PHENOTYPIC ANALYSIS AND GENOMIC ANALYSIS OF RHIZOMATOUSNESS IN SORGHUM

A Thesis

by

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ABSTRACT

Increasingly serious greenhouse gas effects and soil erosion have raised demand for crops with robust belowground systems and carbon sequestration ability. Rhizomes are photoassimilate storage plant tissues, and their biomass can function as important targets for increasing the carbon sequestration capacity of perennial crops. Sorghum is an ideal crop, having rhizomatous wild relatives such as *Sorghum propinquum* (Kunth) Hitchc that have the same ploidy as annual cultivars.

In this study, twelve $F_{3:4}$ heterogeneous inbred families (HIFs) derived from a *Sorghum bicolor* (L.) Moench and *S. propinquum* cross were planted in a greenhouse, and two of their $F_{4:5}$ HIF progeny were employed in field cultivation. Thirteen traits, as well as an additional four traits, were investigated in $F_{4:5}$ and $F_{3:4}$, respectively. High-range variations were found in most of the traits, with many also showing a high heritability. The correlation analysis suggested a positive correlation between rhizome biomass and aboveground biomass, as well as grain yield.

A bulked segregant analysis (BSA) approach was proposed and used for screening the linked markers related to rhizome biomass, whereas no “rhizome biomass” specific simple sequence repeat (SSR) markers were identified and the presence or absence of rhizome was finally analyzed. Twenty linked markers were found for rhizome presence, which roughly defined eight target genomic regions. Three of the

eight regions were overlapped by several rhizome-related quantitative trait loci (QTLs), while four regions partially coincided with vegetative branching QTLs, which were reported in other studies. Five potentially novel regions were found in total.

Our results suggested a situation in which rhizome biomass, aboveground biomass, and grain yield can be potentially improved at the same time, in addition to developing molecular tools both for breeding pipeline and next-step QTL mapping.

DEDICATION

To the selfless love from my mother and father.

（致父母无私之爱）

To penetrate the nature’s mystery, to feed the world.

（为晓天机，为育万民）

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Contributors

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NOMENCLATURE

RHBM	Rhizome Biomass
RTBM	Fibrous Root Biomass
BBM	Belowground Biomass
RTAG	Root Growth Angle
RHAG	Rhizome Growth Angle
RHN	Rhizome Number
RHL	Rhizome Length
FW	Flowering Time
BTN	Basal Tiller Number
RDSN	Rhizome-Derived Shoot Number
ABM	Aboveground Biomass
PH	Plant Height
GY	Grain Yield
HIF	Heterogeneous Inbred Family
BSA	Bulked Segregant Analysis

SSR	Simple Sequence Repeat
QTL	Quantitative Trait Locus

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CHAPTER I

INTRODUCTION

In recent years, the detrimental effects of climate change have become evermore apparent, with a major driver of climate change being the greenhouse effect. This effect is caused by excessive emissions of greenhouse gases (GHGs), which absorb solar radiation reflected by the ground surface and re-emit it, thereby increasing the surface temperature. GHGs mainly include carbon dioxide, methane, and nitrous oxide, among which carbon dioxide is the most prominent. In February 2021, the concentration of atmospheric carbon dioxide had reached 415.88 ppm, an increase of nearly 7% from the same month in 2009 (387.25 ppm). Globally, the average annual growth rate of carbon dioxide in 2019 had reached 2.64 ppm (Ed Dlugokencky, 2021). The continuous increase of the greenhouse effect will have a considerable impact on the stability of the ecosystem and civil society. The impact on agricultural ecosystems will also be significant, as it is widely subject to climate and weather.

An effective way to mitigate the greenhouse effect is increasing our capacity to absorb atmospheric carbon dioxide through plant photosynthesis and stably storing it underground through a process called carbon sequestration (Edenhofer, 2015). According to Edenhofer (2015), one of the critical ways to increase carbon sequestration is to develop new crop varieties with greater carbon storage capacity and similar grain yields. The belowground anatomy of plants is an essential component of carbon storage, including roots, root exudates and rhizomes (Ferchaud et al., 2016). In contrast to annual

row crops that grow fibrous roots, which can store modest amounts of carbon-containing photosynthetic products in their root systems, perennial crops store significant amounts of these nutrients in belowground tissues, including rhizomes and fibrous roots. A rhizome is a subsurface stem developed specifically by perennial plants, which often has a larger volume than the rest of the fibrous root system, thus conferring greater carbon storage capacity to perennial crops than to their annual counterparts. Therefore, developing new varieties of perennial crops that have greater rhizome production but are as productive as annuals in terms of yield is a feasible way to increase carbon sequestration.

The genus *Sorghum* contains both annual and perennial species, among which the cultivated annual *Sorghum bicolor* (L.) Moench (2n=2x=20) is fully interfertile with the perennial relative *Sorghum propinquum* (Kunth) Hitchcock (2n=2x=20) as they have the same ploidy (De Wet, 1978). For another perennial species *Sorghum halepense* (L.) Pers (2n=4x=40), the natural hybridization with *S. bicolor* also can be observed, just with a lower rate of around 11% (Whitmire, 2012). Sorghum is widely grown throughout the United States and diverse regions of the world, which gives this crop the potential to effectively increase carbon sequestration on a global scale. Sorghum is one of the first crops to have protocols for applied maker-assistant-selection (MAS), and numerous molecular markers have been available for aboveground traits in annual sorghum, such as stem diameter (Hart et al., 2001), basal tiller number (Zou et al., 2012), etc. However, rhizome biomass in perennial sorghum is still a novel trait, though it has important

implications in carbon sequestration. Therefore, sorghum is an ideal crop to evaluate the potential to increase rhizome and root biomass production and thereby ecological benefits while maintaining a high grain yield.

CHAPTER II

LITERATURE REVIEW

2.1 Perennial sorghum

Sorghum is the third-largest cereal grain crop in the United States (USGC, 2021). In many regions, including Asia, sorghum grains are mostly consumed by people as a food source, whereas in the United States sorghum is grown mainly as a forage crop to feed livestock. In recent years, ethanol produced from high-biomass sorghum has also become a major source of bioenergy (Whitmire, 2012).

The genus *Sorghum* contains five sections (De Wet, 1978), and both *Sorghum bicolor* (L.) Moench and *Sorghum propinquum* (Kunth) Hitchc. are from the same section *Eu-sorghum* and share the same chromosome number (2n = 2x = 20). *Sorghum bicolor* is an annual, non-rhizome species that expresses very limited perenniality in tropical and subtropical regions and strict annual growth habit in temperate regions (Liang, 1988). This species is also the most commonly cultivated grain sorghum cultivar in the United States (USDA, 2020). *Sorghum propinquum* originates from Southeast Asia (Magoon et al., 1961). This species shares many similar traits - such as relatively small seeds and vigorous tillers -with wild grasses; therefore, it is considered to be the wild relative of *S. bicolor* (Chittenden et al., 1994). Among the rhizomatous sorghum species, *S. propinquum* is the only one that has the same ploidy level as *S. bicolor* (Liang, 1988) and has been proven to produce fertile offspring. Johnsongrass [*Sorghum*

halepense (L.) Pers.] is reported as the hybrid of *S. propinquum* and *S. bicolor*, which has been the primary weed in sorghum cultivation (Paterson, 2008).

Following the initiative of Jackson from Land Institute (Jackson, 1980) to carry out perennial crop breeding in the 1980s, perennial sorghum breeding programs based on hybridization between cultivar and wild grass were conducted. Among them, the johnsongrass [*Sorghum halepense* (L.) Pers.] was preferred as one of the parental lines over *S. propinquum* (Piper & Kulakow, 1994; Arriola & Ellstrand, 1996; Habyarimana et al., 2018) because the former has a greater overwintering ability (Cox et al., 2018). However, in the rhizomatous sorghum species, *S. halepense* is a tetraploid (2n=2x=40) while *S. propinquum* and *S. bicolor* are diploids, meaning that the hybridization between *S. halepense* and *S. bicolor* is more difficult (De Wet, 1978) and often needs chromosome doubling by colchicine treatment. In addition, *S. propinquum* has a weaker rhizome development ability than *S. halepense*, which can reduce the risk of hybrid offspring growing invasive rhizomes like weeds (Jessup et al., 2017). In 2017, Jessup et al. (2017) released a perennial sorghum line PSH12TX09, which was derived from *S. bicolor* x *S. propinquum*, and reported a good overwintering phenotype of this line. The advantages of *S. propinquum* over *S. halepense* provide a new way to introgress perennialism traits into annual cultivars, thus broadening the prospect for perennial sorghum breeding.

2.2 Rhizome development

There exist many rhizomatous and rhizome-like plants in nature, some of which are important crops and some are weeds. In many plants, a rhizome forms a kind of modified swollen stem that functions as storage and vegetative reproduction organs. Rhizomes can be classified into stem tubers in morphology, and this category also includes potato (*Solanum tuberosum* L.) and yam (*Dioscorea alata* L.), etc. The stem tuber is essentially a kind of "modified stem", which is easy to be confounded with the modified lateral root, also known as tuberous roots, including sweet potato [*Ipomoea batatas* (L.) Lam.] and cassava (*Manihot esculenta* Crantz) (Wikipedia, n.d.).

In perennial grasses, rhizomes usually grow horizontally belowground and can confer weediness to some species through invasive vegetative dispersal (Paterson et al., 1995). Rhizomes originate from axillary buds near the soil surface, which is similar to basal tillers growing aboveground. The differentiation of rhizomes and basal tillers is driven by gravitropism (Foster et al., 2020) and along a positional gradient (Kong et al., 2015), and many genes regulating rhizome development overlap with those regulating tiller development, which could be related to the initiation or orientation of axillary buds (Jang et al., 2006; Kong et al., 2015). In perennial plants, the carbon-containing organics produced from photosynthesis in leaves are transported and accumulated in rhizomes (Cheplick & Gutierrez, 2000) so that they can survive underground during the winter, and regrow as new aerial shoots using the stored nutrients. The storage capacity of rhizomes is considerable. In *Miscanthus sinensis* Anderss., the biomass yield of

rhizomes is about 2.42 tons per acre, of which carbon storage is about 1.01 tons per acre (Christensen et al., 2016). And in the triploid hybrid grass *Miscanthus* × *giganteus* Greef et. Deu ex. Hodkinson et Renvoize, the average annual rhizome biomass yield can be even 8.70 tons per acre, with the carbon concentration of 41% (Dohleman et al., 2012). And in another grass, *Arundo donax* L., the rhizome biomass yield is about 6.47 tons per acre, which corresponds to around 2.78 tons carbon per acre (o Di Nasso et al., 2013; Proietti et al., 2017).

The morphological and physiological differences between roots and rhizomes endow rhizomatous plants with ecological significance. As Glover et al. (2010) and DuPont et al. (2014) studied, the root carbon content and biomass of perennial grass are 6.7 times and 3 to 7 times greater, respectively, than annual wheat. In perennial crops such as *Miscanthus*, the dry matter of the rhizome is about twice that of the root, with the carbon content also making up about 2.5 times that of the root (Christensen et al., 2016). The above research proves that perennial rhizomatous crops have higher carbon sequestration potential than annual crops.

2.2.1 Rhizome and other traits

The rhizome is a unique organ of perennial plants; its relationship with other traits - especially important agronomic traits - is an interesting aspect, which cannot be researched in annual plants. Moreover, the life strategies of annual crops and perennial crops are different. Annual crops use seeds for reproduction. In contrast, although perennial crops also produce seeds, they mainly rely on vegetative organs for asexual

reproduction, among which rhizomes that can survive under extreme conditions are the leading option (Silvertown & Dodd, 1996). This may cause the annuals and perennials to be different in developmental physiology, thus changing the correlation between some of the traits.

The fate of rhizome development is variable. It can grow underground throughout the entire growing season, whereas it can also bend upwards and grow as an aboveground shoot (rhizome-derived shoot). In Kansas, the longevity of rhizomes in johnsongrass is one year, which means after overwintering regrowth, the old rhizomes lose their function and decay (Anderson et al., 1960). However, the rhizomes can survive as long as six years in another grass *Phragmites australis* (Cav.) Trin. ex Steud., though the reserves consumption for regrowth begins on old rhizomes (Karunaratne et al., 2004). It was reported that the rhizomes of *Anemone nemorosa* L. could live for seven years (Shirreffs & Bell, 1984). And in grass *Miscanthus*×*giganteus*, the rhizome longevity is even longer, for nine years (Christian et al., 2009). Whether the rhizome develops as a rhizome-derived shoot depends upon both the species characteristic and the environment (Jessup et al., 2017). The rhizome-derived shoots that grow aboveground can normally grow vegetatively, flower, and produce grains like the aerial stems of annual crop. This provides theoretical possibilities for the correlation between rhizomes and aboveground traits, including vegetative traits, like basal tiller number and aboveground biomass, and yield traits, such as grain yield.

As for the relationship with the basal tiller, previous studies have shown that the genetic loci that control rhizomes and those that control the basal tillers partially overlap in sorghum (Jang et al., 2006). The connection between these two traits may be related to the initiation of axillary buds, the same source from which both rhizomes and basal tillers are derived (Kong et al., 2015; Jang et al., 2009). The relationship between rhizomes and flowering time is highly variable, which may change in different species and environments, and its mechanism is still unclear. In bamboo, the elongation of the rhizome is negatively correlated with the flowering time, which is related to the spatial competition of growth (Tachiki et al., 2015). In *S. halepense*, because the rhizome functions as a storage organ, the rapid accumulation of its biomass occurs after flowering (Monaghan, 1980). However, some studies have shown that there is no causal relationship between rhizome growth and flowering in *S. halepense* (Horowitz, 1972). The relationship between rhizomes and aboveground biomass also is not uniform among different plant species and environments. One study has reported that there was a positive correlation between plant height and above and belowground biomass in arbor *Actaea racemose*, a kind of perennial medicinal plant, with the aboveground biomass potentially being used to predict belowground biomass (Chamberlain et al., 2013). However, in another C4 grass species, *Miscanthus* × *giganteus*, rhizome biomass and aboveground biomass were negatively correlated in the early stage of vegetative growth, then showing a positive correlation in the rest of the season (Dohleman et al., 2012). The general opinion regarding the relationship between rhizome biomass and grain yield was

based on a “trade-off” pattern, which was observed in 27 perennial grass species (Wanger, 1990; Cox et al., 2002), yet several more recent studies showed that this trade-off occurs only within limited conditions and time, and some of these conditions can be broken (DeHaan et al., 2005; Cox et al., 2006; Cox et al., 2010).

CHAPTER III

TRAIT PERFORMANCE, CORRELATION, AND GENOMIC EVALUATION

3.1 Introduction

In February 2021, the concentration of the main atmospheric greenhouse gas carbon dioxide reached 415.88ppm, an increase of about 7% over 2019, and it is still increasing at an annual rate of 2.64ppm (Ed Dlugokencky, 2021). This will lead an increasing impact on both the ecosystem and human society.

An effective way to mitigate the greenhouse effect is to increase the absorption and assimilation of atmospheric carbon by plants, and to stably store the assimilation products belowground. This process is called carbon sequestration (Edenhofer, 2015). The root system of plants, including fibrous roots, root exudates and rhizomes, is one of the main carbon sinks of plants (Ferchaud et al., 2016). Among them, the rhizome represents a unique trait capable of significantly increasing the carbon storage and sequestration capacity of the widely grown grain crops in particular.

Rhizomes are modified belowground stems typically developed by perennial plants for resource storage and vegetative reproduction, compared with the annual crops that only grow roots. Morphologically, the rhizome also refers to the stem tuber, which includes some tuberous crops like potato (*Solanum tuberosum* L.) and yam (*Dioscorea alata* L.). Stem tuber can be distinguished from the root tuber, which mainly includes

sweet potato [*Ipomoea batatas* (L.) Lam.] and cassava (*Manihot esculenta* Crantz). Therefore, some common tuberous crops are also rhizomatous crops (Wikipedia, n.d.). The carbon storage capacity of many rhizomatous species is noteworthy. In *Miscanthus sinensis* Anderss., the rhizome biomass is about twice the root biomass, with the carbon content is around 2.5 times. Still in *Miscanthus*, the rhizomes can produce about 2.42 tons of biomass per acre, which corresponds to 1.01 tons of carbon (Christensen et al., 2016), about 0.8 tons more than annual sorghum root carbon content (Myers, 1980). And in the hybrid grass *Miscanthus* × *giganteus* Greef et. Deu ex. Hodkinson et Renvoize, the rhizome biomass yield and carbon content can even reach 8.07 tons per acre and 3.31 tons per acre, respectively, which is 3.35 tons more than annual sorghum root carbon content (Dohleman et al., 2012). In *Arundo donax* L., the rhizome biomass yield is about 6.47 tons per acre and 2.57 tons more carbon storage than annual sorghum (o Di Nasso et al., 2013; Proietti et al., 2017). Besides the important role in solving greenhouse effects, rhizomes also confer perennials several extra ecological benefits. Annual crops have a comparatively weak root system that uses more water, which can lead to the soil erosion of arable land (Huggins et al., 2001). Perennial crops can more effectively prevent soil erosion due to their higher biomass underground root and rhizome systems.

Sorghum is one of the most important crops in the United States and the world. Sorghum is utilized both as a grain and forage and is thus bred for both uses. In regions where sorghum grains are used as food or feed, high-grain yield varieties are the main goal for sorghum breeding. In some countries, including the United States, sorghum is

mainly used to feed animals, including grains, stems, and leaves; thus both high grain yield and high aboveground biomass sorghum varieties are competitive.

At present, the widely planted grain sorghum is entirely composed of annual species. However, the genus sorghum also has perennial species such as *Sorghum propinquum* and *Sorghum halepense*, each of which contains rhizomatous genotypes in their gene pool. Among them, *Sorghum propinquum* and annual grain sorghum (*Sorghum bicolor*) have the same ploidy (Zhang et al., 2013), making it an ideal candidate for breeding new perennial grain sorghum cultivars.

Studying the underlying genetic regulation of rhizome development has a long history. In the beginning, the purpose was to control the damage of perennial weeds like johnsongrass (McWhorter, 1961). In recent years, however, it has been a breeding goal derived from the ecological value of rhizomes in carbon sequestration and preventing soil erosion and water loss (Cox et al., 2010; DeHaan et al., 2005). The genetic loci and corresponding molecular markers that regulate rhizome-related traits, such as rhizomatousness, overwintering, and rhizome number, etc., have been identified in several studies (Paterson et al., 1995; Washburn et al., 2013; Cheng et al., 2013; Kong et al., 2015; Huang et al., 2021) and compared between different species to study the nature of their evolution (Jang et al., 2006; Jang et al., 2009; Hu et al., 2011; Kong et al., 2015). The development and upgrade of molecular tools have accelerated and simplified the breeding process, which also improves the feasibility of molecular marker-assisted selection for perennial crops.

Rhizomatousness was previously thought to be controlled by a pair of dominant alleles by traditional genetic research, as mating annual *S. bicolor* and perennial *Sorghum sudanense* caused a 3:1 segregation ratio in F_2 progeny (Ramaswamy, 1973). Therefore, the existence of rhizomes was considered to be controlled by a dominant gene at that time (Ramaswamy, 1973). However, a later study (Paterson et al., 1995) using RFLP markers to map the F_2 progeny of *S. bicolor* x *S. propinquum* proved that rhizomatousness was controlled by quantitative trait loci (QTL). Three loci on linkage group (LG) C were found for regulating rhizome-derived shoot number, with seven loci distributed across seven LG regulating rhizome number. The rhizome distance was controlled by one locus on LG C and six loci controlled regrowth, with overlap existing between traits. This was the first comprehensive study on the genetic loci of rhizome-related traits. In the following years, Washburn et al. (2013) re-evaluated the sorghum SBI-01 chromosomal region where the QTLs related to overwintering (regrowth) are located and discovered two extra QTLs that controlled this trait. Later, Kong et al. (2015) mapped the QTLs regulating the presence or absence of rhizome, the rhizome number, and rhizome distances based on the study of Paterson et al. (1995) and compared those QTLs with vegetative branching QTLs (Kong et al., 2014).

Many QTLs regulating rhizome-related traits in sorghum closely correspond to homologs in rice, showing the conservation of these genetic regulation loci in the evolutionary process. For instance, *Rhz3* on rice (*Oryza sativa* × *Oryza longistaminata*) chromosome 4 corresponds to the loci on sorghum chromosome 6 (Hu et al., 2003),

QRbn2 QTL on rice chromosome 2 controlling rhizome number corresponds to QTLs on sorghum chromosome 4 controlling regrowth, and the QTLs on sorghum chromosome 10 for rhizomatousness correspond to rice chromosome 6 *QRi6* (Jang et al., 2006). Maize also has some rhizome-regulating QTLs that can correspond to rice and sorghum, but the number of homologs is fewer than either rice or sorghum due to the greater genetic distance between species (Westerbergh & Doebley, 2004). Rhizomatousness is a complex trait that relates to the number of rhizome-derived shoots, the rhizome length, as well as the subterranean rhizome number (Paterson et al., 1995). Further, little research focused on rhizome biomass in sorghum, which is the direct indicator of the carbon sequestration ability of perennial crops.

At present, one of the main obstacles for the large-scale replacement of perennials to their annual counterparts is the potential conflict of resource allocation between rhizomes and grains. Therefore, a case-by-case analysis of grain yield and rhizome biomass is a prerequisite for developing new perennial varieties.

Perenniality is considered an undesired trait in annual crops, as there is a widespread yet unconfirmed opinion that grain yield of perennial crops will be decreased due to reduced photoassimilate transport to grain production (Wagoner & Schaeffer, 1990; Silvertown & Dodd, 1996). However, according to a more recent quantitative trade-off model, changes in environment and genetics can lead two traits to move in the same direction, even if they are negatively correlated (Roff et al., 2002). Just as breeders can combine two traits that appear to be opposed, the environment and targeted trait

selection play an important role that affects plant traits. In this case, numerous physiological studies would suggest that rhizomes do not compete with grains for photoassimilates as they are sourced by proximally different portions of the plant canopy.

Evolutionarily, the trade-off pattern between rhizome and reproduction of perennial plants is not inherent in physiology but the result of long-term natural selection. In the resource-limited environment, perennials develop rhizomes for rapid vegetative reproduction so that they are able to compete for more living space than their neighbors. Whereas this allocation pattern may be changed when the resources are adequate in artificial cropland (DeHaan et al., 2005).

Physiologically, the key assumption supporting the negative trade-off is that the "source" for carbon assimilation is fixed (Jackson & Jackson, 1999; Cox et al., 2002), which happens when most rhizomes grow horizontally belowground. In addition to the leaf canopy that serves as their source of photoassimilates, rhizomes also can bend upwards to transition into rhizome-derived shoots. The rhizome-derived shoots are largely self-sufficient (Jackson & Dewald, 1994); they can develop new roots and rhizomes, form inflorescence, conduct photosynthesis, and produce grains. Therefore, the gram-by-gram trade-off is unnecessary for resource allocation between rhizomes and grains (DeHaan et al., 2005).

From the longevity of rhizomatous perennials, the constructed rhizomes can serve as the carbon source that stores more reserves than seeds (Jackson & Jackson, 1999). As well, perennial sorghum RDS can germinate four weeks earlier than annual

sorghum, which allows the former to have a more extended growth period for photosynthetic carbon assimilation (DeHaan et al., 2005). The root system of perennial crops is more robust than that of annual crops, which can provide higher water and nutrient utilization (Huggins et al., 2001). The overall increase in photosynthetic products brought by a longer growing period and higher water use efficiency may offset the energy allocated to rhizome development. In fact, according to model predictions, if the growth period is three years, perennial crops (rice and wheat) only need to double their biomass every year to achieve the same yield as their annual counterparts (Vico et al., 2016).

The growth and development of annual plants and perennial plants are significantly different. In perennials, autotrophic tillers and rhizome-derived shoots function as independent reproductive units in source-sink relationships (Cox et al., 2006). This difference in developmental strategies may lead to different performances in the same traits between annuals and perennials, even in the same genus. Thus, researching the relationship between rhizomes and other agronomically important traits can facilitate the promotion of perennial crops.

The relationship between rhizome and aboveground traits is mainly connected by two physiological bases. First, the rhizome and the basal tillers have the same developmental origin, both of which originate from the axillary buds on the bottom nodes of the shoots (Kong et al., 2015). Secondly, the apical meristem of the rhizome

and the axillary buds on the rhizome nodes can grow aerial shoots (rhizome-derived shoots) (Gizmawy et al., 1985).

Because the rhizomes and basal tillers have the same origin, their genetic regulatory regions or loci partially overlap. In a perennial sorghum population (*S. bicolor* × *S. propinquum*), five QTL regions that regulate rhizomatousness have been found to overlap with vegetative tillering, including four genes that influence branching. Therefore, the two may share a common physiological and biochemical regulation pathway in the early stages of development (Jang et al., 2009; Kong et al., 2015; Cox et al., 2018). However, rhizome and tiller morphologically and functionally are two distinct organs, thus they have different gene expressions in subsequent differentiation. In perennial rice (*Oryza longistaminata*), several genes related to auxin response have higher expression levels in shoot tip tissues, while encoding chlorophyll-binding and light-harvesting proteins are also down-regulated in rhizomes because they are underground stems (Hu et al., 2011). The overlap of the genomic regulatory region provides the genetic basis for the correlation of rhizome and basal tiller.

Physiologically, the rhizome functions as a storage organ, rapidly accumulating its biomass after flowering. The fresh weight of the rhizomes in johnsongrass can increase from 90 grams to 590 grams within eight days after flowering (Monaghan, 1980; Washburn et al., 2013). However, in the study of Horowitz et al. (1972), the flowering time of johnsongrass had no causal relationship with rhizome development. The possible explanation was that rhizome development might be more related to temperature,

whereas the flowering time of most short-day sorghum varieties (including *S. propinquum*) is more determined by photoperiod (Jagadish et al., 2016).

In this study, two generations, $F_{3:4}$ and $F_{4:5}$ HIFs derived from an original *S.bicolor* × *S. propinquum* crossing, were studied in a greenhouse and sandy field trough over a period of two growing seasons. Thirteen important agronomic traits were investigated in at least one generation. A bulked-segregant-analysis (BSA) approach based on rhizome biomass was employed to screen the linked SSR markers from a 259 SSR set. The trait performance and correlation analysis will estimate the promotion potential of rhizome biomass in sorghum and the physiological basis of rhizome development. Genetic analysis will further define the molecular basis of rhizome development and develop a speed marker-assisted breeding pipeline.

3.2 Materials and Methods

3.2.1 Greenhouse cultivation

The sorghum population cultivated in the greenhouse consisted of twelve $F_{3:4}$ heterogeneous inbred families (HIFs) developed by Paterson et al. (1995). In general, the HIFs were derived from an original crossing between *Sorghum bicolor* (BTx 623) × *Sorghum propinquum* (unnamed line), which were selected based on rhizome number after selfing for three generations. On September 23, 2019, the HIFs with ten individuals per family were planted in the greenhouse at Institute for Plant Genomics and Biotechnology (IPGB), Texas A&M University. Two-gallon pots filled with growth

mixture (Jolly Gardner®, PRO-LINE, C/20 Growing Mix) were arranged according to a randomized complete block design.

The seedlings were irrigated every four days before the boot stage; this frequency was increased to every two days when grains began to accumulate dry weight in order to ensure sufficient water supply for grain filling and rhizome growth. Peters 20-20-20 General Purpose water-soluble fertilizer was used for fertilization with a rate of 1.5 TBSP/gallon (267.75 ppm N) once a week, which was stopped after plants entered the boot stage to stimulate rhizome development and grain filling. The heads of all shoots and tillers were bagged when they completely grew out of the flag-leaf sheath to prevent accidental outcrossing.

3.2.2 Field cultivation

After harvesting $F_{3:4}$ plants, two lines from the same family with the highest and lowest rhizome biomass were selected. On May 12, 2020, 110 $F_{4:5}$ seeds of each line were first germinated in two seedling trays with cells in the IPGB greenhouse to ensure a high germination rate. When most seedlings developed 6-7 leaves, they were transplanted to a field trough at the Texas A&M University Farm (30°31'49.3"N, 96°25'15.4"W) with a planting density of 30,000 plants per acre. The growth matrix filled in trough was Chazos loamy fine sand (fine, smectitic, thermic Udic Paleustalfs), which was beneficial for rhizome growth. The planting method also followed randomized complete block design. The standard sorghum agronomic practices were modified on a case-by-case basis in the sandy trough due to the poor water holding

capacity of sandy soil, increasing the irrigation frequency and reducing the dilution rate of water-soluble fertilizers as needed.

3.2.3 Phenotypic data collection

3.2.3.1 $F_{3:4}$ belowground biomass and grain yield

For $F_{3:4}$ plants, the head on each stem and tiller were cut off and bagged when the color of the hilum changed to black and the grains became hard. All the bags were transported to the Perennial Grass Genetic Lab, TAMU, for threshing and measuring grain yield. After being stored in room temperature for about four weeks for drying, each head was manually threshed on a ribbed rubber surface using a board eraser covered with the same rubber and carefully rubbing and pressing grains. Then the seeds from the same plant were measured for total weight on an electronic balance scale accurate to 0.01 g. The grain yield was defined as the grain weight from a plant basis (g/plant). All the seeds were collected in separate envelopes labeled with a serial number after measurement, then were stored in a cold storage room in approximately 10℃ and 30% relative humidity.

The measurement of belowground traits of $F_{3:4}$ was conducted on February 21, 2020, when the continuous re-growth and re-flowering of plants were terminated. Each plant was removed from the pot with soil intact, then washed by hand in a bucket under high-pressure water flow. The aboveground organs were cut off using garden shears, and the rhizomes were stripped from fibrous roots. Aimed for a better drying effect, all the

belowground organs were packed in paper bags and transported to an isobaric air chamber (Perennial Grass Genetic Lab, TAMU) equipped with a fan exhaust system for air drying. After seven days, all belowground samples were taken out from the air chamber, followed by the rhizomes and fibrous roots being weighed separately using an analytical scale.

3.2.3.2 $F_{4:5}$ aboveground traits

For the two $F_{4:5}$ HIFs grown in the field trough, the flowering date was first recorded for each plant. The consistent observation and recording were conducted once per seven days after July 29, 2020, when plants began to flower. The flowering time was defined as the time each plant takes from germination to exposing the stamens on the first flower, as perennials can continue to develop tillers and rhizome-derived in a continuous blooming process.

Because grains of $F_{4:5}$ plants would not be used to derive subsequent breeding or mapping progenies, the heads were not bagged during the boot stage. Also, due to the constant growth of tiller and rhizome-derived shoots, the heads were not harvested all at once. The grains were observed once a week; when the heads were found to be mature enough, they would be harvested. The earlier harvested heads were placed in paper bags and temporarily stored in indoor room conditions. The last harvest was carried out on October 28, 2020. After that, all heads from the plant were combined and dried naturally at room temperature for about four weeks. The methods used for grain threshing and measuring the grain yield were the same as those described in $F_{3:4}$ generation.

After harvesting all of the heads, the growth and development of aboveground organs ended on November 17, 2020. The harvest of stems and leaves was done by cutting them off at the soil surface, then placing the material into paper bags that were fastened with tape. Aboveground samples were transported to the IPGB greenhouse for primary air drying. Beginning January 18, 2021, a pre-process was conducted as the stems were too long to be put into the oven for drying. At the beginning of the process, the plant height was measured by using a millimeter ruler to 0.1cm. The aboveground organs were then cut into small pieces with a garden shear and bagged again. After all samples were pre-processed, the paper bags were transported to an oven in the Department of Biochemistry & Biophysics for drying, setting the temperature to 55℃ for 24 hours. Once the drying treatment was completed, the aboveground biomass was measured by an electronic balance with an accuracy of 0.01g.

3.2.3.3 $F_{4:5}$ belowground traits

The day after aboveground parts were removed, the belowground samples were dug from the soil. This was done by using a 40 × 40 cm wooden frame to surround the stem segments from the middle, then using an engineer shovel with a surface of about 30 × 40 cm for digging. The width and depth of sampling were set as mentioned because the rhizomes were rarely found to grow long-distance horizontally. Every sandy core containing belowground materials was placed into a bucket, then materials were rinsed with high-pressure water flow by hand to ensure that they were clean to the greatest

extent possible. After washing, all of the belowground organs were packed with paper bags and labeled.

The samples were transported to the Perennial Grass Genetic Lab and dried in an oven at 55℃ for 72 hours. A multiple-step, sequential phenotyping procedure followed drying belowground samples. First, the fibrous (nodal) root growth angle was measured by using a plastic protractor with an accuracy of 1 degree. The fibrous root angle was defined as the angle between the outermost roots and the horizontal soil surface (Figure 1A). For each sample, after the measurement of root angle, the fibrous roots were carefully cut into a tray with a scissor to avoid any damage to the rhizomes. When the whole rhizome system was exposed, the rhizome growth angle would be measured through the same method used in root angle measurement. However, the rhizome growth angle had a different definition from the root angle, which was measured as the angle at which the longest rhizome curves upward rather than the angle between it and the soil surface (Figure 1B). After that, the basal tiller number was counted as represented by the stem segments, along with distinguishing the rhizome-derived shoots (RDSs), which were also counted and recorded afterward. Because the stem segments belonged to the aboveground organs, they were separated from the soil surface position by using a garden scissor and angle grinder in the area where the stems were thickest, then placed into another plastic pan.

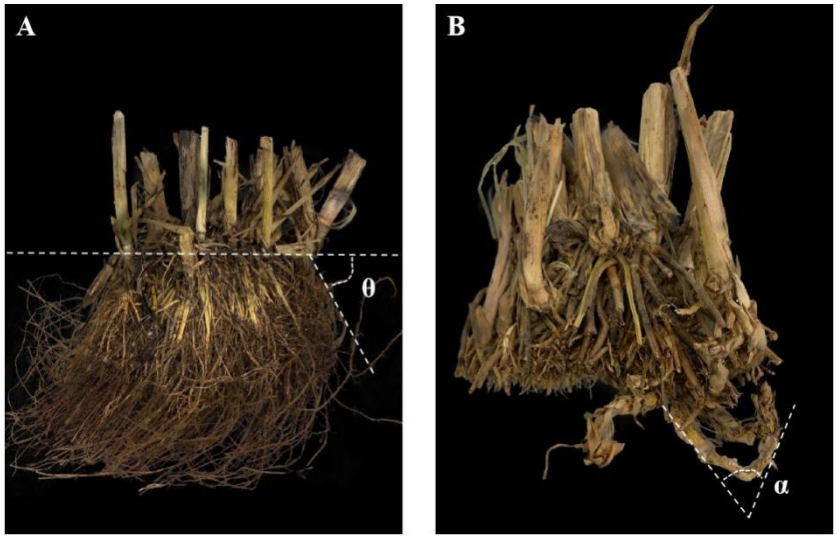

Figure 1 The way to measure the growth angle of belowground organs
(A) Root growth angle (θ); (B) Rhizome bending angle (α).

By the last step, the only existing organ was the rhizome system. First, the rhizome number was counted for each plant. Rhizomes were manually separated with garden shears with the sand concentrated in the gaps cleaned.

By this step, the belowground sample was divided into three parts: rhizomes, fibrous roots with main roots, and stem segments. The different parts were placed in three separate trays. These three parts were weighed with an electronic balance of approximately 0.01g, and the readings were recorded respectively, then packed in three small paper bags and placed in the same large paper bag for storage.

3.2.4 Statistical analysis

A total of thirteen traits were evaluated. For aboveground organs, they were flowering time (FT), number of basal tillers (BTN), number of rhizome-derived shoots (RDSN), plant height (PH), grain yield (GY) and aboveground biomass (ABM). For belowground organs, they were rhizome biomass (RHBM), fibrous root biomass (RTBM), total belowground biomass (BBM), rhizome growth angle (RHAG), root growth angle (RTAG), number of rhizomes (RHN) and rhizome length (RHL). The total belowground biomass was calculated by adding rhizome biomass and fibrous root biomass together. Only RHBM, RTBM, BBM and GY were measured in $F_{3:4}$ in the greenhouse, whereas all of the thirteen traits were measured in $F_{4:5}$ population in field conditions.

The primary analysis phenotypic data for each trait used the distribution function in JMP® Pro 15.0.0 (390308) to construct the histogram graphs. The basic property of each trait was reflected by the population mean, standard deviation, and extreme value. In order to learn the genetic pattern of each trait, a normal fit curve was added to the histogram graph. The more accurate estimation of normality would be reflected by the Shapiro-Wilk test, which was analyzed using Proc Univariate Normal function in SAS® software [SAS (r) 9.4 (9.04.01M2P072314)].

Heritabilities were then calculated for all traits. Because the population constructed in $F_{4:5}$ generation was a genetic mapping population and not a plant breeding population, only two families with two replications were included in the analysis (also

without multiple environments). In this case, only the genotype and replication variance could be divided from total phenotypic variance, whereas other components like environment and all interactions would be integrated into the error variance. Therefore, the heritabilities estimated in this study were broad-sense heritability, which was defined by:

$$\sigma_G{}^2 = (\sigma_F{}^2 - \sigma_e{}^2)/r$$

$$H^2 = \frac{\sigma_G{}^2}{\sigma_G{}^2 + \sigma_e{}^2}$$

Where $\sigma_F{}^2$ is the variance between families; $\sigma_G{}^2$ is the genotypic variance and $\sigma_e{}^2$ is the error variance; H^2 is heritability. The variance components were calculated by conducting an ANOVA in SAS software with Proc GLM function, based on HIFs-mean.

The Pearson correlation coefficient *r* was calculated for each pair of traits that have continuous distribution using the Proc Corr function in SAS. And for the four discrete-distributed traits: RHN, FT, BTN and RDSN, nonparametric Spearman's ρ will be used to replace Pearson's *r*. The results for $F_{4:5}$ would be shown on a family basis.

3.2.5 Genomic evaluation

3.2.5.1 DNA extraction

DNA extractions were carried out for parental lines and two $F_{4:5}$ HIFs. Very young leaf tissues were collected at the five-leaf stage after transplanting the seedlings to the field sandy trough. The vials contained leaf samples that were immediately inserted

into an icebox and transported to the lab. After that, they were frozen in a -80 ℃ freezer for later use. About 100 mg of freeze-dried leaf tissue was taken from each sample using scissors and cut into small pieces in a 2-ml microtube. The rest of the steps followed a normal CTAB procedure (Doyle & Doyle, 1987), but with two modified steps. First, the DNA samples were homogenized by using stainless steel beads and a FastPrep-96™ high-throughput bead beating grinder. Then, except CTAB buffer, another DNA extraction buffer that is consisted of 63.77 g/L Sorbitol, 12.1 g/L Tris, and 1.68g/L EDTA was also added before grinding. The integrity of genomic DNA was checked by running a 1% agarose gel, while a GenesysTM 10 UV Spectrophotometer estimated the concentration.

3.2.5.2 Bulk segregant analysis

The first round of BSA pools were constructed from the top five highest and lowest rhizome biomass individuals. The same amount of DNA from each individual was mixed and diluted with double deionized water to ensure the final concentration of the pool is 10 ng/μL. The second round of BSA pools was constructed in the same manner, only increasing to twelve individuals in each pool. Considering the male parent *S.propinquum* is a heterozygous line, ten randomly selected plants of this line were also mixed and diluted to 10 ng/μL. The DNA of another parent *S.bicolor* was also diluted to this concentration.

3.2.5.3 Molecular marker screening

A total of 259 SSR markers (selected from Winn et al., 2009) that were near-evenly distributed throughout the sorghum genome were employed in this study. First, these markers were analyzed between parents. A 20 μL system was used in PCR amplification, which was made up with: 2μL 10×Taq buffer, 0.5μL 4 mM dNTPs, 1μL 25mM $MgCl_2$, 4μL 10ng/μL DNA, 0.2μL 5U/mL Taq Polymerase, 6.3μL ddH_2O; 3μL+3μL 2μM forward and reverse primers. The PCR program was similar to that described in Winn et al. (2009), only adding the cycle to 50 times for the annealing-extension step. The PCR products were analyzed by running a 3% agarose gel, and the polymorphic markers between parental lines would be selected. Then, those markers were screened across four BSA bulks. The linked markers preliminarily reflected the target genetic regions.

3.3 Results

3.3.1 Basic statistics and genetic pattern

The basic statistic parameters and the Shapiro-Wilk test for normality for both belowground and aboveground traits are shown in Table 1 for $F_{4:5}$ and in Table 7 for $F_{3:4}$. In the $F_{4:5}$ generation, the absolute phenotypic means showed obvious differences between two HIFs in most traits, which means the divergent selection in $F_{3:4}$ worked, though the significance needs to be further analyzed. Almost all traits showed large ranges compared with their standard deviation, which represents great variances within

the family. The trait performance changed when focusing on $F_{3:4}$ data, which included 41 individual plants distributed across seven HIFs. A dramatic decrease in rhizome biomass could be seen in $F_{3:4}$ generations on rhizome biomass comparing $F_{4:5}$. One possible reason could be that the plants were grown in pots in the greenhouse that only provided very limited belowground space and restricted rhizome development. As De Battista and Bouton (1990) reported, the rhizome development of tall fescue (*Festuca arundinacea* Schreb.) was significantly influenced by pot volume.

It is worth noting that the parental line *Sorghum propinquum* did not bloom under greenhouse conditions, so it did not produce grains. This was because this variety was short-day photoperiodic, which usually flowered in mid-September in the latitude of Texas when the daylight was shorter than ~12.5h (Cuevas et al., 2016). Whereas the greenhouse offered supplementary lighting treatment that caused *S. propinquum* not to flower and not grow rhizomes. However, the parental line *S.bicolor* (BTx623) and all the $F_{3:4}$ plants from seven families bloomed. The mean performance of both root biomass and belowground biomass in $F_{3:4}$ was between BTx623 and *S. propinquum*, whereas the $F_{4:5}$ root biomass mean was lower than that in $F_{3:4}$. The possible reason for this is that the plants grown in the trough developed extended rhizomes and thus allocated more photoassimilates into rhizomes but not roots.

According to the distribution of the traits (Figure 5) in $F_{4:5}$, we found that all of the traits in both HIFs had bell-shaped distribution with only one peak, which means that these traits seem to be regulated by quantitative loci. In order to clarify the exact type of

distribution, a Shapiro-Wilk test for normality was done for each trait (Table 1). The result showed that except in the cases of rhizome length and aboveground biomass, all of the other traits were skewed from the normal distribution. The root biomass was normally distributed in the low-rhizome-biomass family but non-normally distributed in the high-rhizome-biomass family; this might be due to the different numbers of individual plants and missing data. We performed logarithmic transformation, arctangent transformation, and reciprocal transformation on all non-normally distributed data, but no traits changed to normal distribution after transformation (Table 8). The possible explanation is that the selection to acquire two extreme $F_{4:5}$ HIFs from $F_{3:4}$ generation reduces heterozygosis on many loci, which will largely decrease genetic segregation in $F_{4:5}$; also, there will be less moderate-performance individuals in F4:5 if most of loci are under additive or partial dominant effect, so that the population distribution will be skewed.

Table 1 The basic statistics of phenotypic performance of thirteen traits in $F_{4:5}$

Traits	Basic Statistics				Test for Normality	
	Mean (g)	Standard Deviation (g)	Minimum (g)	Maximum (g)	W-Value	Pr＜W
RHBM[b]	20.2684[a] 26.1366	16.5614 17.2711	0.03 0.46	77.91 82.47	0.8907 0.9516	<0.0001 0.004
RTBM	12.6267 17.4351	5.4372 7.9278	0.93 1.77	26.76 55.86	0.9878 0.9025	0.549 <0.0001
BBM	32.8952 43.2530	20.0554 24.0538	2.2 2.65	97.3 125.72	0.9337 0.9547	0.0001 0.0057

Table 1 Continued

Traits	Basic Statistics				Test for Normality	
	Mean (g)	Standard Deviation (g)	Minimum (g)	Maximum (g)	W-Value	Pr＜W
RHAG	36.0000 53.5759	16.3367 26.0690	10 15	120 120	0.8244 0.8912	<0.0001 <0.0001
RTAG	31.2634 34.0000	5.2856 5.2410	10 25	42.5 55	0.9388 0.9052	0.0003 <0.0001
RHN	16.1397 16.2025	8.8152 7.6298	2 1	37 40	0.9684 0.9639	0.0239 0.025
RHL	4.8500 4.5769	1.9073 1.8212	0 0.9	9.9 10.3	0.9774 0.9718	0.1365 0.0848
FT	82.9230 84.2682	7.8828 7.0221	73 73	122 108	0.8226 0.8476	<0.0001 <0.0001
BTN	4.2150 2.7317	2.4265 1.7571	1 1	12 9	0.9171 0.8291	<0.0001 <0.0001
RDSN	17.1075 10.6049	11.2108 8.1588	0 0	44 36	0.9415 0.9081	0.0004 <0.0001
PH	69.1637 81.1938	13.6833 18.7543	30.3 15.3	94.2 114.6	0.9685 0.9451	0.0266 0.0017
ABM	97.5618 100.3717	43.6680 49.9360	6.42 9.63	200.06 250.67	0.9778 0.9794	0.1143 0.2126
GY	7.7556 6.7301	7.3675 5.7419	0.14 0.16	46.70 31.58	0.7663 0.8710	<0.0001 <0.0001

[a] The value in upper-half box is the low-rhizome-biomass family, and the value in lower-half box is the high-rhizome-biomass family.

[b] RHBM: Rhizome Biomass; RTBM: Root Biomass; BBM: Belowground Biomass; RHAG: Rhizome Angle; RTAG: Root Angle; RHN: Rhizome Number: RHL: Rhizome Length; FT: Flowering Time; BTN: Basal Tiller Number; RDSN: Rhizome-Derived-Shoots Number; PH: Plant Height; ABM: Aboveground Biomass; GY: Grain Yield.

The first part of the result illustrated that the traits measured both in $F_{3:4}$ and $F_{4:5}$ HIFs had similar basic statistical trends, though some differences existed in the same traits between the two generations. The distributions of traits showed that all of the traits

were quantitatively inherited in the population, yet the non-normal distributions of some traits might be caused by the divergent selection in the previous generation.

3.3.2 Variation analysis and heritability estimation

The ANOVA was constructed for every trait in $F_{4:5}$ based on a randomized complete block design (RCBD), with each family planted as two replications (Table 2). Because most traits other than rhizome length and aboveground biomass were not normally distributed, we conducted nonparametric two-way ANOVA based on the Friedman test for these traits instead of parametric ANOVA. The subsequent heritability estimation was shown in the same table and was defined as broad-sense heritability, because the population was just planted in one environment; thus the environmental effects, as well as genotype by environment interaction (G*E), could not be subtracted from the error term. Therefore, the heritability estimated in this study should be the upper limit of the heritability estimated under multiple locations and multiple years.

The results showed that the replication effects were not significant in all traits, though they occupied a large percentage of variance components in rhizome number (21.51%), rhizome length (62.27%), and aboveground biomass (44.56%). However, the family effect was significant in rhizome biomass, root biomass, belowground biomass, rhizome growth angle, root growth angle, basal tiller number, rhizome-derived shoot number, plant height, aboveground biomass, with the highest accounting for 96.30% of the total phenotypic variance and the lowest at 82.93%. This indicates that there existed significant differences between two families in these traits. Correspondingly, the

Table 2 ANOVA, variance components and heritability estimation of thirteen traits

	Replication	Family	Error	Variance Components (%)			H^2
				Replication	Family	Error	
RHBM	173.90	3857.07**[a]	620.18	3.74%	82.93 %	13.33%	0.7230
RTBM	98.40	13044.31**	573.78	0.72%	95.10 %	4.18%	0.9157
BBM	98.40	6191.86**	613.63	1.43%	89.69 %	8.89%	0.8197
RHAG	10.68	12749.57**	537.47	0.08%	95.88 %	4.04%	0.9191
RTAG	270.08	5259.86**	582.11	4.42%	86.06 %	9.52%	0.8007
RHN	171.90	0.19	626.84	21.51%	0.06%	78.43%	-
RHL	40.49	2.69	553.71	62.27%	17.22 %	20.51%	-
FT	97.28	1046.25	553.81	5.73%	61.64 %	32.63%	0.3078
BTN	98.40	12653.12**	558.05	0.74%	95.07 %	4.19%	0.9155
RDSN	173.90	10504.31**	579.91	1.54%	93.30 %	5.15%	0.8954
PH	42.99	15122.55**	537.93	0.27%	96.30 %	3.43%	0.9313
ABM	98.40	282.18	648.45	44.56%	6.35%	49.09%	-
GY	42.99	141.41	626.60	5.30 %	17.44 %	77.26 %	-

[a] The asterices represents the significant level: $0.01 < \alpha \leq 0.05$ (*); $\alpha \leq 0.01$ (**).

heritabilities of these traits were also relatively high, ranging from 0.72 to 0.93, which means these traits can be improved through selection. This may be because this study did

not use multi-environment estimation, thus environmental effects, as well as the genotype by environment interaction, might not be fully reflected.

Oppositely, rhizome number, rhizome length, flowering time, aboveground biomass and grain yield had insignificant family effects and the heritability of flowering time was also low. In rhizome number, aboveground biomass, and grain yield, we found that the error accounted for a larger proportion of the variance components, while in rhizome length the replication effect was larger. These four traits cannot be calculated for heritability since the genetic variance was much lower than the error variance. The main possible explanation for this is that these traits were more likely affected by environment and genotype by environment interaction, which masks the estimation of genotypic variance.

3.3.3 Correlation analysis

In order to research the relationship between traits, Pearson's correlation analysis was conducted for continuous-distributed traits in $F_{4:5}$ HIFs and $F_{3:4}$ population, and for rhizome number, flowering time, basal tiller number and rhizome-derived shoot number, the Spearman's correlation was used for substitution; the results are shown in Table 3, Table 4, and Table 9, respectively.

In $F_{3:4}$, rhizome biomass (RHBM) was not significantly correlated with belowground biomass (BBM) (Table 9), while root biomass (RTBM) was significantly correlated with belowground biomass ($r = 0.99$). The possible reason is that the limited

space in the pot restricts the elongation of rhizomes so that the root biomass was the main contributor for total belowground biomass. However, the situation completely changed in $F_{4:5}$ (Table 3 and Table 4), in which the rhizome biomass had the strongest correlation with the belowground biomass in both families (Low-rhizome-biomass family r = 0.97; High-rhizome-biomass family r= 0.97), indicating this trait contributed to the most of total belowground biomass. In contrast, although the root biomass was also significantly correlated to the belowground biomass, it was the second contributor in $F_{4:5}$. Furthermore, there also were significant positive correlations between rhizome biomass and rhizome number, rhizome length, rhizome-derived shoot number, aboveground biomass and plant height in the two HIFs. The positive correlations detected above provided a potential developmental pattern for the sorghum population in this study. Under this pattern, the rhizome biomass played a decisive role in the total belowground biomass. When rhizomes initialized, they tended to bend and grow upward as aerial shoots (rhizome-derived shoots) instead of vigorously growing belowground like some sorghum species. After that, the rhizome-derived shoots would continue to grow and significantly contributed to the aboveground biomass.

In $F_{4:5}$, fibrous root biomass (RTBM) kept its essential status in determining belowground biomass following rhizome biomass. Besides that, it also had a significant correlation with the rhizome biomass (Low-rhizome-biomass family r = 0.54; High-rhizome-biomass family r = 0.76), which indicated that the growth and development of the two were mutually synergistic. Therefore, similar to rhizome biomass, root biomass

was also significantly correlated with rhizome number, rhizome length, rhizome-derived shoot number, aboveground biomass and plant height, the only difference being that the correlation coefficients were smaller than rhizome biomass. Due to the strong correlation with rhizome biomass, the belowground biomass also significantly correlated with the five traits mentioned above, and the correlation coefficients were mostly greater than those in root biomass. The analysis of the correlation of fibrous root biomass and belowground biomass indirectly proving that the rhizome biomass was not only the decisive factor of the total belowground biomass, but also widely involved in the aboveground growth as rhizome-derived shoots.

When focusing on root growth angle and rhizome growth angle, a difference was found between two HIFs of some traits. In the high-rhizome-biomass family, both the root growth angle and rhizome growth angle had no significant correlation with almost all other ten traits, indicating their independent relationships in development. However, in the low-rhizome-biomass family, the root growth angle was positively correlated with rhizome biomass, root biomass, belowground biomass, rhizome number and rhizome-derived shoot number, meaning the improvement of rhizome biomass will also increase root growth angle. The rhizome growth angle was significantly correlated with more aboveground traits but the correlation coefficients were also low. The difference between the two families might be caused by the allelic difference caused by divergent selection and the corresponding genotype by environment interaction. Unsurprisingly, there was a significant positive correlation between rhizome number and rhizome length,

rhizome-derived shoot number, aboveground biomass and plant height. The rhizome length was also significantly and positively correlated with rhizome-derived shoot number, aboveground biomass and plant height. This also could be explained by the positive coordinated development of belowground and aboveground organs.

For aboveground traits, rhizome-derived shoot number, aboveground biomass, plant height, and grain yield had significant correlations with each other. The two pairs with the largest correlation coefficients were rhizome-derived shoot number and aboveground biomass (ρ=0.58), and plant height and aboveground biomass (r=0.51). However, the basal tiller number (BTN) did not significantly correlate with most of the other aboveground traits or with a low correlation coefficient, with the possible reason that its role has been competitively replaced by rhizome-derived shoot number, another kind of aerial shoot. No significant relationship was found between flowering time and all underground traits in high-rhizome-biomass family, and most underground traits in low-rhizome-biomass family, which indicates the developmental independence of this trait. However, the relationship between flowering time and basal tiller number and grain yield was negative, which could be explained as the earlier flowering time corresponds to the earlier release from apical dominance, and the extended grain filling time.

It is worth mentioning that, among underground traits, grain yield (GY) was positively correlated with root biomass and belowground biomass in both HIFs. This is because the well-developed root system can provide sufficient water for grain filling. Contrastingly, though rhizome number was also positively correlated with grain yield in

both HIFs, its biomass only significantly contributed to grain yield in high-rhizome-biomass HIF. The possible reason is that the immature rhizome-derived shoots did not significantly contribute to the grain yield in Low-rhizome-biomass HIF. However, the well-developed rhizome-derived shoots increased the grain yield, which could be proven that the rhizome-derived shoot number was positively correlated with grain yield (ρ=0.29; ρ=0.27) in both families.

Table 3 Correlation analysis for thirteen traits in low-rhizome-biomass family

	RHBM	**RTBM**	**BBM**	**RTAG**	**RHAG**	**RHN**	**RHL**	**FT**	**BTN**	**RDSN**	**ABM**	**PH**	**GY**
***RHBM**	1												
RTBM	0.54**	1											
BBM	0.97**	0.72**	1										
RTAG	0.27**	0.23*	0.29**	1									
RHAG	0.47**	0.19	0.44**	0.12	1								
RHN	0.82**	0.34**	0.75**	0.30*	0.37**	1							
RHL	0.60**	0.29**	0.57**	0.17	0.41**	0.62**	1						
FT	0.20*	-0.08	0.14	0.16	0.06	0.22**	0.20	1					
BTN	-0.10	0.24*	0.002	-0.18	-0.23*	-0.23*	-0.06	-0.41**	1				
RDSN	0.84**	0.43**	0.79**	0.21*	0.34**	0.78**	0.58**	0.16	-0.10	1			
ABM	0.61**	0.73**	0.71**	0.19	0.19	0.40**	0.35**	-0.09	0.29**	0.58**	1		

Table 3 Continued

	RHBM	RTBM	BBM	RTAG	RHAG	RHN	RHL	FT	BTN	RDSN	ABM	PH	GY
PH	0.42**	0.53**	0.49**	0.04	0.33**	0.40**	0.29**	-0.06	0.08	0.35**	0.51**	1	
GY	0.19	0.41**	0.27**	0.10	0.01	0.26*	0.08	-0.26*	0.10	0.29**	0.47**	0.40**	1

* RHBM: Rhizome Biomass; RTBM: Root Biomass; BBM: Belowground Biomass; RHAG: Rhizome Angle; RTAG: Root Angle; RHN: Rhizome Number: RHL: Rhizome Length; FT: Flowering Time; BTN: Basal Tiller Number; RDSN: Rhizome-Derived-Shoots Number; PH: Plant Height; ABM: Aboveground Biomass; GY: Grain Yield.

Table 4 Correlation analysis for thirteen traits in high-rhizome-biomass family

	RHBM	RTBM	BBM	RTAG	RHAG	RHN	RHL	FT	BTN	RDSN	ABM	PH	GY
* **RHBM**	1												
RTBM	0.76**	1											
BBM	0.97**	0.88**	1										
RTAG	0.01	0.05	0.03	1									
RHAG	0.16	0.07	0.16	-0.06	1								
RHN	0.79**	0.63**	0.78**	0.18	0.01	1							
RHL	0.37**	0.27*	0.36**	0.04	0.18	0.39**	1						

Table 4 Continued

	RHBM	RTBM	BBM	RTAG	RHAG	RHN	RHL	FT	BTN	RDSN	ABM	PH	GY
FT	-0.13	-0.18	-0.16	-0.10	0.005	-0.13	0.08	1					
BTN	0.25*	0.41**	0.33**	0.12	-0.04	0.09	0.10	-0.24**	1				
RDSN	0.64**	0.66**	0.68**	0.00	0.04	0.62**	0.22*	-0.04	0.07	1			
ABM	0.80**	0.72**	0.82**	0.07	0.19	0.70**	0.30**	-0.28**	0.29**	0.68**	1		
PH	0.58**	0.61**	0.64**	0.15	0.20	0.51**	0.35**	-0.25	0.20	0.40**	0.72**	1	
GY	0.38**	0.31**	0.38**	-0.06	-0.065	0.25*	0.12	-0.59**	0.096	0.27*	0.55**	0.34**	1

* RHBM: Rhizome Biomass; RTBM: Root Biomass; BBM: Belowground Biomass; RHAG: Rhizome Angle; RTAG: Root Angle; RHN: Rhizome Number: RHL: Rhizome Length; FT: Flowering Time; BTN: Basal Tiller Number; RDSN: Rhizome-Derived-Shoots Number; PH: Plant Height; ABM: Aboveground Biomass; GY: Grain Yield.

3.3.4 Genomic evaluation

Among 259 SSR markers, 53 (20.46%) were found to be polymorphic between parental lines. The polymorphism, in this case, was defined as the bands between two parents being different (co-dominant type), or only one parent had PCR products while another is blank ("hemizygous" type) (Figure 2). The rhizome biomass of each individual participated in BSA pool construction was shown in Figure 3, both types of polymorphism were re-evaluated when running BSA pools.

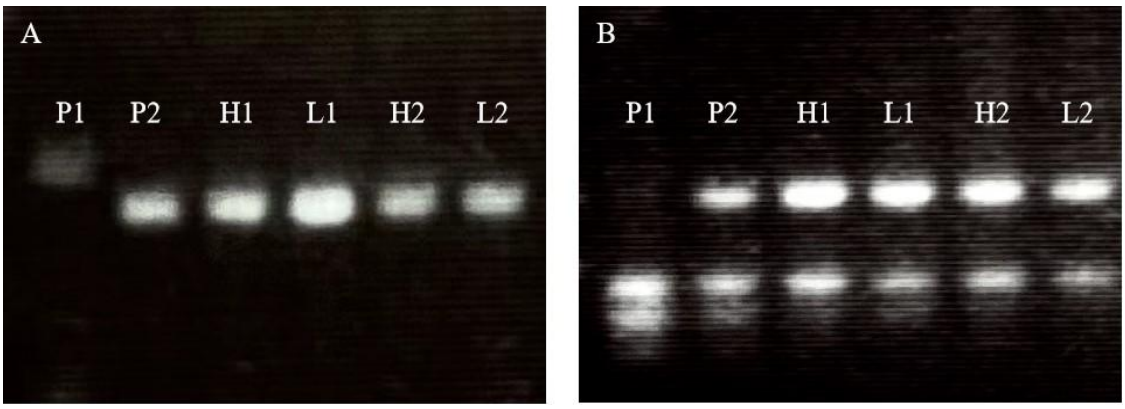

Figure 2 The selected gel figure of the markers linked to rhizome presence

(A) Co-dominant type; (B) Hemizygous type. P1: *S. bicolor*; P2: *S. propinquum*; H1: First-round high-rhizome-biomass bulk; L1: First-round low-rhizome-biomass bulk; H2: Second-round high-rhizome-biomass bulk; L2: Second-round low-rhizome-biomass bulk.

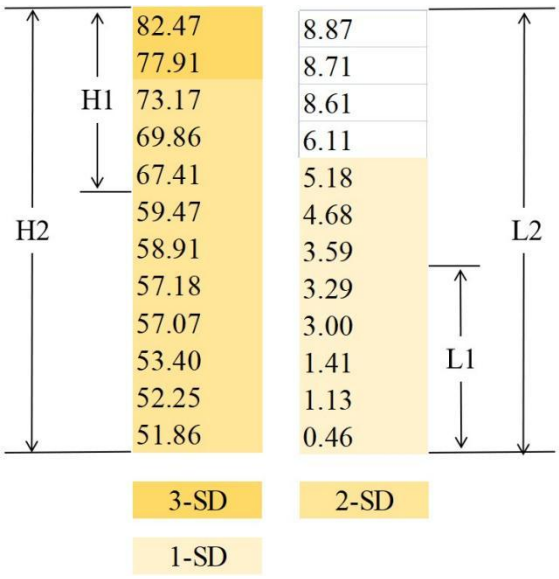

Figure 3 Individuals selected for constructing BSA pools

H1: First-round high-rhizome-biomass bulk; L1: First-round low-rhizome-biomass bulk; H2: Second-round high-rhizome-biomass bulk; L2: Second-round low-rhizome-biomass bulk; SD: Standard deviation; The values in the box are rhizome biomass (g).

All of the 53 markers were then used to analyze two parents, two first-round BSA pools, and two second-round BSA pools. However, the situation we originally hypothesized, in which the high-rhizome-biomass pool could have markers identified that were absent in the low-rhizome-biomass pool, did not occur. Instead, as many as 20 markers had both high and low rhizome biomass BSA pools being consist with *S. propinquum* specific band. Obviously, although the BSA pools were extremely phenotypically differentiated, both of them were composed of individuals that developed rhizomes, which could be distinguished from *S. bicolor* (Figure 2). Therefore, the rhizome biomass was possibly not controlled by a single or small number of large-effect

loci, which greatly reduced the screening ability of the BSA pools that were established based on phenotype. The 20 markers mentioned above (Table 5) might not be sufficient for rhizome biomass, but they were linked to the “presence” of rhizomes and could be used as molecular tools for this trait.

Table 5 Polymorphic markers information

Marker	Chr	Location (Mb)	Sequence*
Xtxp43	1	57.3	GTTTTCCCAGTCACGAGTCACAGCACACTGCTTGTC
			CGTCTCGCGGTCCATTTAA
Xtxp433	1	72.3	GTTTTCCCAGTCACGACAAGCGAGATTACAAGGCCCAACCA
			GCTAGTTAAGAACGTTGACG
Xtxp248	1	79.1	GTTTTCCCAGTCACGACAAGCGGGTGTCCAATGTTGTCTGC
			ACTCATTCCCTGTCATTGCCGG
Xtxp323	1	79.8	GTTTTCCCAGTCACGACAAGCTATATGCATGTTTTAGGTCG
			CCTTCTTTCCTTGTTGTC
Xtxp46	1	80.5	GTTTTCCCAGTCACGACAAGCGGGCAATCTTGATGGCGACAT
			CAAGAGGGGCTCGGTGTGGA
Xtxp471	2	59	GTTTTCCCAGTCACGACAAGCCCGCTTCCTCTCCACTCC
			TTCTGACCCTTCACCCTCAC
Xtxp296	2	70.9	GTTTTCCCAGTCACGCAGAAATAACATATAATGATGGGGTGAA
			TTGAGATGTCCGAGATTTAGTATTGTCGTA
Xtxp26	4	4.9	GTTTTCCCAGTCACGACAAGCAAGTGTAGTAGCAGTTTAGTCTC
			GGAACCAGGAAACTATGGAT
Xtxp41	4	59.2	GTTTTCCCAGTCACGACAAGCTCTGGCCATGACTTATCAC
			GTTCCCTCAGATGCGGTAAA

Table 5 Continued

Xtxp453	5	67.1	GTTTTCCCAGTCACGACAAGCCGACCTGGAATTGGAATGAA
			AGATGCGGCTACAACAAGGA
Xtxp123	5	69.7	GTTTTCCCAGTCACGACAAGCTCGGCGAGCATCTTACA
			TTAGGTTGGCGGATGCAT
Xtxp40	7	0.83	GTTTTCCCAGTCACGACAAGCCAGCAACTTGCACTTGTC
			GATCACGGTTTAACGAGGG
Xtxp295	7	62.3	GTTTTCCCAGTCACGAAATCATGCATCCATGTTCGTCTTC
			ATTCGATACTTACATGAGAACATCGCCCTC
Xtxp354	8	55.5	GTTTCCCAGTCACGACAAGCTGGGCAGGGTATCTAACTGA
			AGTTCCGAGTCTTTTTCCG
Xtxp250	8	58.3	GTTTTCCCAGTCACGACAAGCGCACATCCTCTAAAACTACTTAGT
			TAGATAGTGTAGCAGGACAAG
Xgap34	8	61.8	GTTTTCCCAGTCACGACAAGCAACAGCAGTAATGCCACAC
			CTTCTGTTCAAGAGATGGTTCAGT
Xtxp410	9	2.1	GTTTTCCCAGTCACGACAAGCGGCGCCGTATAAAATAGCAA
			TCTTTTGTTCCTGCGGGAGA
Xgap42	9	3.6	GTTTTCCCAGTCACGTTTTCCTCTTTCAGATAACCGTA
			CTACGGGAACCACCC
Xtxp287	9	4.2	GTTTTCCCAGTCACGGCAAGCGAGCTGACTTATGTAACGAGA
			AAGTGGGACGTATCCAAATCATCGTGAAAC
Xtxp309	10	11.1	GTTTTCCCAGTCACGACAAGCTGCCCTTCAGGAATGATTCGACTACTAC
			AAAAAGAGGATAAACACCGTAAAACGT

* The forward and reverse marker sequence were from -5'~ -3.'

The linked markers could roughly reflect the genetic region of the target trait. Referring to the location of the linked markers on the chromosomes, we found that these markers are distributed on sorghum chromosomes 1, 2, 4, 5, 7, 8, 9, and 10. This seems to confirm the quantitative characteristics of rhizomatousness. In addition, thanks to the increasing and in-depth research on perennial crops in recent years, we were able to compare the genetic regions discovered in this study with previous ones (Table 6). The genomic region on chromosome 1 at 57.3~80.5Mb was high-informative, which partially overlaps with the region of a QTL qRZ1.2 that was reported to regulate the presence or absence of rhizome-derived shoots by Kong et al. (2015), whose plant population was derived from the same parental crossing we used. This region was also overlapped with the rhizome-related QTLs reported by some other studies, like overwintering (Washburn et al., 2013; Kong et al., 2015), rhizome distance (Paterson et al., 1995; Washburn et al., 2013; Kong et al., 2015), rhizome-derived shoots (Paterson et al., 1995; Washburn et al., 2013), and rhizome number (Paterson et al., 1995; Kong et al., 2015). In addition, four vegetative branching QTLs, which included qTL1.1 for basal tiller number, qAX1.1 for axillary branches number, qIM1.1 for immature primary branch number, and qVG1.1 for vegetative branch number, were also partially included in this region (Kong et al., 2014). Besides that, another three regions on chromosome 2, 4, and 9 were also overlapped with the vegetative branching QTLs. Meanwhile, the region on chromosome 4 partially coincided with a rhizomatous QTL qRZ4.2 reported by Kong (2013). And the region on chromosome 7 partially coincided with the QTLs of rhizome-derived shoot number

(qRZ7.1), rhizome number (qRN7.1), and regrowth (pSB067-pSB784) (Kong et al., 2015).

Table 6 Genomic regions and previous QTL

Primer	Chr	Location (Mb)	Rhizomatousness	Vegetative Branching
Xtxp43~Xtxp46	1	57.3~80.5	qRZ1.2; qRN1.2; Xcup73-Xcup22; Over-wintering2011B; Ln2011Dist; Ln2010Dist; Ln2010RDS	qTL1.1; qAX1.1; qIM1.1; qVG1.1
Xtxp471~Xtxp296	2	59.1~70.9		qVG2.1; qM1_2.1; qIM2_2.1
Xtxp26~Xtxp41	4	4.9~59.2	qRZ4.2	qTL4.1
Xtxp453~Xtxp123	5	67.1~69.7		
Xtxp40~Xtxp295	7	0.83~62.3	qRZ7.1; qRN7.1; pSB067-pSB784	
Xtxp35~Xgap34	8	55.5~61.8		
Xtxp410~Xtxp287	9	2.1~4.2		qTR9.1
Xtxp309	10	11.1		

* Paterson et al., (1995); Washburn et al., (2013); Kong, (2013); Kong et al., (2014); Kong et al., (2015)

Except for the overlap with prior studies, five potentially novel genomic regions were also identified in this study. Most of the regions were no larger than 6.3Mb except

the single marker on chromosome 10 and the region on chromosome 1 (11.8 Mb) (Table 5). The target QTL may be located within or on both sides of the interval. And the regions with large intervals may contain more than one target loci.

3.4 Discussion

Rhizomes could be an ideal ecologically beneficial organ for sequestering carbon dioxide from the atmosphere, which is potentially an effective approach to improve the current greenhouse effect. Rhizome biomass is a direct indicator of the carbon sequestration capacity of rhizomes, because 40%-50% of the chemical composition of rhizome dry matter is carbohydrates produced by photosynthesis (McWhorter, 1961). In our $F_{4:5}$ generation high-rhizome-biomass family, the average rhizome biomass yield was 26.13g per plant, and the highest biomass was 82.47 g. This means that under the same planting density of this study (30,000 plants/acre), the population can potentially produce 2638 kg of dry rhizomes per acre, which is equivalent to sequestering 1934.5kg of carbon dioxide from the atmosphere (assuming 50% of the rhizome dry being composed with carbon-containing photoassimilates). The rhizome biomass yield can even be higher as the typical planting density is ~100,000 plants/acre. Approximately 710,000 Americans' annual carbon emissions can be compensated in one growing season if planting our perennial sorghum line based on the current sorghum cropland (USDA, 2021; Hannah and Max, 2020).

Researching the feasibility of developing high-rhizome-biomass varieties is one of the objectives of this study, which depends on the difficulty of improving this trait

itself and the impact on other important traits. The distribution of rhizome biomass (RHBM) is continuous (Table S1), implying this trait should be quantitatively inherited. Though there were no previous studies that researched rhizome biomass heritability in sorghum or other C4 grasses, some related traits have been studied. The heritabilities of rhizome number (0.077) (Paterson et al., 2020) and rhizomatousness (0.34) (Paterson et al., 1995) in johnsongrass were low. Comparatively, the relatively high heritability of rhizome biomass (0.723) in this study shows that it is highly selectable and relatively less affected by the environment as well as genotype by environment interaction. Such huge differences in heritability may be caused by, first, the genetic difference between species *S. propinquum* and *S. halepense* on rhizomes. Johnsongrass features more and longer rhizomes than *S. propinquum* (Warwick et al., 1986; Jessup et al., 2017). The second is the cultivation environment. The above two studies on johnsongrass were carried out in the field and did not clearly specify the agronomic practices and planting density, while our sorghum materials are planted in a sandy trough and followed the standard sorghum agronomy practice. Therefore, adequate nutrition and water greatly alleviate intraspecies competition, which may weaken the environmental impact.

Rhizomes and fibrous roots are two different organs in terms of both morphology and physiological function. Rhizomes mainly exist as storage organs, while one of the main functions of fibrous roots is water absorption and transportation, which is influenced by root growth angle (RHAG) and affects grain yield in grain sorghum cultivar (Mace et al., 2012). In this study, first, we found that rhizome biomass (RHBM)

and fibrous root biomass (RTBM) are significantly positively correlated, indicating that the accumulation of biomass in rhizomes and roots can be improved through selection at the same time. In addition, breeding high-rhizome will not potentially decrease grain yield through water assessment as the rhizome biomass and root growth angle were not negatively correlated.

One of the major uses of sorghum in the US is silage or forage for livestock, and the demand for bioenergy fuel is also increasing in the energy market. For both of these uses, the suitability of the crop is mainly determined by the vegetative aboveground biomass. The average aboveground biomass production in our $F_{4:5}$ populations is 98.96g per plant, with no significant difference between the two families. If regarding the highest aboveground biomass individual plant (250.67g), this population can potentially produce 7520.1kg aboveground dry weight per acre. And more importantly, there exists a significantly positive correlation between aboveground biomass and rhizome biomass ($r= 0.62$, $r= 0.80$ in low and high-rhizome-biomass HIF, respectively), which means the biomass accumulation belowground and aboveground can be improved simultaneously. The highest aboveground biomass plant also has high rhizome biomass (57.07g) that almost exceeds the family mean by two standard deviations (SD). If combining above and belowground biomass as total biomass, our population can potentially produce 9232.2kg dry matter per acre, which is competitive in the current forage market (AgriLife, 2017).

The relationship between rhizome biomass and total aboveground biomass has strong plasticity, which is dynamic, seasonal, varies among different species, and can be changed by several external "mediators". For example, the relationship between the two traits can potentially be linked by the fate of axillary buds. Since both rhizomes and basal tillers develop from basal axillary buds, the differentiation of basal axillary buds will influence the number of basal tillers (Kong et al., 2015; Jang et al., 2006), thereby potentially affecting aboveground biomass. Such a differentiation can be regulated by nitrogen supply, daily temperature, photoperiod (daylight), and varies between different species. Increasing nitrogen supply will induce buds to develop to basal tillers, and will transition initiated rhizomes into rhizome-derived shoots in quackgrass (*Agropyron refiens* L. Be) (McIntyre, 1965; McIntyre, 1967). High day temperature and long photoperiod induce rhizome formation in Kentucky bluegrass (*Poa pratensis* L. Ecotypes) (Moser et al., 1968; Aamlid, 1992) and only long photoperiod is preferred for rhizome initiation in tall fescue [*Lolium arundinaceum* (Schreb.) Darby.] (Saxena et al., 2014). However, the requirement of temperature and photoperiod for rhizome initiation is reversed in quackgrass (McIntyre, 1967).

Another possibility was proposed in this study. The rhizomes in our $F_{4:5}$ population almost did not grow horizontally for a long distance after initiation, instead of bending upwards aboveground as rhizome-derived shoots, which significantly contribute to the aboveground biomass (Figure 4). Such a relationship between aboveground biomass and stem number that includes tillers and rhizome-derived shoots also can be

found in several previous studies in perennial sorghum (Habyarimana et al., 2018; Cox et al., 2002; Habyarimana et al., 2016). Correspondingly, an interesting phenomenon was found in this study. Although the basal tiller number is still significantly correlated with the aboveground biomass, the correlation coefficient is lower than rhizome-derived shoot number, which is different in the study of Kong et al. (2014).

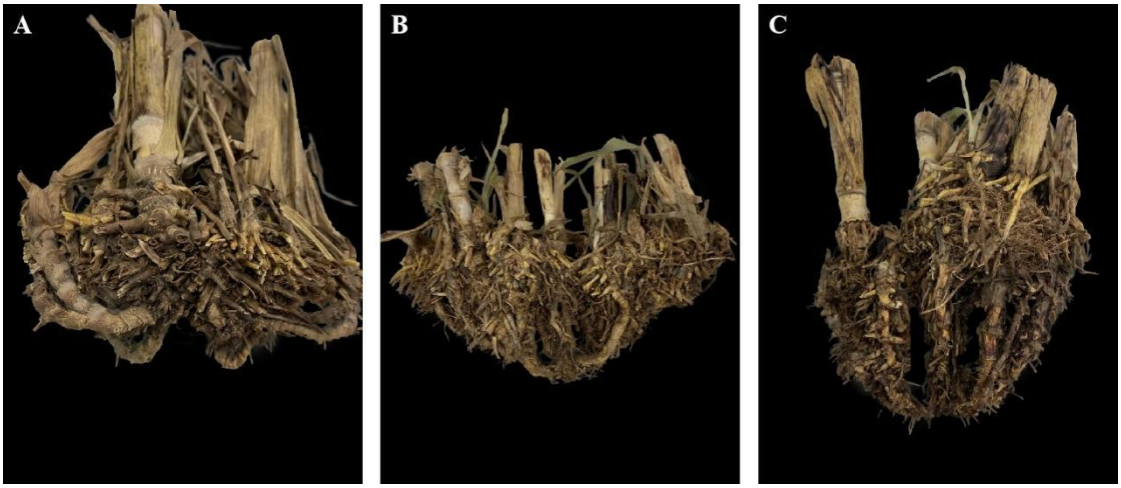

Figure 4 The developing pattern of rhizomes in the $F_{4:5}$ population

Another major use of sorghum is consumption as a grain by humans due to its high antioxidant and other beneficial health properties (Habyarimana et al., 2018). For a long time, annual species have dominated grain sorghum, and the promotion of perennial sorghum has been limited due to rhizome development being considered detrimental to grain filling, as well as a potential weedy risk (Cox et al., 2010; Washburn et al., 2013;

Foster et al., 2020; Paterson et al., 1995; Jessup et al., 2017). However, in our $F_{3:4}$ greenhouse cultivation and $F_{4:5}$ field sandy trough cultivation, we found no negative effects of rhizome biomass on grain yield. And in $F_{4:5}$ high-biomass families, there even was a significant positive correlation between those two traits.

Some breeders have claimed that the grain yield of perennial crops could theoretically not exceed their annual counterparts, based on the "trade-off" of the allocation of resources between grains and underground organs (Jackson and Jackson, 1999; Wagoner, 1990). However, the premise that this trade-off can ultimately reduce grain yield is that the "source" of photosynthate is fixed (Jackson and Jackson, 1999) and incorrectly assume they rhizomes and grain are competing for photoassimilates from the same source leaves. This premise is ungrounded in critical research. In this study, we found that rhizomes can positively affect grain yield by developing large numbers of rhizome-derived shoots (RDS), which can develop their own flowers and produce grains. Noticeably, it has been reported that the grain yield of second-year rhizome-derived shoots (regrowth) was similar to the first-year yield (Nabukalu & Cox, 2016), which proved a great yielding potential of rhizome-derived shoots. However, the grain yield of first-year RDS versus main shoot yield still needs further research. Besides that, flowering asynchrony between crown and RDS can be synchronized through management via mowing or clipping or harvest to reset all to uniform growth stages. The direct evidence of RDS-mediated participation is that in both families, rhizome-derived shoot number has a significant positive correlation with grain yield (ρ=0.29 vs. ρ=0.27).

As is well known, rhizome-derived shoots are largely autotrophic (Jackson and Dewald, 1994); they form their own inflorescences and that produce photoassimilate (Blum, 1985). The "trade-off" was thus broken. Habyarimana et al. (2018) also reported a similar correlation before. The grain yield of their *S.bicolor* × *S.halepense* population was also positively correlated with the number of stems and rhizome development. In addition, they also found a negative correlation between grain yield and maturity, which is also consistent with the negative relationship between flowering time and grain yield in our study (ρ=-0.26 vs. ρ=-0.59). This could be explained by, firstly, delayed flowering causes insufficient time for grain filling; then, the growth and bending-up of rhizomes before flowering is restricted by apical dominance, and this restriction can be broken after flowering (Paterson et al., 2020).

Since rhizome development does not have a negative effect on grain yield, individuals with high yield-high rhizome biomass may exist. The highest grain yield individual appeared in low-rhizome-biomass HIF with 46.70 g, and this individual also produced 22.06 g rhizome biomass. This means that our $F_{4:5}$ population can potentially produce 662 kg dry rhizomes per acre with 1401kg of grains at the same time.

The bulked segregant analysis (BSA) was a convenient approach for marker linkage analysis, which also can be used for narrowing the genetic region, by constructing several rounds of BSA pools and adding more and more individuals into the pool (Michelmore et al., 1991). In this study, we used the BSA method for analyzing and selecting the molecular markers that are tightly linked to rhizome biomass, whereas we

found that the target traits seem not to be controlled by several large-effect loci, which largely weaken the screening ability of BSA pools. This poses more challenges for genetic mapping that requires higher resolution. Thanks to the development of genotyping by sequencing (GBS) technology, either linkage mapping with high-density single-nucleotide polymorphism (SNP) markers, or genome-wide association study (GWAS) is expected to be applied to map the rhizome biomass in the future.

Most previous studies estimated the rhizomatousness, or the presence or absence of rhizome, by counting the rhizome-derived shoots (Paterson et al., 1995; Washburn et al., 2013). This can cause problems for the accuracy of the scoring process, since whether the rhizomes will develop into rhizome-derived shoots highly depends on the development stage (Paterson et al., 2020), environment, and plant species. Also, one rhizome can produce several rhizome-derived shoots both from the apical meristem and axillary buds on rhizome nodes. In this study, we employed a most direct and accurate way to measure all of the belowground traits: destructive harvesting. Benefitting from this, five potentially novel genetic regions regulating the presence or absence of rhizomes were found, among which two of them were overlapped with the previously reported QTLs that are related to tillering and vegetative branching (Kong et al., 2014). This study developed abundant molecular markers for aiding perennial, rhizomatous grain sorghum breeding, and laid the foundation for the following QTL mapping and cloning.

CHAPTER IV

CONCLUSIONS

In this study, we investigated rhizomes through correlation analysis of 13 rhizome-related, belowground, and aboveground traits. Rhizomes were the main contributor to the total belowground biomass, and the improvement of rhizome biomass had a positive effect on most other underground traits. Rhizomes were also found to positively contribute to aboveground vegetative growth and grain production through developing rhizome-derived shoots. Therefore, breeding high-rhizome-biomass varieties also could improve grain yield.

The direct phenotypic performance investigation shows that there exists an extensive range of variation in rhizome biomass, aboveground vegetative biomass, and grain yield. More importantly, individuals with good performance in all three traits exist in the population, which are competitive to the current grain and biomass market.

Rhizome biomass may be a highly quantitative trait that makes the bulked segregant analysis (BSA) approach inefficient for screening the linked molecular markers, whereas the BSA can be used to select the markers related to the presence or absence of rhizome, another trait that is closely related to rhizome biomass. Twenty markers were found to be linked to the presence or absence of rhizome, which defined eight genomic regions, among which three of them overlap with the previously reported QTLs regulating rhizome-related traits and four coincide with vegetative branching

QTLs. The other five regions were potentially novel to the rhizomatousness, which were benefited from the accurate phenotyping method.

The phenotypic analysis results of this study will lay the foundation for studying the physiological regulation of rhizome growth and guide the development of perennial grain sorghum varieties. The results of genomic analysis will facilitate the genetic mapping of rhizome biomass.

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APPENDIX

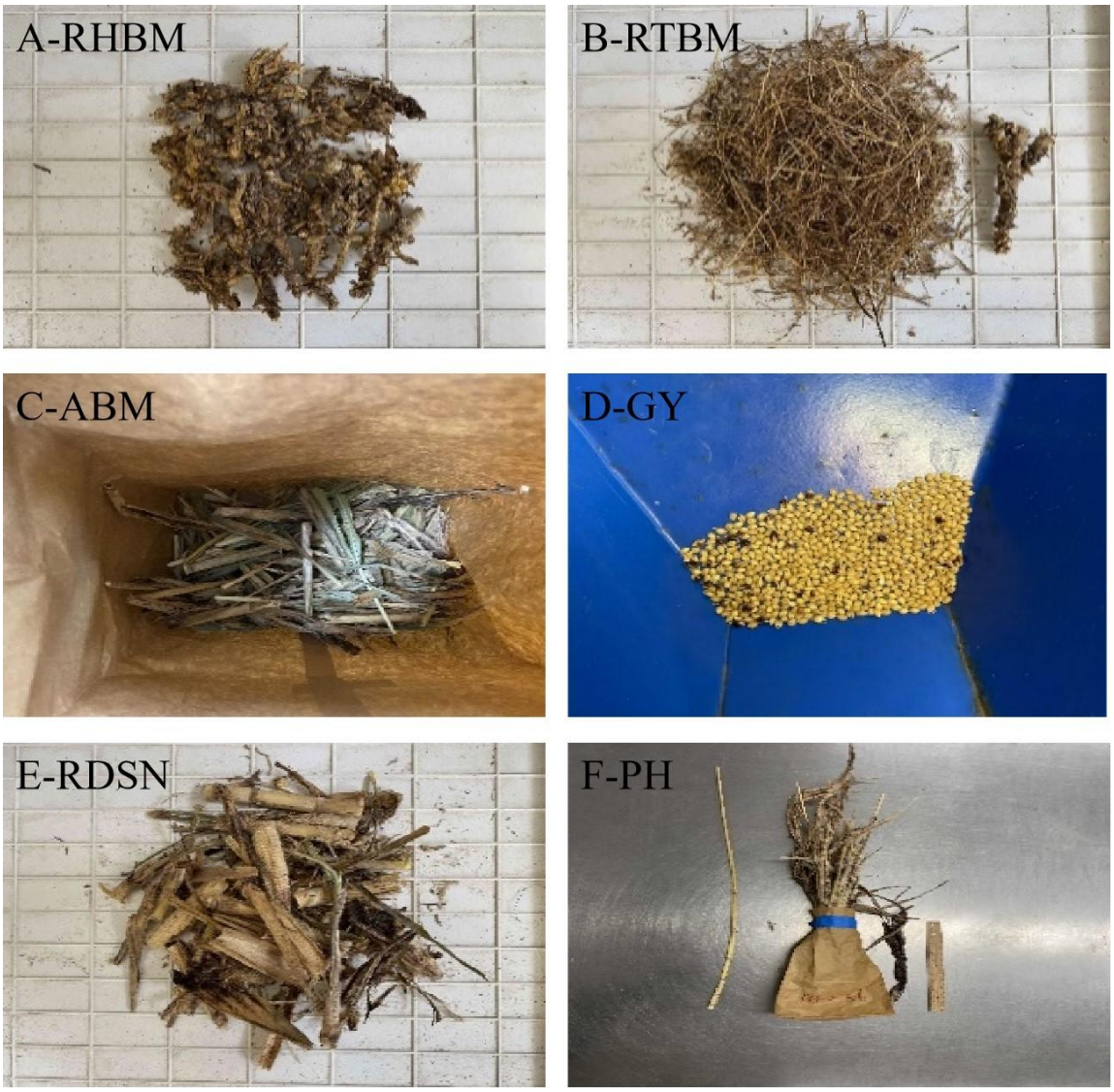

Figure 5 Selected traits in $F_{4:5}$

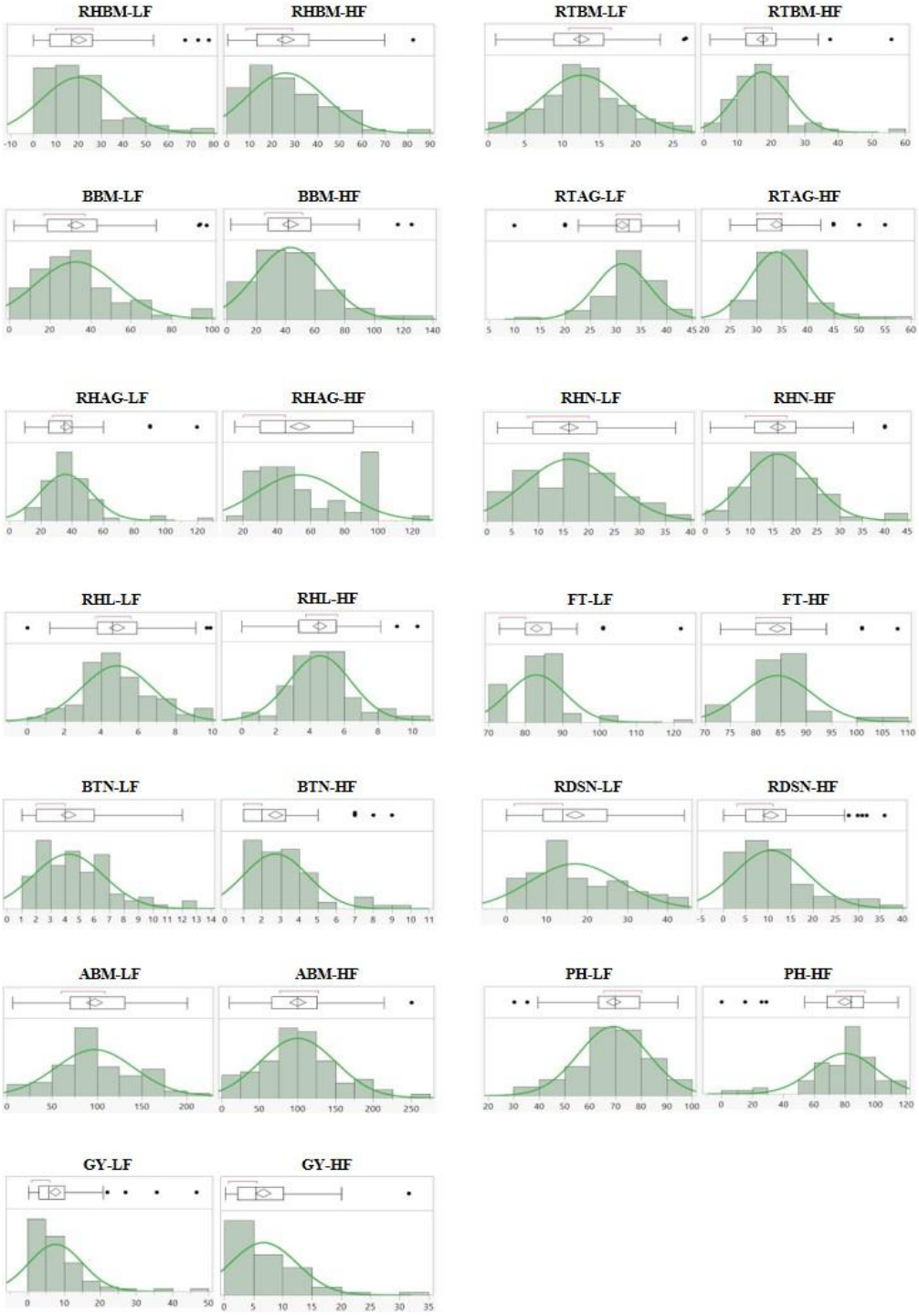

Figure 6 The distribution of thirteen traits in $F_{4:5}$

Table 7 The basic statistics and normality test for $F_{3:4}$

Traits	Basic Statistics						Test for Normality	
	BTX623	*S.Propinquum*	$F_{3:4}$					
	Mean	Mean	Mean	SD	Min	Max	W-value	Pr＜W
RHBM			1.8034	2.142	0	10.40	0.723	<0.001
RTBM	28.01	52.7144	49.381	41.771	14.83	231.26	0.706	<0.001
BBM	28.01	52.7144	51.184	41.441	14.83	232.82	0.706	<0.001
GY			5.1416	3.8947	0	17.75	0.907	0.0027

Table 8 Logarithmic, arctangent and reciprocal transformation of thirteen traits

Traits	Logarithmic		Arctangent		Reciprocal	
	W-Value	Pr＜W	W-value	Pr＜W	W-value	Pr＜W
RHBM	0.8596	<0.0001	0.5596	<0.0001	0.1167	<0.0001
	0.8876	<0.0001	0.4323	<0.0001	0.3144	<0.0001
RTBM	0.8459	<0.0001	0.4747	<0.0001	0.4130	<0.0001
	0.8976	<0.0001	0.5131	<0.0001	0.4855	<0.0001
BBM	0.9169	<0.0001	0.5373	<0.0001	0.5241	<0.0001
	0.9073	<0.0001	0.4421	<0.0001	0.4299	<0.0001
RHAG	0.9689	0.0268	0.8630	<0.0001	0.8623	<0.0001
	0.9368	0.0007	0.8978	<0.0001	0.8975	<0.0001
RTAG	0.8265	<0.0001	0.6019	<0.0001	0.6011	<0.0001
	0.9428	0.0014	0.9464	0.0022	0.9467	0.0022
RHN	0.9184	<0.0001	0.7079	<0.0001	0.6928	<0.0001
	0.9041	<0.0001	0.4522	<0.0001	0.3759	<0.0001
FT	0.8576	<0.0001	0.8768	<0.0001	0.8765	<0.0001
	0.8667	<0.0001	0.8780	<0.0001	0.8773	<0.0001
BTN	0.9419	0.0004	0.8302	<0.0001	0.7687	<0.0001
	0.8975	<0.0001	0.8421	<0.0001	0.8014	<0.0001
RDSN	0.9455	0.0008	0.4546	<0.0001	0.5732	<0.0001
	0.9502	0.0044	0.5464	<0.0001	0.5962	<0.0001
PH	0.9094	<0.0001	0.8097	<0.0001	0.8090	<0.0001
	0.7592	<0.0001	0.4602	<0.0001	0.4598	<0.0001
GY	0.9700	0.0325	0.4034	<0.0001	0.4015	<0.0001
	0.9636	0.0227	0.5455	<0.0001	0.5449	<0.0001

Table 9 Correlation analysis for $F_{3:4}$ traits

	RHBM	**RTBM**	**BBM**	**GY**
RHBM	1.0000			
RTBM	-0.1791	1.0000		
BBM	-0.1288	0.99871**	1.0000	
GY	-0.0270	0.0985	0.0979	1.0000