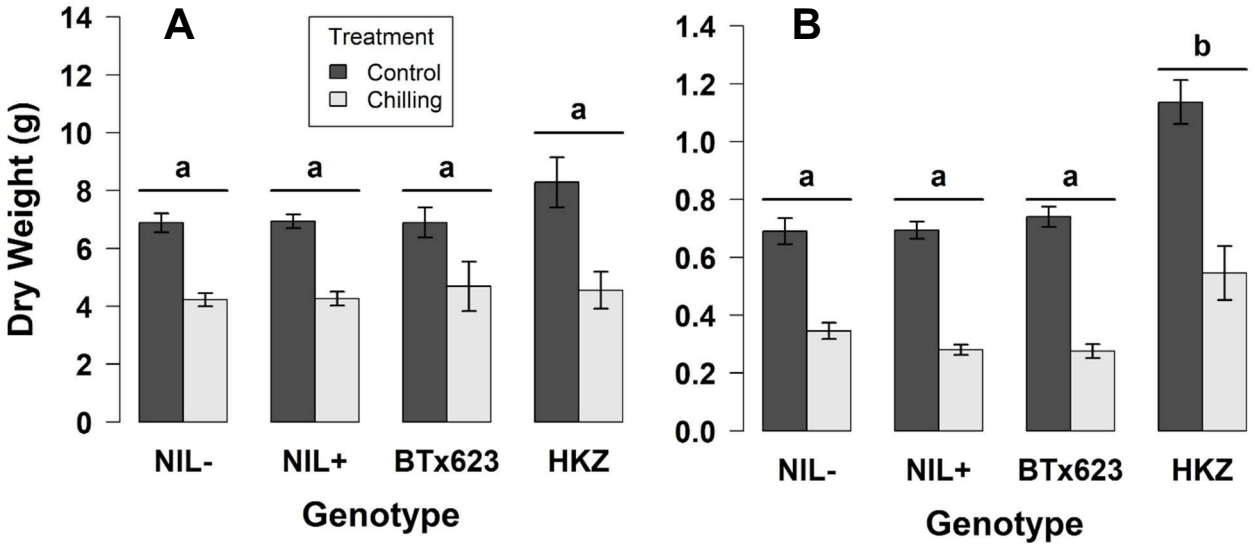

Figure 2.7. The effect of chilling *per se* on dry weight in NILs and parent lines. Error bars span +/- one standard error. NIL data are means of families 1-3. (**A**) Five day old seedlings subjected to month long chilling treatment. Genotype effect $p > 0.05$, Treatment effect *** $p < 0.001$, Genotype x Treatment effect $p > 0.05$ (**B**) One week old seedlings subjected to three day chilling shock and seven day recovery. Genotype effect *** $p < 0.001$, Treatment effect *** $p < 0.001$, Genotype x Treatment effect $p > 0.05$. The *p*-values were calculated using two-way ANOVA. Pairwise comparisons were made using Tukey HSD.

compared to BTx623 or NILs (33%) but does not perform better than expected under chilling conditions.

Comparison of photosynthetic parameters under chilling reveals photoprotection between NILs

To test the hypothesis that *pCT04.62/Tan1* governs chilling tolerance through the regulation of NPQ, plants were subjected to a time course analysis of photosynthetic parameters under controlled-environment chilling stress. At the same time, the photochemical function was fluorometrically tracked for significant differences across genotypes. The baseline for each photosynthetic measurement was established day one before chilling stress (Fig. 2.8 A-C). There were significant differences among genotypes for PhiNO and PhiNPQ (Fig. 2.8 A-B). NPQ, a central photoprotection pathway, regulates the dispersal of excess light energy as heat. Under chilling, all genotypes exhibit increased NPQ, measured as PhiNPQ, with kaoliangs exhibiting consistently higher levels than BTx623 across all days, with statistical significance days seven, eight and nine post-chilling ($p < 10^{-4}$, $p < 10^{-4}$, $p < 10^{-4}$). Further, BTx623 returned to baseline during the recovery period, while HKZ became elevated from baseline on days eight and nine, suggesting a post-chilling reaction to chilling stress in HKZ. Both NILs grouped with BTx623 across all days and did not exhibit the HKZ's adaptive capacity (Fig. 2.8A). Non-regulated light energy dissipation, a central photodamage driver, was measured here as PhiNO. This data reinforces the trends uncovered when measuring NPQ. Across all days, HKZ sustains the lowest levels of PhiNO, while both NILs remain grouped with BTx623. Significant differences exist on days one, eight, and nine (-20%, -30%, -45%; $p = 0.02$, $p < 10^{-4}$, $p < 10^{-4}$). On day one, HKZ shows relatively lower levels of PhiNO. During chilling, all lines have decreased PhiNO, corresponding with increases in NPQ, with HKZ exhibiting the lowest levels.

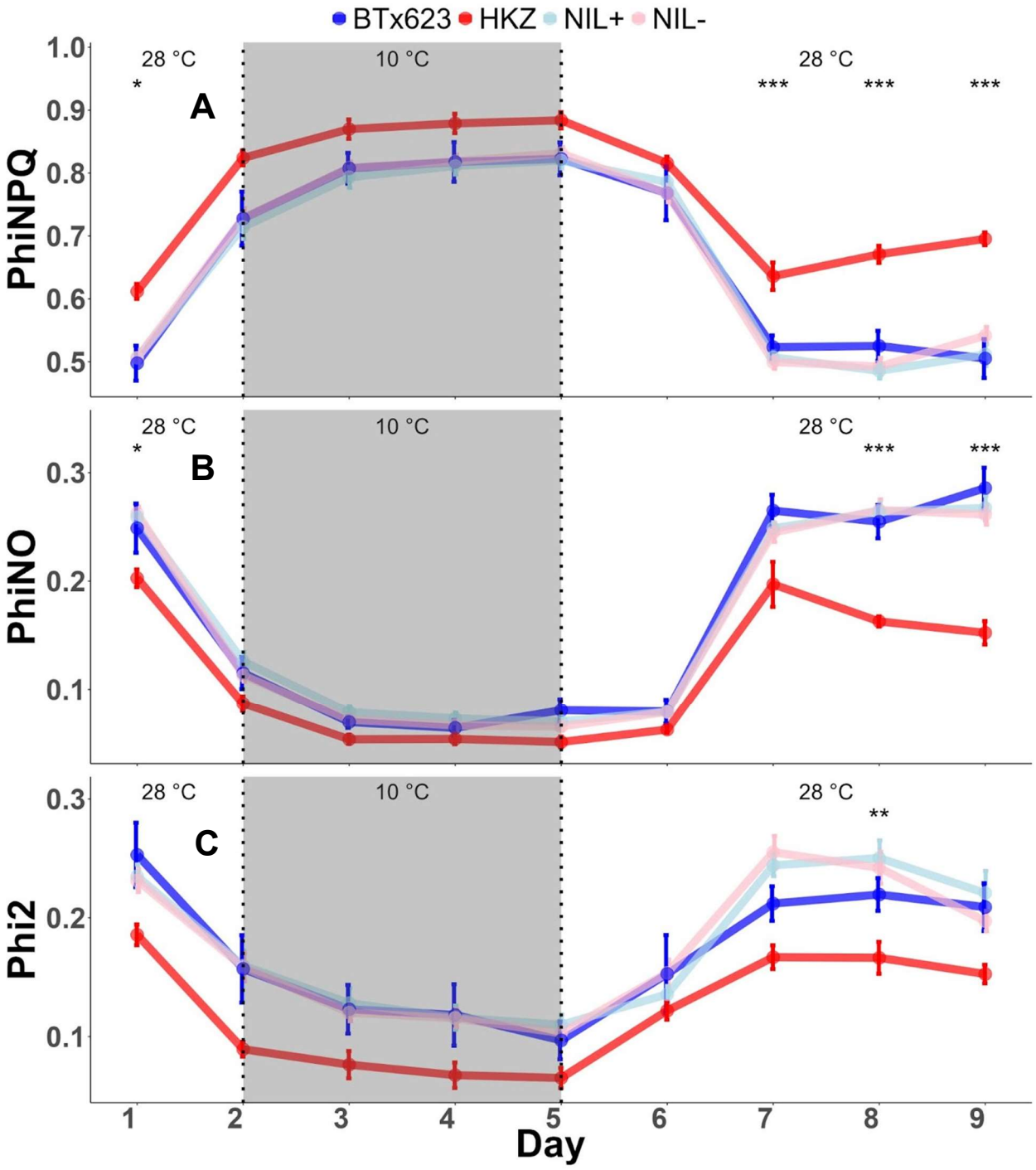

Figure 2.8. Photosynthetic parameters under chilling and recovery for NILs and parent lines. Shaded area represents chilling conditions at 10°C while non-shaded area represents control conditions at 28°C. Significant difference among genotypes is measured by 1 way ANOVA for each day independently and is denoted: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PhiNPQ, PhiNO, and Phi2 measurements were taken simultaneously using a MultiSpeQ fluorometer. NIL data are means of families 1-3. (**A**) PhiNPQ (**B**) PhiNO (**C**) Phi2.

During recovery on days six-nine, BTx623 and NILs are slightly elevated from baseline, while HKZ drops below baseline. This data also suggests that HKZ sustains decreased levels of photodamage at all points of the time course analysis (Fig. 2.8B). Interestingly, total input into photosynthesis, measured as phi2, was lower in HKZ relative to BTx623 and NILs (0.05-0.1) with statistical significance on day 8 (p=0.001). Both NIL lines are consistently grouped with BTx623. Phi2 dramatically decreased in all lines under chilling, with HKZ at about half the rate of BTx623 (Fig. 2.8C). Overall, HKZ shows a more reserved photosynthetic strategy which prioritizes protection over maximum photosynthetic capacity, a trend which is amplified upon exposure to chilling stress.

Discussion

NILs as a genetic resource to study Tannin1

In this study, we used NILs to investigate the association between *Tannin1* and *qCT04.62* and understand whether it is based in linkage or pleiotropy. NILs can be a powerful tool in forward genetics research, particularly when paired with high-resolution genomic data. Here, whole genome re-sequencing of the NILs (Fig. 2.3) shows very little segregation between lines, which allows us to be confident of results and exclude lines from experiments with off-target introgressions, which may have confounding effects from other QTL, ex. NIL3 family from germination tests. From the genomic data, we could also identify the size of on-target introgressions in NIL+ and examine what pertinent genetic features are included (Fig. 2.4). This detailed genomic assessment already allows us to begin excluding specific candidate genes and is a starting point for continued fine mapping at this locus.

With the NILs, we were able to mendelize the effect of *pCT04.62+/Tan1* to run controlled genetic experiments on the introgressions function in chilling tolerance.

Mendalization is an effective forward genetics strategy for mapping genotype to phenotype in complex traits. In this case, we can only narrow the overall understanding of *qCT04.62* regulatory function as *pCT04.62+/Tan1* had no apparent regulatory effect on chilling tolerance (Fig. 2.6, Fig. 2.7, Fig. 2.8), which does not functionally correspond to *qCT04.62.* This remains useful information and provides a foundation to build on for future functional genetic experiments.

pCT04.62+/Tan1 likely does not contain the QTN driving chilling tolerance in qCT04.62

From these experiments (Fig. 2.4; Fig. 2.6; Fig. 2.7; Fig. 2.8), it is likely that the NIL+ are not chilling tolerant as expected with the introgression of the NAM peak SNPS and functional *Tannin1.* In all assays performed, NIL+ showed no evidence for induction of a response indicative of chilling tolerance, though we did see differing chilling responses between BTx623 and HKZ, the positive and negative controls for chilling tolerance (Fig. 2.6 A-C, Fig. 2.7B, Fig. 2.8 A-C). HKZ exhibits more rapid germination and initial growth than BTx623, but surprisingly, in response to chilling stress, HKZ seems to have reduced growth, germination, and photosynthesis. This trade-off of growth may be a protective mechanism that allows the plant to avoid exacerbating stress under poor growing conditions. In the case of photosynthesis, this strategy likely leads to a reduction in photosynthetic input and a reduction in photodamage.

These limited observations show that HKZ employs different responses to chilling, though it is difficult to conclude the overall mechanism. While HKZ displayed phenotypes suggestive of chilling tolerance for vigor, germination, and photosynthesis, NIL+ and NIL- lines, which are in BTx623 genetic background, behave nearly identically to each other and BTx623 (Fig. 2.6 A-C, Fig. 2.7B, Fig. 2.8 A-C). This result is unexpected if the QTN was within *pCT04.62/Tan1* as *qCT04.62* underlay 17% of the phenotypic variation in the HKZ family in

field trials for early season vigor (Marla et al., 2019). These conclusions are further strengthened by the genotyping data, which shows that only 25% of the *qCT04.62* HKZ confidence interval was introgressed with *pCT04.62/Tan1* (Fig. 2.4A), which makes it possible that the QTN driving chilling tolerance regulation was not included in the introgression leaving all NIL lines homozygous for the BTx623 allele.

It is also possible that the chilling tolerance QTN was introgressed. One explanation is that *CT04.62* functions epistatically with another QTL that was not introgressed, rendering *CT04.62* non-functional in the NILs. Also, the controlled environment assays may be insufficient to elucidate the chilling tolerance mechanism regulated by *CT04.62* under field conditions. Natural environments are extremely temporally variable for temperature and light, two critical factors in chilling stress (Taylor & Rowley, 1971). The chilling tolerance function of *qCT04.62* may be induced by environmental variability of these or other factors, which would result in the negative results we observed. It is also possible that the light intensity and temperature were insufficient to provide adequate stress. The experimental light intensity was set at 700 μmol m^{-2} $s^{-1,}$ which is relatively low compared to natural sunlight, which can exceed 1500 μmol m^{-2} s^{-1} on sunny summer days (Bilger et al., 1995).

Another explanation is that *qCT04.62* does not regulate chilling tolerance *per se* but instead regulates early-season vigor independently of chilling tolerance. The region around *qCT04.62* has been associated with early season vigor in several field-based mapping studies (Fig. 2.1; Burow et al., 2011; Knoll et al., 2008; Marla et al., 2019), which makes it unlikely to be a spurious association. Chilling tolerance is commonly assumed to be the trait driving early season vigor, but this has not been studied. It is possible that some other trait, such as mold resistance, herbivore resistance, or other unknown factors, might be influencing the field

phenotype and is, in fact, a significant contributor (Esele et al., 1993; Wu et al., 2019). Finally, reports in maize of chilling-induced changes on root structure (Richner et al., 1996) and relatively high levels of CBF expression in roots (Liu et al., 2013) suggest the possibility that *qCT04.62* regulates root-specific chilling tolerance, a tissue we did not investigate in this set of experiments and so would not have detected.

The co-inheritance between Tan1 and chilling tolerance is likely due to linkage

If *pCT04.62+/Tan1* introgression does not contain the QTN, this would indicate that the functional *Tan1* allele is not contributing to the chilling tolerance phenotype at *qCT04.62*, and the association is caused by a linkage instead of pleiotropy. Genetic data supporting this claim is mixed, as the peak JLM SNPs precisely co-localized with *Tannin1*, while the HKZ confidence interval was located upstream (Fig. 2.5). Also, previous studies found *Tannin2* to be associated with chilling tolerance (Marla et al., 2019), further strengthening the evidence that either grain tannins or the MBW complex play a role in chilling tolerance regulation.

Tannin1 is the WD40 subunit of the MBW complex, further suggesting the potential for pleiotropic regulation. The MBW complex is a trimeric gene regulatory complex composed of Mybs, bHLH repeats, and a WD40 subunit. In Arabidopsis, a single W subunit, *TTG1*, has regulatory functions across all known MBW phenotypes (Tian & Wang, 2020). Though only one subunit of each class participates in the complex at a time, multiple B and M subunits are interchangeable within their class, each specific to one or several phenotypes (Chen & Wang, 2019). In this way, the MBW complex can regulate multiple phenotypes independently. In sorghum, *Tannin1* is the WD40 subunit, and *Tannin2* is a bHLH subunit, presumably regulating grain tannins with an as yet unidentified myb (Wu et al., 2012, 2019). Further, *Tannin1* and *Tannin2* were found to co-localize closely with chilling tolerance QTL (Marla et al., 2019). Two

recent studies in Arabidopsis have identified further *TTG1* regulatory roles with flowering time and carbon partitioning in seeds (Airoldi et al., 2019; C. Li et al., 2018; Paffendorf et al., 2020), which could be suggestive of chilling tolerance regulation. Colocalization and homology also suggest a pleiotropic regulatory function of chilling tolerance for the MBW complex in sorghum.

Alternatively, our experimental testing displays a lack of effect by differing *Tannin1* alleles on chilling tolerance, suggesting *Tan1* linkage with chilling tolerance. The NIL families used in chilling tolerance testing were segregating for grain tannins and *Tannin1* alleles, which had no measurable effect on germination, vigor, or photosynthesis (Fig. 2.6 A-C, Fig. 2.7A-B, Fig. 2.8 A-C). If the association between *Tan1* and chilling tolerance is truly a linkage, the result is advantageous from a breeding perspective, as the respective alleles can be recombined to break the cosegregation of the two traits, unlocking the potential to use *qCT04.62* as a target for marker assisted selection.

Another hypothesis we tested is whether the CBF ortholog within the introgression regulates chilling tolerance at *qCT04.62.* In Arabidopsis, the CBF regulon is a major cold acclimation regulator and functions through JA upregulation of CBF genes (Fowler & Thomashow, 2002; Hu et al., 2017). In sorghum, chilling induces CBF upregulation (Marla et al., 2017), and a CBF ortholog precisely colocalized with *qCT04.62* (Fig. 2.4B; Marla et al., 2019). This CBF gene is an ortholog with known rice chilling tolerance regulator *OsDREB1G* and maize CBF *ZmDREB1.9* (Liu et al., 2013; Moon et al., 2019). Our data suggest that this CBF also does not underlie *qCT04.62*, as chilling tolerant vs chilling sensitive alleles were present in the NILs but had no discernible effect on chilling tolerance or acclimation.

Future avenues for breeding and genetics research using Tan1 NILs

There are several areas of research for which these NILs are particularly apt. First, the chilling tolerance work conducted in this study should be followed up. For the pleiotropy-linkage hypothesis to be conclusively decided, laboratory phenotypes must be confirmed with field-based experiments. As the QTL was originally mapped in the field, we cannot know how field conditions affect chilling tolerance or if we are missing a critical piece of the stress.

In sorghum, controlled genetic studies using mutants or NILs are relatively rare, making the NILs investigated in this study a valuable genetic resource (Xin et al., 2021). There are several avenues of sorghum research on which these NILs could shed light. One is the adaptive role of proanthocyanidins. In plants, tannins are known to be important defense molecules. They are a deterrent to birds, fungi, and herbivory (Constabel et al., 2014). Proanthocyanidins are rare in cultivated grains, as they are generally lost during domestication, and their function is not yet fully understood (Zhu, 2019). They may be beneficial in certain environments or circumstances (Xie et al., 2019; Zhu, 2019). Controlled experiments might help elucidate their function and adaptive potential.

Another area of research is the function of *Tan1* in sorghum. *TTG1* has a broad regulatory function, which may be conserved by *Tan1* (Tian & Wang, 2020). Maize research suggests that subfunctionalization events occurred as maize *TTG1* ortholog *PAC1* only regulates anthocyanins in seeds but not leaf anthocyanidins, root hair development, or trichomes (Selinger & Chandler, 1999). Further, if there are additional *tan1* phenotypes, it is unknown how these might affect yield and fitness in commercial sorghum varieties.

Material and Methods

Plant material

Sorghum cultivars were originally chosen for this study based on published field evaluation of each line for early season chilling tolerance (Franks et al., 2006; Kapanigowda et al., 2013). For NIL development, all crosses were made at Kansas State University. Three RILs from the chilling tolerant NAM BTx623 x Hong Ke Zi (PI 567946) family were used as starting material to reduce subsequent backcrossing effort (Marla et al., 2019). The RILs were then crossed to BTx623. F1 progeny were selected on two criteria: heterozygosity at the QTL of interest using a KASP marker system and visually for resemblance to BTx623, the recurrent parent. Selected progeny were then backcrossed to BTx623. Selection and backcrossing were repeated four times. 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).

Cold Tolerance QTL Map

Cold tolerance QTL data was downloaded from the Sorghum QTL Atlas (Mace et al., 2019). Using custom R v4.1.2 scripts (R Core Team, 2021), QTL were filtered for biparental and NAM mapping studies and plotted by genomic location.

Genotyping by Whole Genome Resequencing

Leaf tissue was collected from two-week-old seedlings and frozen at -80°C until DNA extractions. Following the manufacturer's instructions, DNA extractions were performed using Quick-DNA Plant/Seed Miniprep Kit (ZYMO, D6020). DNA was quantified using a Thermo Scientific NanoDrop 2000/2000c Spectrophotometer. Library Preparation and DNA sequencing

were performed by the Kansas State University Integrated Genomics Facility (https://www.k-state.edu/igenomics/index.html). DNA was sequenced to ~1x depth on Illumina NextSeq 500 using 300 cycles and 151 paired-end chemistry. Low-quality read sequences were trimmed using Trimmomatic v0.32 (Bolger et al., 2014), and the remaining reads were mapped to BTx623 v3.1.1 reference genome (McCormick et al., 2018) using BWA-MEM (H. Li, 2013). Picard v2.26 MarkDuplicates was then used to merge bam files from common read groups and flag duplicate reads (Picard Toolkit, 2019). SNPs were then called using GATK v4.2.5.0 suite of tools, including Haplotype Caller to create gVCF files, GenomicsDBImport to create gVCF database, and GenotypeGVCF to create final VCF (GA Van der Auwera & BD O'Connor, 2020). BCFtools v1.15.1 was then used to sort variants and filter for high-quality biallelic SNPs (Danecek et al., 2021). A custom script was written using R v4.1.2 to analyze genome-wide sliding windows and plot alternate allele frequencies using 10000 Kb windows (R Core Team, 2021). Two biological replicates were analyzed independently. Red is $alternate^{x}/alternate^{HKZ} >= 0.2$; blue is $alternate^{x}/alternate^{HKZ} > 0.2$; yellow is when a color call differs between biological replicates.

Bleach Test for Grain Tannin Presence

The bleach test was performed as previously described (Marla et al., 2019; Waniska et al., 1992). Briefly, fifteen seeds from each genotype were placed in a 50 mL centrifuge tube. One mL of bleach/sodium hydroxide solution was added (3.75% NaOCl and 5% NaOH) to the seeds and left for 30 minutes. Seeds containing proanthocyanidins became dark, while non-proanthocyanidin seeds became white.

Characterization of Controlled Environment Chilling Stress

The experiments were carried out in controlled environment chambers (Conviron Model CMP6050, Manitoba, Canada) at the Plant Growth Facilities at Colorado State University in Fort Collins, CO. Experiment designs were created and randomized using a custom R v4.1.2 script (R Core Team, 2021). Each genotype/treatment combination had six replicates. Two temperature treatments were applied in parallel, chilling and control, in discrete growth chambers. For the long temperature treatment, control is defined as 30°C/20°C day/night temperature treatment and chilling 20°C/10°C. For the short temperature treatment control is defined as 28°C/25°C day/night temperature treatment and chilling 10°C/4°C. All other parameters were kept as consistent as possible between treatments; a 12h photoperiod and 700 μmol m^{-2} s^{-1} light intensity. All plants were potted in 1.5-inch Cone-tainers using Lambert LM-HP potting soil and given 3g Osmocote controlled-release fertilizer. Water was provided in excess using a bottom watering system. For the long treatment, all pots were germinated under control temperature conditions for approximately five days. Following germination, conditions for control plants remained unchanged, while chilling conditions were applied to chilling plants. After six weeks under treatment conditions, plant shoots were harvested, dried, and analyzed for dry weight. For the short treatment, all pots were germinated under control temperature conditions and grown for approximately seven days when chilling conditions were applied to chilling plants. After three days under treatment conditions, plants were again allowed to grow at control temperatures for seven more days. Plant shoots were then harvested, dried, and analyzed for dry weight.

Photosynthetic Characterization of Stress Response

Experiment designs were created and randomized using a custom R v4.1.2 script (R Core Team, 2021). Each genotype/treatment combination had six replicates. Photosynthetic

components were measured using MultiSpeQ (Kuhlgert et al., 2016) and analyzed using R v4.1.2 (R Core Team, 2021). All plants were potted in 1.5-inch Cone-tainers using Lambert LM-HP potting soil. Photoperiod was a 12 h day-night cycle with transits at 6:00 am and 6:00 pm. Light intensity was 700 μmol m^{-2} s^{-1}, and water was provided in excess using a bottom watering system. Two temperature treatments were applied consecutively over a nine-day time course, optimal (28°C/25°C) and chilling (10°C/4°C) day/night. Throughout the time course, treatment changes occurred at 5:30 am on the scheduled day. After planting, seedlings were allowed to germinate and grow at an optimal temperature until large enough for accurate leaf measurements to be taken for approximately ten days. The final day of the growth phase is day one for our time course analysis. Measurements were taken each day of the time course beginning at 10:00 am. On day two, seedlings were subjected to chilling treatment until day six. From day six through day nine, seedlings were again grown at optimal temperatures. Graphs were constructed using ggplot2 v3.4.2 r package (Hadley Wickham, 2016).

Characterization of Low-Temperature Germination

Four temperature treatments were used to measure the genotypic effect on low-temperature germination, increasing from 10°C to 25°C in 5° increments. There were three replicates per temperature. For each replicate, twelve seeds from each genotype were placed in a 90-mm petri dish lined with filter paper and moistened with 2 mL distilled water. There were three petri dishes per genotype, totaling 36 seeds per replicate. Dishes were sealed with parafilm and placed in a dark growth chamber at the treatment temperature. Each day for four days, petri dishes were opened, and a picture was taken. Pictures were then scored for germination (Schneider et al., 2012) and analyzed using R v4.1.2 (R Core Team, 2021). Graphs were created using ggplot2 v3.4.2 r package (Hadley Wickham, 2016).

REFERENCES: CHAPTER II

Airoldi, C. A., Hearn, T. J., Brockington, S. F., Webb, A. A. R., & Glover, B. J. (2019). TTG1 proteins regulate circadian activity as well as epidermal cell fate and pigmentation. *Nature Plants*, *5*(11), Article 11. https://doi.org/10.1038/s41477-019-0544-3

Berry, E. M., Dernini, S., Burlingame, B., Meybeck, A., & Conforti, P. (2015). Food security and sustainability: Can one exist without the other? *Public Health Nutrition*, *18*(13), 2293–2302. https://doi.org/10.1017/S136898001500021X

Bilger, W., Schreiber, U., & Bock, M. (1995). Determination of the Quantum Efficiency of Photosystem II and of Non-Photochemical Quenching of Chlorophyll Fluorescence in the Field. *Oecologia*, *102*(4), 425–432.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Burow, G., Burke, J. J., Xin, Z., & Franks, C. D. (2011). Genetic dissection of early-season cold tolerance in sorghum (Sorghum bicolor (L.) Moench). *Molecular Breeding*, *28*(3), 391–402. http://dx.doi.org.ezproxy2.library.colostate.edu/10.1007/s11032-010-9491-4

Chen, S., & Wang, S. (2019). GLABRA2, a Common Regulator for Epidermal Cell Fate Determination and Anthocyanin Biosynthesis in Arabidopsis. *International Journal of Molecular Sciences*, *20*(20), Article 20. https://doi.org/10.3390/ijms20204997

Constabel, C. P., Yoshida, K., & Walker, V. (2014). Diverse Ecological Roles of Plant Tannins: Plant Defense and Beyond. In *Recent advances in polyphenol research* (p. 464). https://doi.org/10.1002/9781118329634.ch5

Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., Whitwham, A., Keane, T., McCarthy, S. A., Davies, R. M., & Li, H. (2021). Twelve years of SAMtools and BCFtools. *GigaScience*, *10*(2), giab008. https://doi.org/10.1093/gigascience/giab008

Esele, J., Frederiksen, R., & Miller, F. (1993). The association of genes controlling caryopsis traits with grain mold resistance in sorghum. *Phytopathology*, *83*(5), 490–495.

Fowler, S., & Thomashow, M. F. (2002). Arabidopsis Transcriptome Profiling Indicates That Multiple Regulatory Pathways Are Activated during Cold Acclimation in Addition to the CBF Cold Response Pathway[W]. *The Plant Cell*, *14*(8), 1675–1690. https://doi.org/10.1105/tpc.003483

Franks, C. D., Burow, G. B., & Burke, J. J. (2006). A Comparison of U.S. and Chinese Sorghum Germplasm for Early Season Cold Tolerance. *Crop Science*, *46*(3), 1371–1376. https://doi.org/10.2135/cropsci2005.08-0279

GA Van der Auwera & BD O'Connor. (2020). *Genomics in the Cloud: Using Docker, GATK, and WDL in Terra* (1st Edition). O'Reilly Media.

Hadley Wickham. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. https://ggplot2.tidyverse.org

Hu, Y., Jiang, Y., Han, X., Wang, H., Pan, J., & Yu, D. (2017). Jasmonate regulates leaf senescence and tolerance to cold stress: Crosstalk with other phytohormones. *Journal of Experimental Botany*, *68*(6), 1361–1369. https://doi.org/10.1093/jxb/erx004

Kapanigowda, M. H., Perumal, R., Aiken, R. M., Herald, T. J., Bean, S. R., & Little, C. R. (2013). Analyses of sorghum [*Sorghum bicolor* (L.) Moench] lines and hybrids in response to early-season planting and cool conditions. *Canadian Journal of Plant Science*, *93*(5), 773–784. https://doi.org/10.4141/cjps2012-311

Knoll, J., & Ejeta, G. (2008). Marker-assisted selection for early-season cold tolerance in sorghum: QTL validation across populations and environments. *Theoretical and Applied Genetics*, *116*(4), 541–553. https://doi.org/10.1007/s00122-007-0689-8

Knoll, J., Gunaratna, N., & Ejeta, G. (2008). QTL analysis of early-season cold tolerance in sorghum. *Theoretical and Applied Genetics*, *116*(4), 577–587. https://doi.org/10.1007/s00122-007-0692-0

Kuhlgert, S., Austic, G., Zegarac, R., Osei-Bonsu, I., Hoh, D., Chilvers, M. I., Roth, M. G., Bi, K., TerAvest, D., Weebadde, P., & Kramer, D. M. (2016). MultispeQ Beta: A tool for large-scale plant phenotyping connected to the open PhotosynQ network. *Royal Society Open Science*, *3*(10), 160592. https://doi.org/10.1098/rsos.160592

Li, C., Zhang, B., Chen, B., Ji, L., & Yu, H. (2018). Site-specific phosphorylation of TRANSPARENT TESTA GLABRA1 mediates carbon partitioning in Arabidopsis seeds. *Nature Communications*, *9*(1), Article 1. https://doi.org/10.1038/s41467-018-03013-5

Li, H. (2013). *Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM* (arXiv:1303.3997). arXiv. http://arxiv.org/abs/1303.3997

Liu, S., Wang, X., Wang, H., Xin, H., Yang, X., Yan, J., Li, J., Tran, L.-S. P., Shinozaki, K., Yamaguchi-Shinozaki, K., & Qin, F. (2013). Genome-Wide Analysis of ZmDREB Genes and Their Association with Natural Variation in Drought Tolerance at Seedling Stage of Zea mays L. *PLOS Genetics*, *9*(9), e1003790. https://doi.org/10.1371/journal.pgen.1003790

Long, S. P., & Spence, A. K. (2013). Toward Cool C4 Crops. *Annual Review of Plant Biology*, *64*(1), 701–722. https://doi.org/10.1146/annurev-arplant-050312-120033

Mace, E., Innes, D., Hunt, C., Wang, X., Tao, Y., Baxter, J., Hassall, M., Hathorn, A., & Jordan, D. (2019). The Sorghum QTL Atlas: A powerful tool for trait dissection, comparative genomics and crop improvement. *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, *132*(3), 751–766. https://doi.org/10.1007/s00122-018-3212-5

Maggio, A., Van Criekinge, T., & Malingreau, J. P. (2015). *Global food security 2030: Assessing trends with a view to guiding future EU policies.* Publications Office of the European Union. https://data.europa.eu/doi/10.2788/5992

Marla, Burow, G., Chopra, R., Hayes, C., Olatoye, M. O., Felderhoff, T., Hu, Z., Raymundo, R., Perumal, R., & Morris, G. P. (2019). Genetic Architecture of Chilling Tolerance in Sorghum Dissected with a Nested Association Mapping Population. *G3 Genes|Genomes|Genetics*, *9*(12), 4045–4057. https://doi.org/10.1534/g3.119.400353

Marla, S., Terry Felderhoff, Hayes, C., Perumal, R., Wang, X., Poland, J., & Morris, G. P. (2023). Genomics and Phenomics Enabled Prebreeding Improved Early-Season Chilling Tolerance in Sorghum. *G3 Genes|Genomes|Genetics*. https://doi.org/10.1101/2022.10.31.514536

Marla, Shiva, S., Welti, R., Liu, S., Burke, J. J., & Morris, G. P. (2017). Comparative Transcriptome and Lipidome Analyses Reveal Molecular Chilling Responses in Chilling-Tolerant Sorghums. *The Plant Genome*, *10*(3), plantgenome2017.03.0025. https://doi.org/10.3835/plantgenome2017.03.0025

McCormick, R. F., Truong, S. K., Sreedasyam, A., Jenkins, J., Shu, S., Sims, D., Kennedy, M., Amirebrahimi, M., Weers, B. D., McKinley, B., Mattison, A., Morishige, D. T., Grimwood, J., Schmutz, J., & Mullet, J. E. (2018). The Sorghum bicolor reference genome: Improved assembly, gene annotations, a transcriptome atlas, and signatures of

genome organization. *The Plant Journal*, *93*(2), 338–354. https://doi.org/10.1111/tpj.13781

Moghimi, N., Desai, J. S., Bheemanahalli, R., Impa, S. M., Vennapusa, A. R., Sebela, D., Perumal, R., Doherty, C. J., & Jagadish, S. V. K. (2019). New candidate loci and marker genes on chromosome 7 for improved chilling tolerance in sorghum. *Journal of Experimental Botany*, *70*(12), 3357–3371. https://doi.org/10.1093/jxb/erz143

Monk, R., Franks, C., & Dahlberg, J. (2014). Sorghum. In *Yield Gains in Major U.S. Field Crops* (pp. 293–310). John Wiley & Sons, Ltd. https://doi.org/10.2135/cssaspecpub33.c11

Moon, S.-J., Min, M. K., Kim, J.-A., Kim, D. Y., Yoon, I. S., Kwon, T. R., Byun, M. O., & Kim, B.-G. (2019). Ectopic Expression of OsDREB1G, a Member of the OsDREB1 Subfamily, Confers Cold Stress Tolerance in Rice. *Frontiers in Plant Science*, *10*. https://www.frontiersin.org/articles/10.3389/fpls.2019.00297

Moose, S. P., & Mumm, R. H. (2008). Molecular Plant Breeding as the Foundation for 21st Century Crop Improvement. *Plant Physiology*, *147*(3), 969–977. https://doi.org/10.1104/pp.108.118232

Mosier, A., Kroeze, C., Nevison, C., Oenema, O., Seitzinger, S., & van Cleemput, O. (1998). Closing the global N2O budget: Nitrous oxide emissions through the agricultural nitrogen cycle. *Nutrient Cycling in Agroecosystems*, *52*(2), 225–248. https://doi.org/10.1023/A:1009740530221

Ortiz, D., Hu, J., & Salas Fernandez, M. G. (2017). Genetic architecture of photosynthesis in Sorghum bicolor under non-stress and cold stress conditions. *Journal of Experimental Botany*, *68*(16), 4545–4557. https://doi.org/10.1093/jxb/erx276

Paffendorf, B. A. M., Qassrawi, R., Meys, A. M., Trimborn, L., & Schrader, A. (2020). TRANSPARENT TESTA GLABRA 1 participates in flowering time regulation in Arabidopsis thaliana. *PeerJ*, *8*, e8303. https://doi.org/10.7717/peerj.8303

Picard Toolkit (2.26). (2019). Broad Institute. https://broadinstitute.github.io/picard/

R Core Team. (2021). *R: A language and environment for statistical computing.* (4.1.2). R Foundation for Statistical Computing. https://www.r-project.org/

Raymundo, R., Sexton-Bowser, S., Ciampitti, I. A., & Morris, G. (2021). *Crop modeling defines opportunities and challenges for drought escape, water capture, and yield increase using chilling-tolerant sorghum* [Preprint]. Plant Biology. https://doi.org/10.1101/2021.01.27.428532

Richner, W., Soldati, A., & Stamp, P. (1996). Shoot-to-Root Relations in Field-Grown Maize Seedlings. *Agronomy Journal*, *88*(1), 56–61. https://doi.org/10.2134/agronj1996.00021962008800010012x

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), Article 7. https://doi.org/10.1038/nmeth.2089

Selinger, D. A., & Chandler, V. L. (1999). A Mutation in the pale aleurone color1 Gene Identifies a Novel Regulator of the Maize Anthocyanin Pathway. *The Plant Cell*, *11*(1), 5–14. https://doi.org/10.1105/tpc.11.1.5

Taylor, A. O., & Rowley, J. A. (1971). Plants under Climatic Stress: I. Low Temperature, High Light Effects on Photosynthesis. *Plant Physiology*, *47*(5), 713–718. https://doi.org/10.1104/pp.47.5.713

Tian, H., & Wang, S. (2020). TRANSPARENT TESTA GLABRA1, a Key Regulator in Plants with Multiple Roles and Multiple Function Mechanisms. *International Journal of Molecular Sciences*, *21*(14), 4881. https://doi.org/10.3390/ijms21144881

Waniska, R. D., Hugo, L. F., & Rooney, L. W. (1992). Practical Methods to Determine the Presence of Tannins in Sorghum. *Journal of Applied Poultry Research*, *1*(1), 122–128. https://doi.org/10.1093/japr/1.1.122

Wheeler, T., & von Braun, J. (2013). Climate Change Impacts on Global Food Security. *Science*, *341*(6145), 508–513. https://doi.org/10.1126/science.1239402

Wu, Y., Guo, T., Mu, Q., Wang, J., Li, X., Wu, Y., Tian, B., Wang, M. L., Bai, G., Perumal, R., Trick, H. N., Bean, S. R., Dweikat, I. M., Tuinstra, M. R., Morris, G., Tesso, T. T., Yu, J., & Li, X. (2019). Allelochemicals targeted to balance competing selections in African agroecosystems. *Nature Plants*, *5*(12), 1229–1236. https://doi.org/10.1038/s41477-019-0563-0

Wu, Y., Li, X., Xiang, W., Zhu, C., Lin, Z., Wu, Y., Li, J., Pandravada, S., Ridder, D. D., Bai, G., Wang, M. L., Trick, H. N., Bean, S. R., Tuinstra, M. R., Tesso, T. T., & Yu, J. (2012). Presence of tannins in sorghum grains is conditioned by different natural alleles of Tannin1. *Proceedings of the National Academy of Sciences*, *109*(26), 10281–10286. https://doi.org/10.1073/pnas.1201700109

Xie, P., Shi, J., Tang, S., Chen, C., Khan, A., Zhang, F., Xiong, Y., Li, C., He, W., Wang, G., Lei, F., Wu, Y., & Xie, Q. (2019). Control of Bird Feeding Behavior by Tannin1 through Modulating the Biosynthesis of Polyphenols and Fatty Acid-Derived Volatiles in Sorghum. *Molecular Plant*, *12*(10), 1315–1324. https://doi.org/10.1016/j.molp.2019.08.004

Xin, Z., Wang, M., Cuevas, H. E., Chen, J., Harrison, M., Pugh, N. A., & Morris, G. (2021). Sorghum genetic, genomic, and breeding resources. *Planta*, *254*(6), 114. https://doi.org/10.1007/s00425-021-03742-w

Zhu, F. (2019). Proanthocyanidins in cereals and pseudocereals. *Critical Reviews in Food Science and Nutrition*, *59*(10), 1521–1533. https://doi.org/10.1080/10408398.2017.1418284

SORGHUM *TANNIN1* LOST PLEIOTROPIC REGULATORY FUNCTION IN THIS ORTHOLOG OF ARABIDOPSIS MASTER REGULATOR *TTG1*

Introduction

Creating sustainable agricultural systems is one of the major challenges facing the developed world. In many areas, climate instability and depletion of crucial resources such as water and soil fertility are spurring investment in finding solutions to these challenges (Berry et al., 2015). One important tool in shaping agricultural systems is crop adaptation. Breeders and geneticists can create crop varieties that maintain productivity in new and changing environments or under novel management practices (Islam et al., 2016; Qaim, 2020). Chilling tolerance is a trait with considerable positive potential for sustainability by maintaining yields with reduced inputs. Chilling tolerant crops are planted earlier in the season, which benefits yield through a lengthened growing season (Long & Spence, 2013). In addition, early sowing shifts plant development to more favorable evapotranspirative conditions allowing more efficient use of natural precipitation (Raymundo et al., 2021). Further, earlier planting creates shorter fallow periods, which may reduce nitrogen loss through runoff and N_2O emissions (Mosier et al., 1998). Also, earlier planting can reduce weed pressure on seedlings, limiting the need for hand weeding or herbicide application (McDonald & Gill, 2009; Vida et al., 2006).

Many molecular and physiological mechanisms enable plant adaptation to chilling stress. Cold affects the molecular function of plants in several ways. It reduces the rates of enzymatic function, slowing molecular processes and producing a buildup of toxic metabolic intermediates (Lyons, 1973). Furthermore, cold also reduces membrane fluidity, creating leakages and the reduction of chemical potentials, dramatically reducing photosynthetic capacity, and creating an excess buildup of ROS, eventually leading to irreversible photodamage (Allen & Ort, 2001;

Lyons, 1973). Plants have developed strategies to mitigate these effects, including growth repression, membrane lipids remodeling, increased ROS scavenging, and photoprotection (Liu et al., 2019; Mira et al., 2021; Shi et al., 2018). Further, the CBF group of transcription factors is widely conserved and is known as master regulators of cold acclimation in many plants (Guo et al., 2019; Ito et al., 2006; Jaglo-Ottosen et al., 1998; Marozsán-Tóth et al., 2015; Qin et al., 2004; Savitch et al., 2005; Zhang et al., 2004). However, the roles of chilling tolerance mechanisms are mainly understood in model systems, and it is unclear to what extent they are conserved across species.

Sorghum is an important crop commercially and for agricultural sustainability in the U.S. and globally. Sorghum is the fourth-highest-produced grain and is particularly well adapted to semi-arid environments (Monk et al., 2014). Like many tropical-origin crops, sorghum is chilling sensitive, with stress at temperatures under 15°C (Lyons, 1973). Further, the co-localization of chilling tolerance with agronomic traits such as height and grain tannins may have contributed to purging chilling tolerance alleles from commercial germplasm (Marla et al., 2019). Several landraces have been identified as sources of chilling tolerance, most notably the Chinese-origin Kaoliangs (Franks et al., 2006). However, because of their divergent genetic backgrounds, molecular breeding is likely required to isolate the chilling tolerant alleles and transfer them to commercially relevant varieties (Burow et al., 2011; Franks et al., 2006; Marla et al., 2019).

In mapping studies of early-planted seedling vigor and chilling tolerance, a major effect QTL, *qCT04.62*, has been repeatedly identified at the end of chromosome 4 (Schuh 2023). It is associated with many different phenotypic markers of chilling tolerance, including early germination, early emergence, early vigor, early leaf appearance, total plant biomass, and root biomass (Burow et al., 2011; Fiedler et al., 2012, 2014; Knoll et al., 2008; Marla et al., 2019;

Moghimi et al., 2019). Unfortunately, using *qCT04.62* for breeding chilling tolerant sorghum is difficult because of its tight co-localization with *Tannin1*, an important grain tannin regulator, and the co-inheritance of chilling tolerance and grain tannins that this colocalization causes (Marla et al., 2019). In commercial grain sorghum in the U.S., grain tannins reduce livestock feed efficiency and have been subsequently purged from commercial germplasm (Nyachoti et al., 1997; Wu et al., 2012). The segregation of chilling tolerance with grain tannins could have two potential causes, linkage or pleiotropy. If linkage underlies the association, breeding is possible through recombining the alleles. But if *Tan1* pleiotropically regulates the two traits, the alleles are the same and cannot be separated via recombination. To empower future breeding efforts, understanding the association's cause is imperative.

Orthology of *Tannin1* with *TTG1* suggests *Tannin1* may also be a pleiotropic regulator. Sorghum *Tannin1* is a WD40 subunit and an ortholog of the Arabidopsis *TTG1* (Wu et al., 2012). *TTG1* is a known master regulator and the universal subunit in the MBW regulatory complex, with a known function regulating the biosynthesis of flavonoids, seed coat mucilage, development of root hair, and leaf trichomes (Tian & Wang, 2020). The MBW complex is also conserved in maize, though *PAC1*, the WD40 subunit, has a reduced regulatory function from *TTG1* (Carey et al., 2004; Selinger & Chandler, 1999). Though *Tan1* orthology with *TTG1* suggests pleiotropic function, during the 20th century, *Tan1* function was meticulously investigated by classical geneticists as the *B1* gene, and pleiotropic function was not reported (Doggett, 1970). Further, non-functional *tan1* is ubiquitous as a grain tannin inhibitor in commercial sorghum germplasm (Wu et al., 2012), and if pleiotropically regulating major developmental pathways, a moderate to strong deleterious effect would be expected from *tan1* loss of function alleles, though this has also never been reported. This suggests a more narrow

regulatory function for *Tannin1,* similar to *PAC1*, possibly relegated to grain tannins alone. Currently, the degree of functional conservation between *Tannin1* and *TTG1* remains largely unstudied.

To investigate the role of *Tannin1* in chilling tolerance, NILs were bred using a chilling sensitive background with heterozygosity for a Kaoliang haplotype, including part of *qCT04.62* and *Tan1* (Marla et al., 2023; Schuh, 2023). A previous study found that chilling tolerance did not co-segregate with the Kaoliang introgression, suggesting the introgression does not contain the *qCT04.62* QTN and that the association between *Tan1* and chilling tolerance is underlined by linkage (Schuh, 2023). In this study, we use transcriptomics to validate the prior research and investigate the functional conservation between *Tan1* and *TTG1*. We looked at major cold tolerance pathways for signs of regulation by *Tan1* (Fig. 3.1). Consistent with the previous study, we found no evidence of chilling tolerance regulation at the introgression. Further, we also found no other significant pathway upregulation which might be expected of a functionally conserved

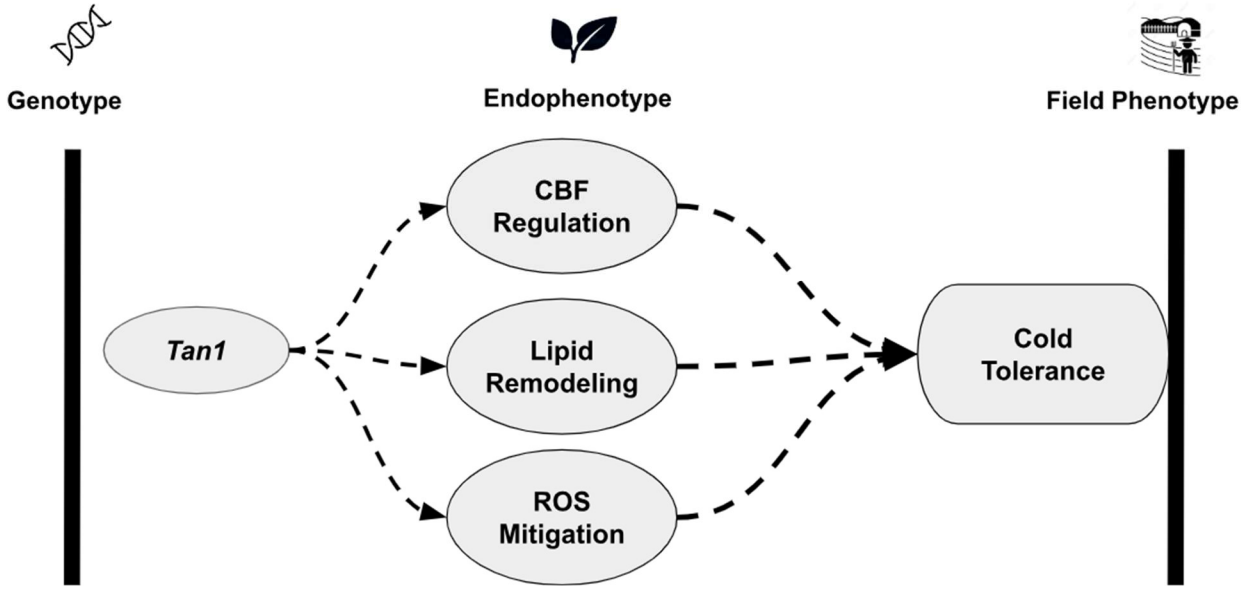

Figure 3.1. Hypothetical regulation of major chilling tolerance pathways by *Tan1*. Genotype to phenotype map explaining hypothetical association between *Tan1* and cold tolerance in sorghum.

TTG1 ortholog. Overall, the findings of this study take a step towards unlocking the potential of *qCT04.62* for molecular breeding and explaining the apparent lack of deleterious phenotypes in *tan1* elite lines.

Results

Tannin1 is widely expressed, while the other sorghum co-ortholog of TTG1 is not

The NILs carry part of *CT04.62+* and *Tan1*. Results from the previous study suggest that *pCT04.62+/Tan1* introgression is not regulating chilling tolerance, though this is inconclusive and needs further validation (Schuh, 2023). As *Tannin1* is a major gene candidate for regulating chilling tolerance at *qCT04.62,* we looked at *Tannin1* expression in the NILs to see if it is regulating chilling tolerance. *Tannin1* is expressed in NIL+ and NIL- with no significant difference between the lines. Interestingly, there is a highly significant downregulation of *Tannin1* (p<10-4) under chilling conditions in both lines suggesting it may not be a chilling tolerance regulator (Fig. 3.2A). *Tannin1* orthologs are major pleiotropic regulators in Arabidopsis and other plants, so we hypothesize this function may be conserved in sorghum. To test this and identify candidates for possible subfunctionalization, we looked at the expression for *Tannin1* and the *Tannin1* paralogs in our samples and across other tissues using publicly available data. There is no expression for Sobic.004G161600, the *Tannin1* paralog with the greatest similarity to Arabidopsis *TTG1*, in either NIL+ or NIL- lines (Fig. 3.2B). Across other sorghum tissues, *Tannin1* is widely expressed, while there is no evidence that the other *TTG1* co-ortholog Sobic.004G161600 is expressed in any tissue or time point (Fig. 3.2C). Other genes with lower similarity to *TTG1* are not expressed in most tissues, but several (Sobic.003G427100,

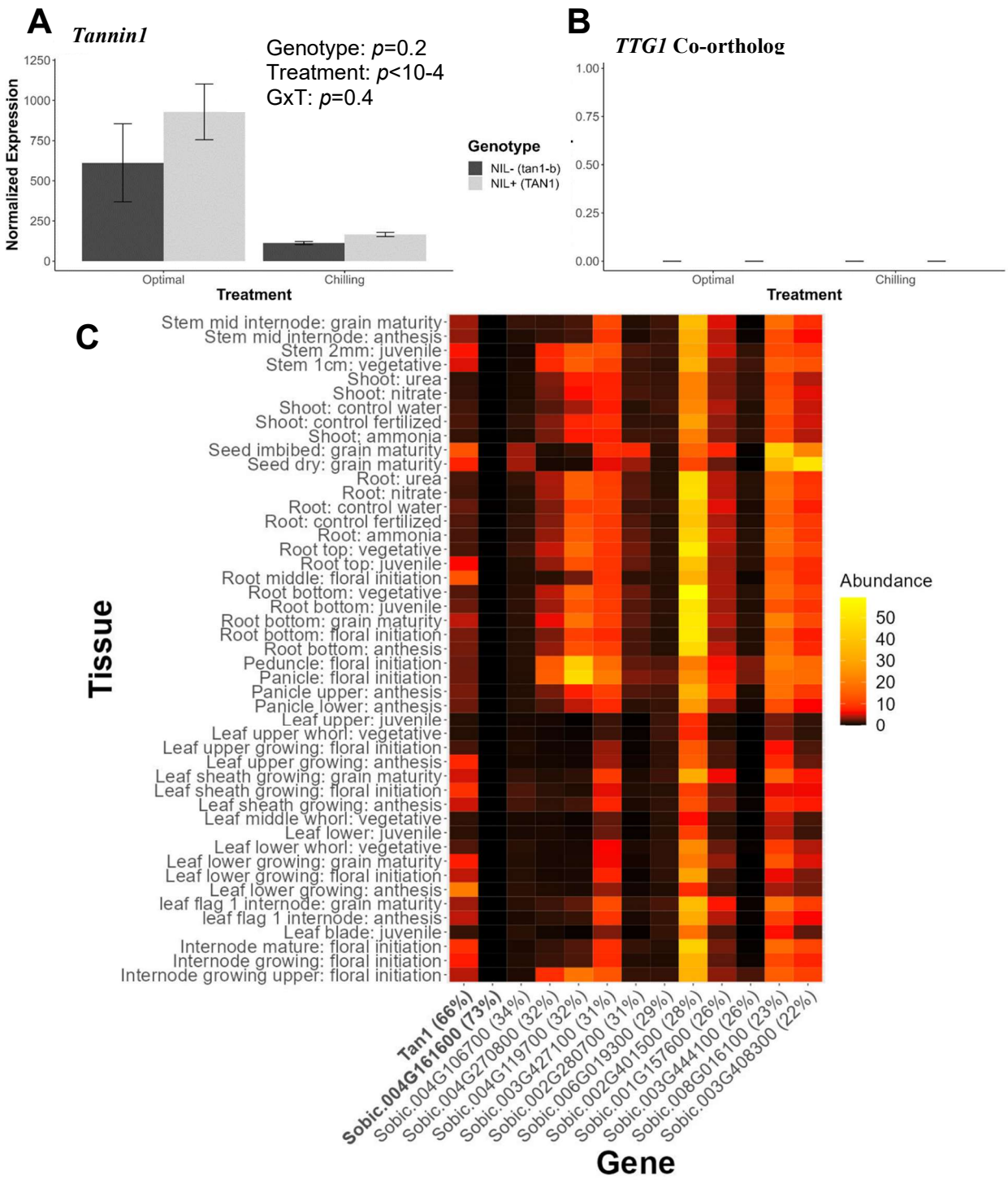

Figure 3.2. Expression patterns of *Tan1* and other *TTG1* homologs. (**A**) *Tan1* expression in NILs under control and chilling treatments. Error bars span +/- one standard error. P-values are calculated by one-way ANOVA without correction. (**B**) Expression of *TTG1* co-ortholog, *Sobic.004G161600*, in NILs under control and chilling treatments. All means are 0. (**C**) Expression of *Tan1* and other *TTG1* homologs in diverse sorghum tissues. Black is 0, red is 7, orange is 25, yellow is >50. Bolded genes are *TTG1* co-orthologs, non-bolded genes share *TTG1* homology. Percent similarity with *TTG1* is in parentheses after gene name/ID. Tissue expression data is publicly available on phytozome.

Sobic.002G401500, Sobic.008G016100, and Sobic.003G408300) were highly and widely expressed. This data suggests that *Tannin1* may be a critical regulatory WD40 paralog in sorghum, but subfunctionalization or recruitment of other expressed WD40 sorghum proteins can not be ruled out, particularly the paralogs with the highest expression.

No differential expression in major pathways involved in chilling response between NILs

To confirm previous negative chilling tolerance results in our NIL lines, we looked for transcriptional enrichment in genes and pathways previously indicated to play a role in sorghum chilling tolerance (Li et al., 2015; Marla et al., 2017; Moghimi et al., 2019). No *CBF* orthologs were significantly differentially expressed between NILs ($p = 1$), though several were up or down regulated due to chilling (Fig. 3.3A, Table 1). Further, no statistically significant upregulation for previously identified genes involved in lipid remodeling, NPQ, or phytohormone biosynthesis in NIL+ compared to NIL-. These findings provide further evidence

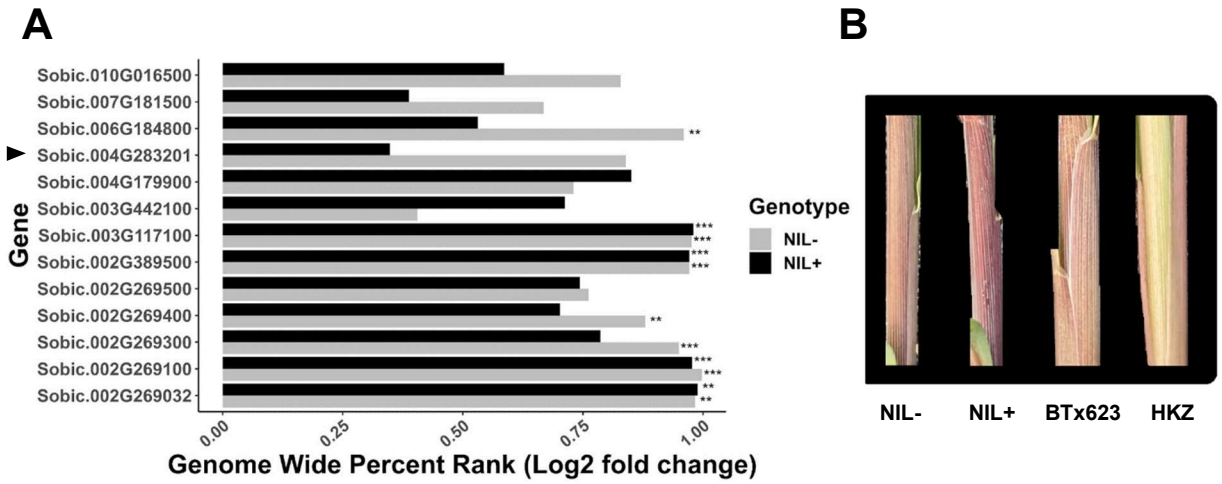

Figure 3.3. Absence of *Tan1* pleiotropic regulation of CBFs or anthocyanins. (**A**) Genome-wide percent rank of log2 fold change in predicted CBF orthologs. Significance values are calculated for log2 fold change per NIL. There is no significant difference for log2 fold change between NILs in any CBF ortholog. The triangle indicates the CBF homolog that is in the NIL+ introgressions. The *p*-values were calculated using the Wald test and corrected for multiple testing bias using the Benjamini-Hochberg correction. Significance codes are: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (**B**) Anthocyanin accumulation in stem tissue by genotype.

Table 3.1. Significance of log2 fold change for predicted CBF orthologs in NIL+ and NIL-

Annotation	Gene ID	Corrected *p*-value		
		Ctrl+/Chl+	Ctrl-/Chl-	Genotype
C-repeat/DRE binding factor 2				
	Sobic.002G269032	0.001	0.007	1
	Sobic.002G269400	0.2	0.001	1
	Sobic.004G283201	0.8	0.1	1
	Sobic.010G016500	0.8	0.2	1
	Sobic.002G269400	0.2	0.001	1
	Sobic.002G269032	0.001	0.007	1
	Sobic.004G283201	0.8	0.1	1
	Sobic.010G016500	0.8	0.2	1
C-repeat-binding factor 4				
	Sobic.002G269100	$<10^{-4}$	$<10^{-4}$	1
	Sobic.002G269500	0.2	0.1	1
	Sobic.003G442100	1	1	1
	Sobic.004G179900	1	1	1
	Sobic.006G184800	0.1	0.003	1
	Sobic.007G181500	0.9	0.6	1
	Sobic.002G269100	$<10^{-4}$	$<10^{-4}$	1
	Sobic.002G269500	0.1	0.1	1
	Sobic.003G442100	1	1	1
	Sobic.004G179900	1	1	1
	Sobic.006G184800	1	0.003	1
	Sobic.007G181500	1	0.6	1
homologue of NAP57				
	Sobic.002G389500	$<10^{-4}$	$<10^{-4}$	1
	Sobic.003G117100	$<10^{-4}$	$<10^{-4}$	1
	Sobic.002G389500	$<10^{-4}$	$<10^{-4}$	1
	Sobic.003G117100	$<10^{-4}$	$<10^{-4}$	1

The *p*-values were calculated using the Wald Test and corrected for multiple testing using the Benjamini-Hochberg correction.

that no major chilling tolerance mechanisms are regulated by *Tan1* or the introgressed portion of *qCT04.62*.

If *Tan1* has a conserved function with *TTG1*, we would expect regulatory changes between the NILs in other major pathways, particularly flavonoids. However, GO analysis showed no significant enrichment for any terms, though only 17 genes had a significant genotype or genotype x treatment effect at $p = 0.05$. Further, no genes with known or predicted function in flavonoid biosynthesis had DE between NILs. Additionally, anthocyanins visibly accumulate in leaf and stem tissue for all genotypes (Fig. 3.3B). This suggests that *Tan1* does not regulate anthocyanin accumulation in leaf or stem tissue in sorghum, in contrast to Arabidopsis *TTG1*.

Expression pattern suggests independent regulation among DE genes

To visualize the transcriptomic influence of genotype and treatment on the samples and test the validity of our experimental methodology, we generated a PCA plot with NILs and NIL parents as coordinates (Fig. 3.4). The PC1 axis separates chilling and control groups, accounting

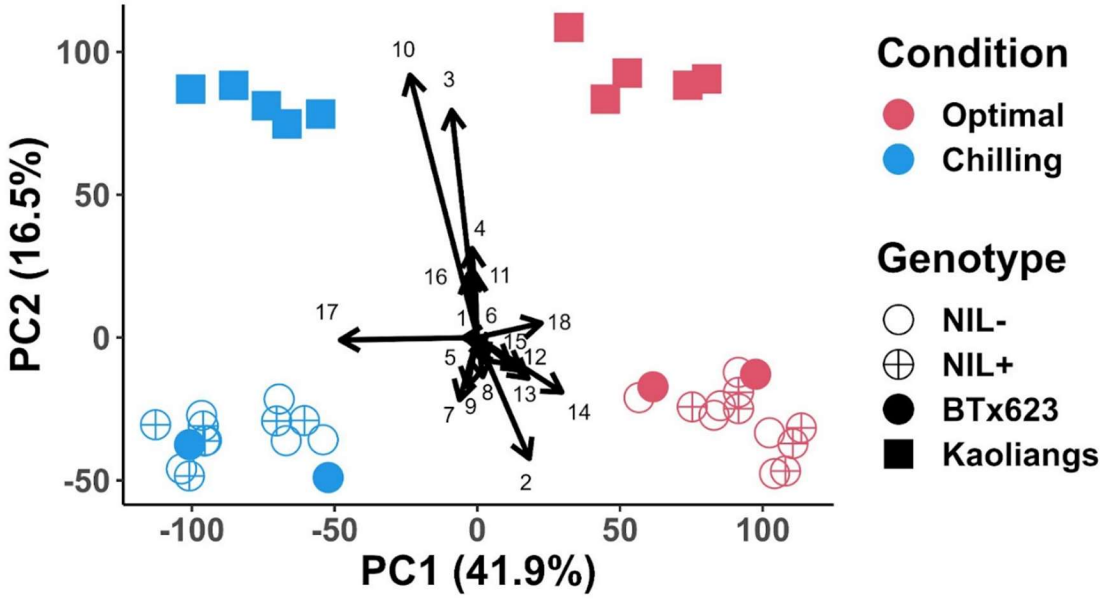

Figure 3.4. Principal coordinate plot with component analysis of genes regulated by introgressed polymorphisms. PC1 axis resolves treatment while PC2 genetic background. Circle represents BTx623 genetic background, square represents Kaoliangs, filled shape represents parents. DE genes are scaled up by 3000. Direction of arrow shows effect of upregulation.

Table 3.2. Differentially expressed genes with a significant G or GxT interaction

Ind.	Gene ID	Annotation	PC1	PC2	*p*-value	
					G	GxT
1	Sobic.002G051300	Flavin-dependent monooxygenase 1	-0.0017	-0.000040	$<10^{-4}$	$<10^{-4}$
2	Sobic.004G284001		0.0061	-0.014	$<10^{-4}$	1
3	Sobic.004G282800	Glucose-methanol-choline (GMC) oxidoreductase family	-0.0030	0.027	$<10^{-4}$	0.05
4	Sobic.009G248801		-0.00060	0.010	$<10^{-4}$	1
5	Sobic.004G278650	BURP domain containing protein	0.0011	-0.0030	0.001	1
6	Sobic.004G184800		0.00059	-0.0019	0.02	0.7
7	Sobic.006G073400	F-box family protein	-0.0021	-0.0073	0.002	0.4
8	Sobic.004G291200		0.00064	-0.0046	0.04	1
9	Sobic.006G249100		-0.0011	-0.0058	0.02	0.9
10	Sobic.010G208750	Ribosomal protein L20	-0.0079	0.031	$<10^{-4}$	$<10^{-4}$
11	Sobic.010G085500		-0.00030	0.0076	0.7	$<10^{-4}$
12	Sobic.007G092900	Cytochrome P450, family 71, subfamily A, polypeptide 25	0.0052	-0.0038	1	$<10^{-4}$
13	Sobic.008G183900	NAC domain containing protein 42	0.0060	-0.0048	0.9	$<10^{-4}$
14	Sobic.002G323500	Alpha/beta-Hydrolases superfamily protein	0.0099	-0.0064	1	$<10^{-4}$
15	Sobic.002G100200		0.0038	-0.0031	0.9	0.008
16	Sobic.002G278800	Ribonuclease T2 family protein	-0.0011	0.0074	0.2	$<10^{-4}$
17	Sobic.004G274700	SNARE associated Golgi protein family	-0.016	-0.00030	0.9	$<10^{-4}$
18	Sobic.004G270000	Clathrin adaptor complexes medium subunit family protein	0.0075	0.0017	$<10^{-4}$	$<10^{-4}$

*Indexes (Ind.) are given as reference to Fig. 3.4 as well as pre-scaled coordinates. Annotations are predicted Arabidopsis orthologs. The *p*-values were calculated using the Wald Test and corrected for multiple testing using the Benjamini-Hochberg correction.

for ~42% of the total variance. The PC2 axis separates genotypes with BTx623 genetic background from Kaoliangs, accounting for ~17% of the total expression variance. There is a clear structure with four discrete clusters. Starting in the top left corner and moving clockwise, these are Kaoliang-Chilled, Kaoliang-Control, BTx623 background-Control, and BTx623 Background-Chilled. Both NIL+ and NIL- group with BTx623 for control and chilling treatments. This is not surprising based on their shared genetic background and is consistent with previous results showing no significant pathway regulation by the introgression.

To identify co-regulated gene response in the NILs and investigate regulatory function, we examined the expression patterns of top DE genes relative to NIL parents. First, to identify the significant differentially regulated genes, we filtered for genes with a log2 fold change >2 and a Benjamini-Hochberg corrected *p*-value < 0.05 for genotype or GxT effects between NIL+ and NIL-. After filtering, 17 genes remained out of ~30,000 total. We then rescaled component vectors for the 17 genes and plotted them over the coordinates. The direction of the arrow shows the influence of the upregulation of the gene in the NILs relative to the parents. Because we only filtered for differentially expressed genes between NILs, we can assume that differential regulation in all plotted genes originates from polymorphisms in the introgressed region (Fig. 3.4).

Of the 17 plotted genes, nine are negative on the PC2 axis, suggesting that upregulation contributes to NIL similarity to BTx623 and that the introgression induces down-regulation in these genes. Of the nine genes with strongly negative PC2, one gene, 8, is near zero on PC1, suggesting upregulation across treatments in BTx623, while five genes (15, 12, 13, 14, 2) are positive on PC1, indicating upregulation in BTx623 under control conditions. Three genes have negative PC1 (5, 7, 9), indicating upregulation in BTx623 during chilling conditions.

Conversely, eight genes are positive or near zero for PC2, showing that upregulation contributes to similarity to Kaoliang for NILs and that the introgression induces upregulation. Five positive PC2 genes (16, 10, 3, 4) are near 0 for PC1, indicating that these genes are expressed across treatments in the Kaoliangs and are upregulated regardless of treatment by the introgression. While one gene, 18, has a strongly positive PC1 suggesting upregulation in the Kaoliangs under control conditions. The final two genes (17, 1) are negative at PC1, suggesting the up-regulation of these genes in response to chilling in the Kaoliangs is controlled somewhere within the introgression. Overall, there appears to be little regulatory coordination among genes which suggests independent regulation. Further, out of the 17 DE genes, only two (17, 1) have expression patterns suggesting chilling tolerance adaptation.

Cis-regulation underlies expression changes in almost half of differentially expressed genes

The introgression regulates the differential expression of the 17 significant genes in either *cis* or *trans* fashion. To identify which of the genes' expression is controlled by *cis*-regulation, we looked at expression changes in genes located within the introgression. To do this, we filtered for genes located within the NIL+ introgressions. These genes were further filtered for log2 fold change > 2 and a corrected *p*-value < 0.1 for genotype or GxE (Fig. 3.5). Out of all the 225 total introgressed genes, we found eight with significant genotypic or GxT interactions in their expression pattern, with only Sobic.004G270000 and Sobic.004G274700 exhibiting significant GxT interactions. Sobic.004G270000 is predicted as a sorghum ortholog for a clathrin adaptor complex subunit and is very significantly upregulated in the chilling tolerant NILs under control conditions, while Sobic.004G274800 is a predicted ortholog of SNARE-associated Golgi protein and is upregulated in both NILs under chilling treatment, but much more highly in NIL+. Overall, of the 17 genes with introgression-induced expression changes, seven are likely due to

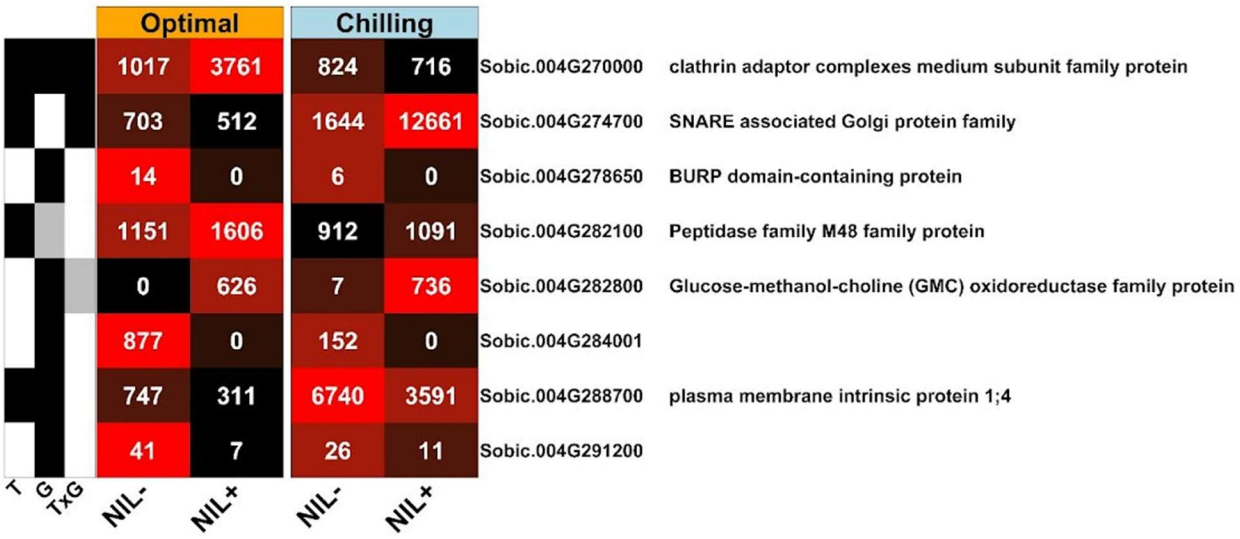

Figure 3.5. Introgressed genes with *cis*-regulatory changes. Expression of introgressed genes under optimal (orange) and chilling (blue) conditions, which display significant G or GxT interactions. Horizontally, red represents highest expression rank, while black represents lowest. Median of ratios normalized mean expression is displayed in the heatmap cell. Labels are gene ID and predicted gene annotation. Left sidebar shows significance of interaction: black<0.05, 0.05<grey<0.1, white>0.1. The *p*-values were calculated using the Wald test and corrected for multiple testing bias using the Benjamini-Hochberg correction.

polymorphisms in *cis*-regulatory elements within the introgression, while the other ten are *trans*-regulated.

Discussion

Multiple unrelated polymorphisms within introgression likely underlie DE between NILs

In this study, we confirmed previous results that *Tannin1* does not regulate tolerance to controlled chilling stress and investigated *Tannin1*'s functional conservation with Arabidopsis *TTG1*. From the presented data, genes with differential expression between NILs appear to be mainly regulated independently and not by a single transcription factor such as *Tannin1*. Overall, expression patterns in NILs overwhelmingly reflect BTx623 genetic background and show no major regulatory differences. Moreover, we only detected 17 total genes which are regulated by polymorphisms at the introgression (Fig. 3.4). Of the 17 differentially regulated genes, seven are located within the introgression and display variable expression patterns, suggesting control by

independent *cis*-regulatory mutations (Fig. 3.5). Though this seems disproportionate, it is unsurprising as the introgression exhibits a disproportionately high polymorphic density between NILs compared to the rest of the genome which is overwhelmingly isogenic (Schuh, 2023). Further, as there appears to be little regulatory organization in the ten *trans*-regulated genes (Fig. 3.4), it is probable that they also are primarily regulated independently, though by protein-coding polymorphisms located within the introgression.

Differential gene expression suggests that QTN for qCT04.62 are not in NIL+

Data from this study supports previous conclusions that *pCT04.62+/Tan1* introgression likely does not contain the QTN underlying *qCT04.62* regulation of chilling tolerance. We found no upregulation of chilling tolerant pathways in NIL+ and only 17 differentially regulated genes. Further, for the DE genes, there was little evidence to point to any of them contributing to chilling tolerance. Although it is possible that the chilling stress applied by growth chambers was not sufficient to induce *qCT04.62* chilling tolerance mechanisms, we know chilling stress was applied as there was clear induction of chilling response for all lines as well as significant ($p < 10^{-4}$) chilling-induced upregulation of CBF orthologs (Fig. 3.3A, Fig. 3.4). It is also possible that some other mechanism contributes to chilling tolerance in the Kaoliangs, which is not induced by chilling stress. Further studies will have to be conducted to exclude this possibility.

Variation at Tan1 does not regulate chilling response in sorghum

As the NIL pairs differ for *Tan1* wildtype allele versus the *tan1b* loss of function allele but exhibit no chilling tolerance mechanisms, we can conclude with fair certainty that *Tan1* does not regulate chilling tolerance in our study system. Though this conclusion can not be applied to field-based systems without further studies, this data does point towards a linkage between *Tan1* and a chilling tolerance regulator, with recombination breaking the association during NIL

creation. This conclusion is unexpected as both *Tannin1* and *Tannin2* were located near significant peaks in multiple chilling tolerance mapping studies, which suggests that grain tannins or MBW pleiotropy are involved in chilling tolerance regulation. Additionally, as this experiment was conducted under controlled stress conditions, we cannot entirely reject *Tannin1* as the causal gene underlying *qCT04.62* since unaccounted environmental stressors like mold, herbivores, or environmental variability may still drive the association (Esele et al., 1993; Wu et al., 2019).

Field experiments with current NILs or laboratory experiments with different NILs with introgressions encompassing *qCT04.62* but lacking the wildtype *Tan1* allele could be conducted to validate these results. Further, epistatic interactions with unintrogressed chilling tolerance loci could also inhibit *Tan1* function in the NIL+, though this hypothesis could be ruled out by looking for QTL by QTL interactions involving *qCT04.62* in previous mapping studies. If the association between *Tan1* and chilling tolerance is due to linkage, breeders can recombine the alleles to break the association and use *qCT04.62* to improve chilling tolerance and take advantage of early planting in the cropping system. Further, if *qCT04.62* regulatory function is not controlled by *Tan1*, it must be controlled by an unidentified cold tolerance gene, which though useful in sorghum, if cloned, could have implications for cold tolerance improvement in other crops such as maize and rice.

Tan1 has lost master regulatory functions of TTG1

This study also allowed us to study the function of the sorghum ortholog of *TTG1*. *TTG1* is the W subunit (WD40) of the trimeric MBW complex, which also includes Mybs and bHLH subunits (Tian & Wang, 2020). In Arabidopsis, the MBW ternary complex regulates many phenotypes. The current model of MBW complex function states that WD40 subunit regulates all

phenotypes, while the BHLH and Myb subunits each have multiple paralogs with different paralogs having specificity for one or several regulatory phenotypes (Chen & Wang, 2019). In rice, the MBW complex likely has a similar function. The complex has been shown to regulate leaf trichome development and flavonoids in several tissues, with studies of *TTG1* homolog, OsTTG1 regulating leaf and shoot anthocyanidins and the bHLH transcription factor *Rc* regulating grain proanthocyanidins (Gu et al., 2011a; Yang et al., 2021). Further, *Rc* has also been shown to pleiotropically regulate seed dormancy though regulation of ABA biosynthesis, a metabolic product of flavonoid biosynthesis (Gu et al., 2011b).

Though studies of Arabidopsis *TTG1* and rice *OsTTG1* suggest that *Tannin1* should be a highly pleiotropic master regulator, this has not been examined in a controlled manner. As expected, *Tannin1* expression matches *TTG1*, *PAC1*, and *OsTTG1* and broadly exists across tissues (Yang et al., 2021). Though *TTG1* and *OsTTG1* regulate flavonoids in leaves, our experiments indicate that *Tannin1* does not, which is consistent with *PAC1* regulation of flavonoids in maize (Selinger & Chandler, 1999). These are interesting results, as it suggests a loss of function in the *PAC1*/*Tannin1* ancestor after speciation from rice but before speciation between sorghum and maize (Fig. 3.6). Furthermore, as *Tan1* and *PAC1* are both sufficient to rescue anthocyanin pigmentation in Arabidopsis *ttg1* mutants (Carey et al., 2004; Wu et al., 2012), it is unlikely that the loss of functionality is due to a mutation affecting the function of either *Tan1* or *PAC1*. Instead, the loss of function likely originates further downstream in the regulatory pathway.

This finding begins to unravel the paradox of non-functional *Tan1* alleles having become fixed in many sorghum populations without deleterious pleiotropic effects that might be expected

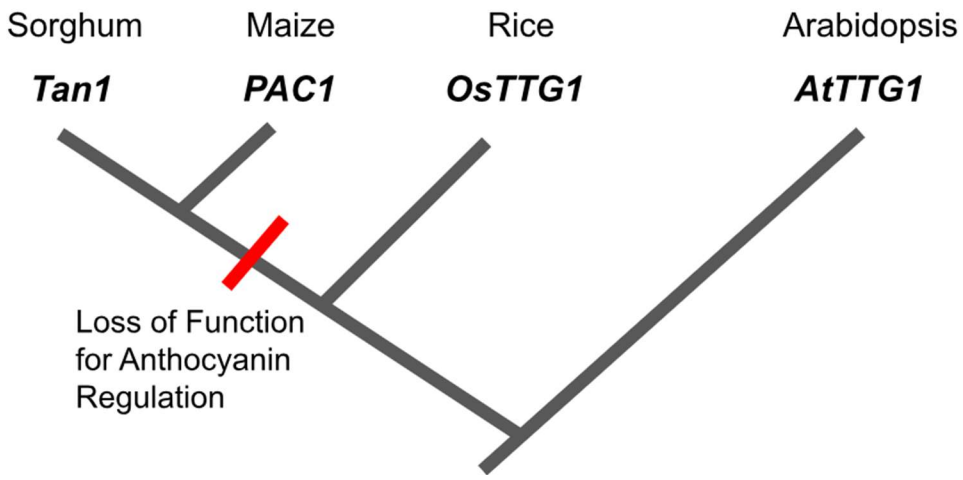

Figure 3.6. Hypothesis on loss of pleiotropic functions in *TTG1* orthologs in the Andropogonae after the split from rice. Loss of pleiotropic function occurring before sorghum-maize speciation, but after speciation with rice.

if *Tan1* was a master regulator. Also, it sheds more light on the evolutionary origin of sorghum chilling tolerance as either ancestral or derived (Marla et al., 2019). The lack of pleiotropic control over chilling tolerance by *Tan1* precludes the possibility of coinheritance through pleiotropy and suggests a derived origin for chilling tolerance, where the Kaoliangs adapted to chilling tolerance through novel mutations. It does not, however, fully discard the ancestral hypothesis, which posits the inadvertent purging of ancestral chilling tolerance by American breeders when selecting for colocalized traits. As chilling tolerance is a complex trait, it is possible to have a mixed origin for the trait, with both derived and ancestral alleles. Also, though pleiotropic coinheritance is not likely for *Tan1* and chilling tolerance, a tight linkage may still produce meaningful co-inheritance, depending on recombination frequency and selection pressure, resulting in the loss of ancestral chilling tolerant alleles. Overall, this study confirms previous work that *Tan1* does not regulate chilling tolerance and suggests that *qCT04.62* may be useful as a breeding target. Further, it takes a step towards understanding MBW function in sorghum and illuminating the evolutionary history of *Tanin1* and chilling tolerance.

Material and Methods

Plant materials and development of near isogenic lines

Sorghum genotypes were chosen for this study based on published field evaluation of each line for early season chilling tolerance (Franks et al., 2006; Kapanigowda et al., 2013). For NIL development, all crosses were made at Kansas State University. Three RILs from the chilling tolerant NAM BTx623 x Hong Ke Zi (PI 567946) family were used as starting material to reduce subsequent backcrossing (Marla et al., 2019). The RILs were then crossed to BTx623. F1 progeny were selected on two criteria, for heterozygosity at the QTL of interest using a KASP marker system and visually for resemblance to BTx623, the recurrent parent. Selected progeny were then backcrossed to BTx623. Selection and backcrossing were repeated four times. 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).

Chilling treatment and RNA sequencing

The experiment was carried out in controlled environment chambers (Conviron Model CMP6050, Manitoba, Canada) at the Plant Growth Facilities at Colorado State University in Fort Collins, CO. Experiment designs were created and randomized using a custom R v4.1.2 script (R Core Team, 2021). All plants were potted in 1.5-inch Cone-tainers using Lambert LM-HP potting soil and grown using a 12 h photoperiod and 700 μmol m^{-2} s^{-1} light intensity. After sowing ten 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 a bottom watering system.

After a 36 hour chilling treatment, 2g 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. 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.

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. Heatmap was 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 Wickham, 2016). Mean expression was displayed in the heatmap cell. *P*-values were included in the sidebar with white $p \geq 0.1$, gray $0.1 > p > 0.05$, and black $p \leq 0.05$. 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).

REFERENCES: CHAPTER III

Allen, D. J., & Ort, D. R. (2001). Impacts of chilling temperatures on photosynthesis in warm-climate plants. *Trends in Plant Science*, *6*(1), 36–42. https://doi.org/10.1016/S1360-1385(00)01808-2

Berry, E. M., Dernini, S., Burlingame, B., Meybeck, A., & Conforti, P. (2015). Food security and sustainability: Can one exist without the other? *Public Health Nutrition*, *18*(13), 2293–2302. https://doi.org/10.1017/S136898001500021X

Burow, G., Burke, J. J., Xin, Z., & Franks, C. D. (2011). Genetic dissection of early-season cold tolerance in sorghum (Sorghum bicolor (L.) Moench). *Molecular Breeding*, *28*(3), 391–402. http://dx.doi.org.ezproxy2.library.colostate.edu/10.1007/s11032-010-9491-4

Carey, C. C., Strahle, J. T., Selinger, D. A., & Chandler, V. L. (2004). Mutations in the pale aleurone color1 Regulatory Gene of the Zea mays Anthocyanin Pathway Have Distinct Phenotypes Relative to the Functionally Similar TRANSPARENT TESTA GLABRA1 Gene in Arabidopsis thaliana [W]. *The Plant Cell*, *16*(2), 450–464. https://doi.org/10.1105/tpc.018796

Chen, S., & Wang, S. (2019). GLABRA2, a Common Regulator for Epidermal Cell Fate Determination and Anthocyanin Biosynthesis in Arabidopsis. *International Journal of Molecular Sciences*, *20*(20), Article 20. https://doi.org/10.3390/ijms20204997

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics*, *29*(1), 15–21. https://doi.org/10.1093/bioinformatics/bts635

Doggett, H. (1970). *Sorghum*. Longmans.

Du, Z., Zhou, X., Ling, Y., Zhang, Z., & Su, Z. (2010). agriGO: A GO analysis toolkit for the agricultural community. *Nucleic Acids Research*, *38*(suppl_2), W64–W70. https://doi.org/10.1093/nar/gkq310

Esele, J., Frederiksen, R., & Miller, F. (1993). The association of genes controlling caryopsis traits with grain mold resistance in sorghum. *Phytopathology*, *83*(5), 490–495.

Fiedler, K., Bekele, W. A., Duensing, R., Gründig, S., Snowdon, R., Stützel, H., Zacharias, A., & Uptmoor, R. (2014). Genetic dissection of temperature-dependent sorghum growth during juvenile development. *Theoretical and Applied Genetics*, *127*(9), 1935–1948. https://doi.org/10.1007/s00122-014-2350-7

Fiedler, K., Bekele, W. A., Friedt, W., Snowdon, R., Stützel, H., Zacharias, A., & Uptmoor, R. (2012). Genetic dissection of the temperature dependent emergence processes in sorghum using a cumulative emergence model and stability parameters. *Theoretical and Applied Genetics*, *125*(8), 1647–1661. https://doi.org/10.1007/s00122-012-1941-4

Franks, C. D., Burow, G. B., & Burke, J. J. (2006). A Comparison of U.S. and Chinese Sorghum Germplasm for Early Season Cold Tolerance. *Crop Science*, *46*(3), 1371–1376. https://doi.org/10.2135/cropsci2005.08-0279

Goodstein, D. M., Shu, S., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., & Rokhsar, D. S. (2012). Phytozome: A comparative platform for green plant genomics. *Nucleic Acids Research*, *40*(Database issue), D1178–D1186. https://doi.org/10.1093/nar/gkr944

Gu, X.-Y., Foley, M. E., Horvath, D. P., Anderson, J. V., Feng, J., Zhang, L., Mowry, C. R., Ye, H., Suttle, J. C., Kadowaki, K., & Chen, Z. (2011a). Association Between Seed Dormancy and Pericarp Color Is Controlled by a Pleiotropic Gene That Regulates

Abscisic Acid and Flavonoid Synthesis in Weedy Red Rice. *Genetics*, *189*(4), 1515–1524. https://doi.org/10.1534/genetics.111.131169

Gu, X.-Y., Foley, M. E., Horvath, D. P., Anderson, J. V., Feng, J., Zhang, L., Mowry, C. R., Ye, H., Suttle, J. C., Kadowaki, K., & Chen, Z. (2011b). Association Between Seed Dormancy and Pericarp Color Is Controlled by a Pleiotropic Gene That Regulates Abscisic Acid and Flavonoid Synthesis in Weedy Red Rice. *Genetics*, *189*(4), 1515–1524. https://doi.org/10.1534/genetics.111.131169

Gu, Z., Eils, R., & Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics*, *32*(18), 2847–2849. https://doi.org/10.1093/bioinformatics/btw313

Guo, J., Ren, Y., Tang, Z., Shi, W., & Zhou, M. (2019). Characterization and expression profiling of the ICE-CBF-COR genes in wheat. *PeerJ*, *7*, e8190. https://doi.org/10.7717/peerj.8190

Hadley Wickham. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. https://ggplot2.tidyverse.org

Islam, S., Cenacchi, N., Sulser, T. B., Gbegbelegbe, S., Hareau, G., Kleinwechter, U., Mason-D'Croz, D., Nedumaran, S., Robertson, R., Robinson, S., & Wiebe, K. (2016). Structural approaches to modeling the impact of climate change and adaptation technologies on crop yields and food security. *Global Food Security*, *10*, 63–70. https://doi.org/10.1016/j.gfs.2016.08.003

Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2006). Functional Analysis of Rice DREB1/CBF-type

Transcription Factors Involved in Cold-responsive Gene Expression in Transgenic Rice. *Plant and Cell Physiology*, *47*(1), 141–153. https://doi.org/10.1093/pcp/pci230

Jaglo-Ottosen, K. R., Gilmour, S. J., Zarka, D. G., Schabenberger, O., & Thomashow, M. F. (1998). Arabidopsis CBF1 Overexpression Induces COR Genes and Enhances Freezing Tolerance. *Science*, *280*(5360), 104–106. https://doi.org/10.1126/science.280.5360.104

Kapanigowda, M. H., Perumal, R., Aiken, R. M., Herald, T. J., Bean, S. R., & Little, C. R. (2013). Analyses of sorghum [*Sorghum bicolor* (L.) Moench] lines and hybrids in response to early-season planting and cool conditions. *Canadian Journal of Plant Science*, *93*(5), 773–784. https://doi.org/10.4141/cjps2012-311

Knoll, J., Gunaratna, N., & Ejeta, G. (2008). QTL analysis of early-season cold tolerance in sorghum. *Theoretical and Applied Genetics*, *116*(4), 577–587. https://doi.org/10.1007/s00122-007-0692-0

Li, Q., Zheng, Q., Shen, W., Cram, D., Fowler, D. B., Wei, Y., & Zou, J. (2015). Understanding the Biochemical Basis of Temperature-Induced Lipid Pathway Adjustments in Plants. *The Plant Cell*, *27*(1), 86–103. https://doi.org/10.1105/tpc.114.134338

Liao, Y., Smyth, G. K., & Shi, W. (2014). featureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, *30*(7), 923–930. https://doi.org/10.1093/bioinformatics/btt656

Liu, X., Ma, D., Zhang, Z., Wang, S., Du, S., Deng, X., & Yin, L. (2019). Plant lipid remodeling in response to abiotic stresses. *Environmental and Experimental Botany*, *165*, 174–184. https://doi.org/10.1016/j.envexpbot.2019.06.005

Long, S. P., & Spence, A. K. (2013). Toward Cool C4 Crops. *Annual Review of Plant Biology*, *64*(1), 701–722. https://doi.org/10.1146/annurev-arplant-050312-120033

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Lyons, J. M. (1973). Chilling Injury in Plants. *Annual Review of Plant Physiology*, *24*(1), 445–466. https://doi.org/10.1146/annurev.pp.24.060173.002305

Marla, Burow, G., Chopra, R., Hayes, C., Olatoye, M. O., Felderhoff, T., Hu, Z., Raymundo, R., Perumal, R., & Morris, G. P. (2019). Genetic Architecture of Chilling Tolerance in Sorghum Dissected with a Nested Association Mapping Population. *G3 Genes|Genomes|Genetics*, *9*(12), 4045–4057. https://doi.org/10.1534/g3.119.400353

Marla, S., Terry Felderhoff, Hayes, C., Perumal, R., Wang, X., Poland, J., & Morris, G. P. (2023). Genomics and Phenomics Enabled Prebreeding Improved Early-Season Chilling Tolerance in Sorghum. *G3 Genes|Genomes|Genetics*. https://doi.org/10.1101/2022.10.31.514536

Marla, Shiva, S., Welti, R., Liu, S., Burke, J. J., & Morris, G. P. (2017). Comparative Transcriptome and Lipidome Analyses Reveal Molecular Chilling Responses in Chilling-Tolerant Sorghums. *The Plant Genome*, *10*(3), plantgenome2017.03.0025. https://doi.org/10.3835/plantgenome2017.03.0025

Marozsán-Tóth, Z., Vashegyi, I., Galiba, G., & Tóth, B. (2015). The cold response of CBF genes in barley is regulated by distinct signaling mechanisms. *Journal of Plant Physiology*, *181*, 42–49. https://doi.org/10.1016/j.jplph.2015.04.002

McDonald, G. K., & Gill, G. S. (2009). Improving crop competitiveness with weeds: Adaptations and trade-offs. In *Crop Physiology: Applications for Genetic Improvement and Agronomy* (pp. 449–488). Academic Press.

Mira, M. M., Ibrahim, S., Hill, R. D., & Stasolla, C. (2021). Cold stress in maize (Zea mays) is alleviated by the over-expression of Phytoglobin 1 (ZmPgb1.1). *Plant Physiology and Biochemistry*, *167*, 901–910. https://doi.org/10.1016/j.plaphy.2021.08.046

Moghimi, N., Desai, J. S., Bheemanahalli, R., Impa, S. M., Vennapusa, A. R., Sebela, D., Perumal, R., Doherty, C. J., & Jagadish, S. V. K. (2019). New candidate loci and marker genes on chromosome 7 for improved chilling tolerance in sorghum. *Journal of Experimental Botany*, *70*(12), 3357–3371. https://doi.org/10.1093/jxb/erz143

Monk, R., Franks, C., & Dahlberg, J. (2014). Sorghum. In *Yield Gains in Major U.S. Field Crops* (pp. 293–310). John Wiley & Sons, Ltd. https://doi.org/10.2135/cssaspecpub33.c11

Mosier, A., Kroeze, C., Nevison, C., Oenema, O., Seitzinger, S., & van Cleemput, O. (1998). Closing the global N2O budget: Nitrous oxide emissions through the agricultural nitrogen cycle. *Nutrient Cycling in Agroecosystems*, *52*(2), 225–248. https://doi.org/10.1023/A:1009740530221

Nyachoti, C. M., Atkinson, J. L., & Leeson, S. (1997). Sorghum tannins: A review. *World's Poultry Science Journal*, *53*(1), 5–21. https://doi.org/10.1079/WPS19970002

Qaim, M. (2020). Role of New Plant Breeding Technologies for Food Security and Sustainable Agricultural Development. *Applied Economic Perspectives and Policy*, *42*(2), 129–150. https://doi.org/10.1002/aepp.13044

Qin, F., Sakuma, Y., Li, J., Liu, Q., Li, Y.-Q., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2004). Cloning and Functional Analysis of a Novel DREB1/CBF Transcription Factor Involved in Cold-Responsive Gene Expression in Zea mays L. *Plant and Cell Physiology*, *45*(8), 1042–1052. https://doi.org/10.1093/pcp/pch118

R Core Team. (2021). *R: A language and environment for statistical computing.* (4.1.2). R Foundation for Statistical Computing. https://www.r-project.org/

Raymundo, R., Sexton-Bowser, S., Ciampitti, I. A., & Morris, G. P. (2021). Crop modeling defines opportunities and challenges for drought escape, water capture, and yield increase using chilling-tolerant sorghum. *Plant Direct*, *5*(9), e349. https://doi.org/10.1002/pld3.349

Savitch, L. V., Allard, G., Seki, M., Robert, L. S., Tinker, N. A., Huner, N. P. A., Shinozaki, K., & Singh, J. (2005). The Effect of Overexpression of Two Brassica CBF/DREB1-like Transcription Factors on Photosynthetic Capacity and Freezing Tolerance in Brassica napus. *Plant and Cell Physiology*, *46*(9), 1525–1539. https://doi.org/10.1093/pcp/pci165

Schuh, A. (2023). Sorghum *Tannin1* lost pleiotropic regulatory function in this ortholog of Arabidopsis master regulator *TTG1*. Colorado State University.

Selinger, D. A., & Chandler, V. L. (1999). A Mutation in the pale aleurone color1 Gene Identifies a Novel Regulator of the Maize Anthocyanin Pathway. *The Plant Cell*, *11*(1), 5–14. https://doi.org/10.1105/tpc.11.1.5

Shi, Y., Ding, Y., & Yang, S. (2018). Molecular Regulation of CBF Signaling in Cold Acclimation. *Trends in Plant Science*, *23*(7), 623–637. https://doi.org/10.1016/j.tplants.2018.04.002

Tian, H., & Wang, S. (2020). TRANSPARENT TESTA GLABRA1, a Key Regulator in Plants with Multiple Roles and Multiple Function Mechanisms. *International Journal of Molecular Sciences*, *21*(14), 4881. https://doi.org/10.3390/ijms21144881

Vida, F. B. P. de, Laca, E. A., Mackill, D. J., Fernández, G. M., & Fischer, A. J. (2006). Relating rice traits to weed competitiveness and yield: A path analysis. *Weed Science*, *54*(6), 1122–1131. https://doi.org/10.1614/WS-06-042R.1

Wu, Y., Guo, T., Mu, Q., Wang, J., Li, X., Wu, Y., Tian, B., Wang, M. L., Bai, G., Perumal, R., Trick, H. N., Bean, S. R., Dweikat, I. M., Tuinstra, M. R., Morris, G., Tesso, T. T., Yu, J., & Li, X. (2019). Allelochemicals targeted to balance competing selections in African agroecosystems. *Nature Plants*, *5*(12), 1229–1236. https://doi.org/10.1038/s41477-019-0563-0

Wu, Y., Li, X., Xiang, W., Zhu, C., Lin, Z., Wu, Y., Li, J., Pandravada, S., Ridder, D. D., Bai, G., Wang, M. L., Trick, H. N., Bean, S. R., Tuinstra, M. R., Tesso, T. T., & Yu, J. (2012). Presence of tannins in sorghum grains is conditioned by different natural alleles of Tannin1. *Proceedings of the National Academy of Sciences*, *109*(26), 10281–10286. https://doi.org/10.1073/pnas.1201700109

Yang, X., Wang, J., Xia, X., Zhang, Z., He, J., Nong, B., Luo, T., Feng, R., Wu, Y., Pan, Y., Xiong, F., Zeng, Y., Chen, C., Guo, H., Xu, Z., Li, D., & Deng, G. (2021). OsTTG1, a WD40 repeat gene, regulates anthocyanin biosynthesis in rice. *The Plant Journal*, *107*(1), 198–214. https://doi.org/10.1111/tpj.15285

Zhang, X., Fowler, S. G., Cheng, H., Lou, Y., Rhee, S. Y., Stockinger, E. J., & Thomashow, M. F. (2004). Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant Arabidopsis. *The Plant Journal*, *39*(6), 905–919. https://doi.org/10.1111/j.1365-313X.2004.02176.x

APPENDIX I: SUPPLEMENTAL FIGURES

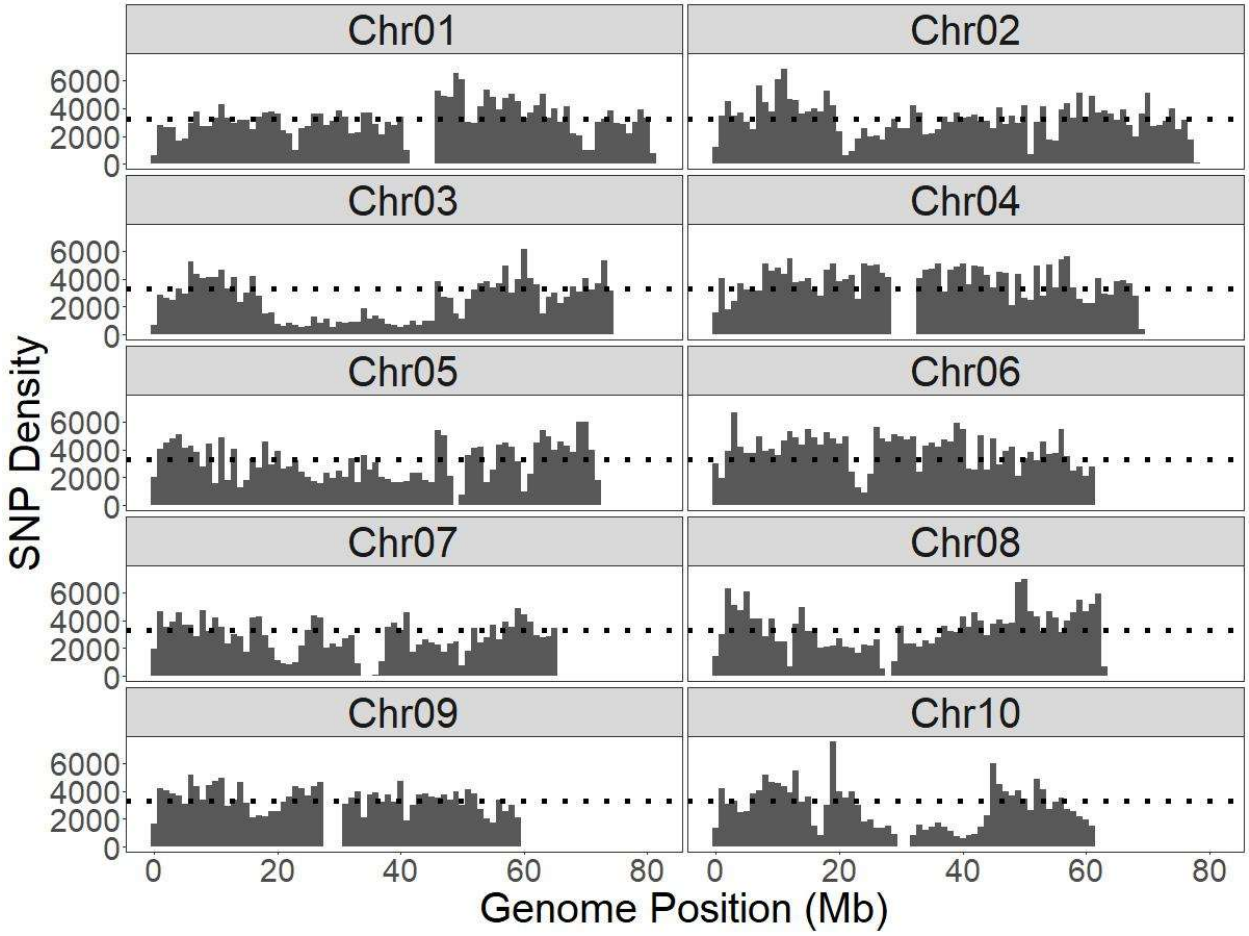

Fig S1. SNP density across the genome for all sorghum lines. Window size is 1 Mb. Dotted line is average genome wide SNP density.

LIST OF ABBREVIATIONS

CBF

C-repeat Binding Factor

CI

Confidence Interval

COR

Cold Regulated Genes

CRT/DRE

C-repeat/dehydration Responsive Element

***CT04.62*$^{+}$**

The chilling tolerant allele of *qSBCT04.62* which originated in HKZ

***CT04.62*$^{-}$**

The chilling sensitive allele of *qSBCT04.62* which originated in BTx623

DE

Differential Expression

ESV

Early Season Vigor

GO

Gene Ontology

GxT

Genotype by Treatment

HKZ

Hong Ke Zi

ICE

Inducer of CBF Expression

JA

Jasmonic Acid

JLM

Joint Linkage Mapping

LD

Linkage Disequilibrium

MAS

Marker Assisted Selection

Mb

Megabase (1,000,000 base pairs)

MBW Complex

MYB-bHLH-WD40 Complex

mL

Milliliter

NAM

Nested Association Mapping

NIL

Near Isogenic Line

NIL+

Near Isogenic Line with *pCT04.62+/Tan1* introgression

NIL-

Near Isogenic Line without chilling tolerant haplotype at introgression site

NPQ

Non-Photochemical Quenching

PA

Phosphatidic Acid

PAC1

Pale Aleurone Color1

PC

Phosphatidyl Choline

pCT04.62+/Tan1

Partial *CT04.62+* and *Tan1* introgression

PVE

Percentage of variance explained

QTL

Quantitative Trait Locus

QTN

Quantitative Trait Nucleotide

RIL

Recombinant Inbred Line

ROS

Reactive Oxygen Species

SNP

Single Nucleotide Polymorphism

Tan1

Functional *Tannin1* allele

Tannin1

Tannin1 gene

tan1

Non-functional *Tannin1* allele

TTG1

TRANSPARENT TESTA GLABRA1

VCF

Variant Call File