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A novel regulator of wheat tillering *LT1* identified by using an upgraded BSA method, uni-BSA

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A novel regulator of wheat tillering *LT1* identified by using an upgraded BSA method, uni-BSA

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Promoteurs: Pierre Delaplace

Yonghong Wang

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Résumé

Résumé:

Le tallage (ramification) est un processus crucial pour l'architecture des plantes et participe à l'établissement d'une composante essentielle du rendement en grains. Cependant, le tallage est complexe et contrôlé de manière intriquée à la fois par des facteurs endogènes et environnementaux. Les mécanismes sous-jacents du tallage chez le blé restent mal compris. Dans cette étude, nous avons identifié le gène *LT1* comme un nouveau régulateur du tallage chez le blé en utilisant une méthode d'analyse de ségrégation en vrac (BSA) nouvellement améliorée appelée uni-BSA, bien adaptée à cette espèce. La perte de fonction de *LT1* réduit le nombre de talles en raison de défauts dans l'initiation du méristème axillaire et la croissance des bourgeons. Nous avons cartographié *LT1* dans une région de 6 Mb sur le petit bras du chromosome 2D et validé un gène codant pour un domaine de liaison à des nucléotides (NB) comme *LT1* en utilisant une approche CRISPR/Cas9. De plus, la concentration plus faible en saccharose dans les bases des pousses du mutant *lt1* pourrait entraîner une croissance inadéquate des bourgeons en raison de perturbations dans les voies de biosynthèse du saccharose. L'analyse de co-expression suggère que *LT1* contrôle le tallage en régulant *TaROX/TaLAX1*, l'orthologue du régulateur de tallage d'*Arabidopsis REGULATOR OF AXILLARY MERISTEM FORMATION* (*ROX*) ou du régulateur de méristème axillaire du riz *LAX PANICLE1* (*LAX1*). Cette étude offre non seulement une nouvelle ressource génétique pour établir une architecture végétale optimale, mais souligne également l'importance de notre méthode BSA innovante. Cette méthode uni-BSA permet l'identification rapide et précise de gènes pivots associés à des traits agronomiques significatifs, accélérant ainsi les processus de clonage de gènes et de sélection végétale chez le blé.

Mots clés: Blé Tallage Auxine Cytokinine Saccharose Reséquençage de l'exome complet Analyse des ségrégants en vrac, HSP90

Abstract

Summary:

Branching/tillering is a critical process for plant architecture and grain yield. However, branching is intricately controlled by both endogenous and environmental factors. The underlying mechanisms of tillering in wheat remain poorly understood. In this study, we identified the *Less Tiller 1* (*LT1*) gene as a novel regulator of wheat tillering using an enhanced bulked segregant analysis (BSA) method, uni-BSA. This method effectively reduces alignment noise caused by the high repetitive sequence content in the wheat genome. Loss-of-function of *LT1* results in fewer tillers due to defects in axillary meristem initiation and bud outgrowth. We mapped *LT1* to a 6 Mb region on the chromosome 2D short arm and validated a nucleotide-binding (NB) domain encoding gene as *LT1* using CRISPR/Cas9. Furthermore, the lower sucrose concentration in the shoot bases of *lt1* might result in inadequate bud outgrowth due to disturbances in the sucrose biosynthesis pathways. Co-expression analysis suggests that *LT1* controls tillering by regulating *TaROX/TaLAX1*, the ortholog of the *Arabidopsis* tiller regulator *REGULATOR OF AXILLARY MERISTEM FORMATION* (*ROX*) or the rice axillary meristem regulator *LAX PANICLE1* (*LAX1*). This study not only offers a novel genetic resource for cultivating optimal plant architecture but also underscores the importance of our innovative BSA method. This uni-BSA method enables the swift and precise identification of pivotal genes associated with significant agronomic traits, thereby hastening gene cloning and crop breeding processes in wheat.

Keywords: Wheat, tillering, auxin, cytokinin, sucrose, whole-exome resequencing, bulked segregant analysis, HSP90

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List of Abbreviations

CKs: Cytokinins

BRs: Brassinosteroids

LFY: LEAFY

EXB1: EXCESSIVE BRANCHES1

LOF1/LOF2: LATERAL ORGAN FUSION1/2

RAX1: REGULATOR OF AXILLARY MERISTEM1

BZR1: BRASSINAZOLE-RESISTANT1

BAS1: PHYB ACTIVATION TAGGED SUPPRESSOR1

LOB: LATERAL ORGAN BOUNDARIES

DRN: DORNROSCHEN

CUCs: CUP-SHAPED-COTYLRDON genes

BRC1: BRANCHED1

STM: SHOOT MERISTEMLESS

LAS: LATERAL SUPPRESSOR

SPL9: SQUAMOSA PROMOTER BINDIN PROTEIN-LIKE9

GA: Gibberellin

ATH1: *ARABIDOPSIS* THALIANA HOMEOBOX GENE1

GA2ox4: gibberellin 2- oxidase4

DELLA: aspartic acid–glutamic acid–leucine–leucine–alanine

AXR1: *Arabidopsis* auxin-resistant 1

ROX: REGULATOR OF AXILLARY MERISTEM FORMATION

REV: REVOLUTA

PAGO10: ARGONAUTE10

MP: monopteros

ARF5: Auxin Response Factor 5

IPT: ADENYLATE ISOPENTENYLTRANSFERASE

ARR1: *Arabidopsis* response regulator1

WUS: WUSCHEL

CLV3: CLAVATA3

PRC: Polycomb repressive complex

DPA4: NGATHA-LIKE transcription factors DEVELOPMENT-RELATED PcG TARGET IN THE APEX4

SOD7I: SUPPRESSOR OF DA1-1 7

UBP15: UBIQUITIN-SPECIFIC PROTEASE15

B-ARR: type-B *Arabidopsis* response regulator

SL: Strigolactone

ABA: Abscisic acid

TB1: Teosinte Branched1

BRC1: BRANCHED1

PIN1: PIN-FORMED1

SCF: Skp-Cullin-F-box

Aux/IAA: Auxin/Indole-3-Acetic Acid

CYP735A: cytochrome P450 monooxygenase 735A

PAT: Polar Auxin Transport

LOG: LONELY GUY

tRNA-IPT: transfer RNA isopentenyltransferase

CKX: cytokinin oxidase

PCNA1: PROLIFERATING CELL NUCLEAR ANTIGEN1

SPL14 and SPL15: SQUAMOSA PROMOTER BINDIN PROTEIN-LIKE14 and 15

NCED3: 9-CIS-EPOXYCAROTENOID DIOXYGENASE3

HB21: HOMEOBOX PROTEIN 21

HB40: HOMEOBOX PROTEIN 40

HB53: HOMEOBOX PROTEIN 53

R:FR: Red:Far-red light ratio

D14: DWARF 14

MAX3, 2, 1 and 4: more axillary growth 3, 2, 1 and 4

TPRs: TOPLESS-RELATED PROTEINs

TFs: transcription factors

D53-like SMXLs: DWARF53-LIKE SMAX1-LIKEs

BES1: bri1-EMS-suppressor 1

AM: axillary meristem

SAM: Shoot apical meristem

OsCYP19: Oryza sativa cytochrome P450 family 19

OsMAD57: Oryza sativa MADS-box protein 57

miR159: microRNA 159

PhyB: phytochrome B

OsNPF7.7: peptide transporters family 7.7

OsNRT1.1B: Oryza sativa Nitrate Transporter 1.1B

OsNR2: Oryza sativa NADH/NADPH-DEPENDENT NITRATE REDUCTASE 2

OsWRKY94: Oryza sativa WRKY transcription factor 94

TaNAC2-5A: Triticum aestivum NAC transcription factor 2-5A

miR393: microRNA 393

OsAFB: Oryza sativa AUXIN SIGNALING F-BOX 2

OsTIR1: Oryza sativa TRANSPORT INHIBITOR RESPONSE

OsTCP19: TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR 19

DLT: DWARF AND LOW-TILLERING

NSP1/2: Nodulation Signaling Pathway 1/2

Pi: Phosphorus

OsHAK5: Oryza sativa High-Affinity K+ Transporter 5

K^+: Potassium

CCD7/8: Carotenoid cleavage dioxygenase 7/8

PCA: Principal Component Analysis

ARR5 and ARR6: RESPONSE REGULATOR5 and 6

NRT1.2: nitrate transporter 1.2

Chapter I

General Introduction

Section 1 from this chapter is adapted from Yuan Y, Khourchi S, Li S, Du Y, Delaplace P (2023) Unlocking the multifaceted mechanisms of bud outgrowth: advances in understanding shoot branching. Plants-Basel 12 (20):3628-3652, Yuan Y, Du Y, Delaplace P (2024) Unraveling the molecular mechanisms governing axillary meristem initiation in plants. Planta 259 (5):101, and Yuan Y, LB, Qi Juan, Liu Xin, Wang Yuanzhi, Delaplace Pierre, Du Yanfang (2024) A novel regulator of wheat tillering *LT1* identified by using an upgraded BSA method, uni-BSA. Mol. Breed 44 (7):47.

1. Background

Plants exhibit remarkable shoot plasticity to ensure survival and propagation, particularly under challenging conditions. This adaptability is largely manifested through branching, a process that significantly influences shoot architecture in seed plants by determining the number, position, orientation, and size of shoot branches. Branching, common in both dicots and monocots, involves the development of new shoots from axillary buds along the main stem or existing branches, resulting in tree-like or bush-like structures (Figure 1). While dicotyledons typically display more pronounced and complex branching patterns, monocotyledons, especially grasses like wheat, often exhibit a specialized form of branching called tillering. This process involves the production of lateral shoots (tillers) from the base of the main stem, near or below the soil surface, often developing their own root systems. Although true tillering is primarily a monocot trait, some dicots, such as sunflowers, may produce basal shoots resembling tillers. The key distinctions between branching and tillering lie in their growth patterns (tree-like versus multiple parallel stems) and prevalence (branching being more common in dicots like *Arabidopsis*, while tillering is characteristic of monocots such as wheat, barley, and rice). The precise regulation of shoot branching, orchestrated by a complex regulatory network, represents a critical adaptive strategy that allows plants to optimize their growth and reproduction in response to varying environmental conditions.

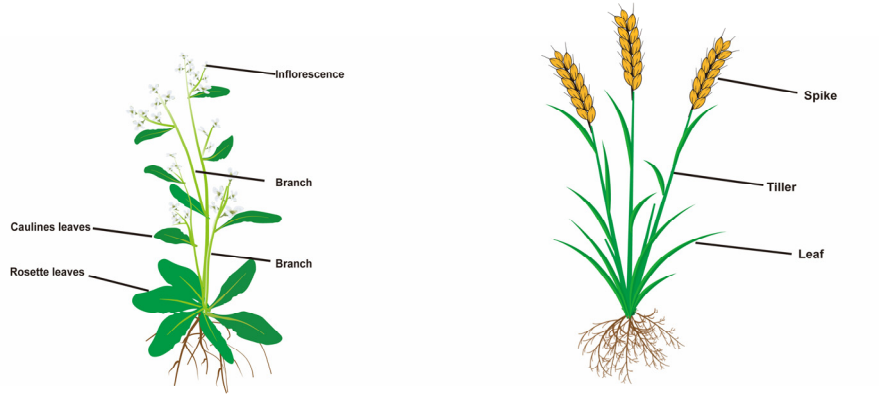

Figure 1. Models of branching and tillering. Branching, as illustrated by the left *Arabidopsis*, common in dicots and monocots, is the development of new shoots from axillary buds along the main stem or existing branches, resulting in a tree-like or bush-like structure. Tillering, on the other right, portrayed as wheat, is a specialized form of branching primarily observed in monocotyledons, especially grasses, such as wheat and rice. It involves the production of lateral shoots (tillers) from the base of the main stem, near or below the soil surface, often forming their own root systems.

The development of the primary shoot axis originates from the activity of the shoot apical meristem (SAM), a group of mitotic cells formed during embryogenesis. Subsequently, derivatives of this meristem generate all above-ground portions of

plants (Bowman and Eshed, 2000). The SAM continuously produces aerial organs by adding growth units called phytomere, which typically consist of an internode, a leaf, and an axillary meristem (AM), which is initiated in the leaf axil (Wang et al., 2018a). The AM, acting as a new SAM of the secondary growth axis, differentiates into, such as in rice, tiller bud, leaf sheath primordium, and leaf primordium (Wang, 2021; Yan et al., 2023). Therefore, the scalable branching across multiple levels is enabled by the specification and activity of AMs, leading to the generation of diverse architectural forms. Notably, AM activity has long been a target of breeding selection due to its significant contribution to crop yield through effects on tiller/branch number, panicle number, and panicle branches (Wang and Li, 2008; Springer, 2010; Wang and Li, 2011; Shao et al., 2019).

Branching in crops is a process involving tiller bud formation related to AM initiation and then its outgrowth or dormancy depending on its internal or external situations (Yuan et al., 2023b). Numerous studies over several decades have sought to elucidate AM initiation mechanisms. The prevailing model proposes that main endogenous and developmental cues interact to regulate AM initiation (Yuan et al., 2024a). For instance, the *LATERAL SUPPRESSOR* (*LAS*), *REVOLUTA* (*REV*), and *CUP-SHAPED-COTYLEDON* (*CUC*) genes (Aida et al., 1999; Otsuga et al., 2001; Greb et al., 2003a; Hibara et al., 2006) can function together in a regulatory cascade controlling AM initiation. An Auxin minimum niche is required to sustain *SHOOT MERISTEMLESS* (*STM*) expression in the boundary zone, thereby maintaining AM identity (Guo et al., 2015). Subsequently, *WUSCHEL* (*WUS*) follows the expression of *STM* and then activates the expression of *CLAVATA3* (*CLV3*) (Shi et al., 2016; Cao and Jiao, 2020; Cao et al., 2020b), forming a *WUS-CLV3* loop in the center of leaf axils to maintain stem cell activity (Xin et al., 2017), indicating the completion of AM initiation and *de novo* formation of new stem cell niches in the leaf axils (Yang et al., 2023). Following the auxin minimum, a cytokinin pulse occurs in the leaf axil during AM formation (Wang et al., 2014). In addition to auxin and cytokinin, many other phytohormones (e.g., strigolactones, brassinosteroids, gibberellins, and abscisic acid) participate and interplay in shoot AM formation (Napoli and Ruehle, 1996; Beveridge et al., 1997; Beveridge, 2000; Chatfield et al., 2000; Foo et al., 2001; Morris et al., 2001; Turnbull et al., 2002; Sorefan, 2003; Beveridge, 2006; Reddy et al., 2013; Zhang et al., 2020b). These factors intricately interact and regulate AM initiation through shared or distinct mechanisms.

Despite the AM formation, the outgrowth of the bud to form a branch/tiller is also crucial for branching (Yuan et al., 2023b). Many research studies conducted have aimed to unravel the mechanisms underlying this process. The prevailing understanding is that various inputs, such as endogenous factors, developmental cues, and environmental signals, interlock to regulate shoot branching. For instance, among multiple essential genes controlling shoot branching in plants, *TEOSINTE BRANCHED 1* (*TB1*) acts locally in buds to inhibit bud outgrowth and is considered to be an integrator of diverse phytohormonal, trophic, and environmental signaling networks (Aguilar-Martínez et al., 2007; Wang et al., 2019c). In addition, the signals defined by phytohormones, nitrogen, light, and sugars have been shown to affect tiller

bud outgrowth significantly (Helliwell et al., 2001; Dharmasiri et al., 2005a; Lo et al., 2008; Bayer et al., 2009; Lin et al., 2009; Finlayson et al., 2010; Wang et al., 2013; Mason et al., 2014; Tegeder, 2014; Tian et al., 2014; Wang et al., 2014; González-Grandío et al., 2017; Zhang et al., 2020b).

Tillering, also referred to as shoot branching, is a crucial trait in cereal crops such as rice and wheat. It plays a vital role in generating an adequate number of panicles, which is essential for ensuring optimal grain production. The mechanism underlying tillering in crops like rice is relatively well-studied. For example, the two processes (Figure 2), including tiller bud formation and its outgrowth, are influenced by genetic factors, endogenous hormones, and exogenous environmental cues (Yan et al., 2023). The identification of rice tillering mutants and their corresponding genes over past decades has accelerated the speed of the establishment of tiller regulation. The mutants have been grouped into AM formation and tiller bud growth defect mutants (Sato et al., 1996; Li et al., 2003; Zou et al., 2006; Arite et al., 2007; Lin et al., 2009; Oikawa and Kyozuka, 2009; Xu et al., 2012; Lu et al., 2015b). The molecular mechanisms in AM formation and tiller bud outgrowth are becoming more refined (Yan et al., 2023; Yuan et al., 2023b; Yuan et al., 2024a). Regarding AM initiation, *MONOCULM1* (*MOC1*), the first gene isolated in rice, coordinates with *MONOCULM3* (*MOC3*) **(**Lu et al., 2015b**)**, *LAX PANICLE1* (*LAX1*) (Komatsu et al., 2003b), *LAX PANICLE2* (*LAX2*) (Tabuchi et al., 2011), and their interaction factor MOC1-interacting protein1 (MIP1) to promote the formation of rice tillers (Sun et al., 2010). In addition, MOC1 interacts with Tillering and dwarf1 (TAD1) to form the E3 ubiquitin ligase complex, which is further degraded (Xu et al., 2012). SLR1 binds to MOC1 and protects it from degradation (Liao et al., 2019). *O. sativa homeobox1* (*OSH1*), the common downstream gene of *MOC1* and *MOC3*, is strongly expressed in the early stage of AM formation (Sato et al., 1996). MOC3 promotes the formation of AM by increasing the expression of *OSH1* and Oryza sativa *WUSCHEL-RELATED HOMEOBOX4* (*OsWOX4*), and also interacts with MOC1 to promote the expression of *FLORAL ORGAN NUMBER1* (*FON1*), thus promoting the growth of tiller buds (Shao et al., 2019). For the tiller bud outgrowth, a well-studied network is appearing. For instance, *OsTB1*, as the main negative regulator of tillering bud growth in rice, acts downstream in the strigolactone (SL) signaling pathway to regulate rice tiller bud growth (Wang et al., 2018a). The synthesis of SL from carotenoid is catalyzed by *O. sativa MORE AXILLARY GROWTH1* (*OsMAX1*), *DWARF17* (*D17*), *DWARF27* (*D27*) and *DWARF10* (*D10*) (Wai and An, 2017). As a receptor, DWARF14 (D14) is negatively regulated by *O. sativa* MADS-domain transcription factor 57 (OsMADS57), interacts with D3, and forms a complex with SCF, which transmits SL signals and causes DWARF53 (D53) protein degradation. D53 further binds to a DLT-RLA1-OsBZR1 complex to inhibit *OsTB1* expression. OsTB1 interacts with OsMADS57 to reduce its inhibition of D14 transcription and regulate the growth of tiller buds in rice. *IDEAL PLANT ARCHITECTUTRE1* (*IPA1*), a well-known ideal plant type gene, is a target gene of *O. sativa* CIRCADIAN CLOCK ASSOCIATED1 (OsCCA1) along with OsTB1, D10 and D14. IPA1 interacts with and is inhibited by OSHI1. These two genes competitively bind to the promoters of *OsTB1*, which inhibit

the formation of tillers in rice. Of note, *FON1*, which impacts AM initiation also promotes the growth of tiller buds.

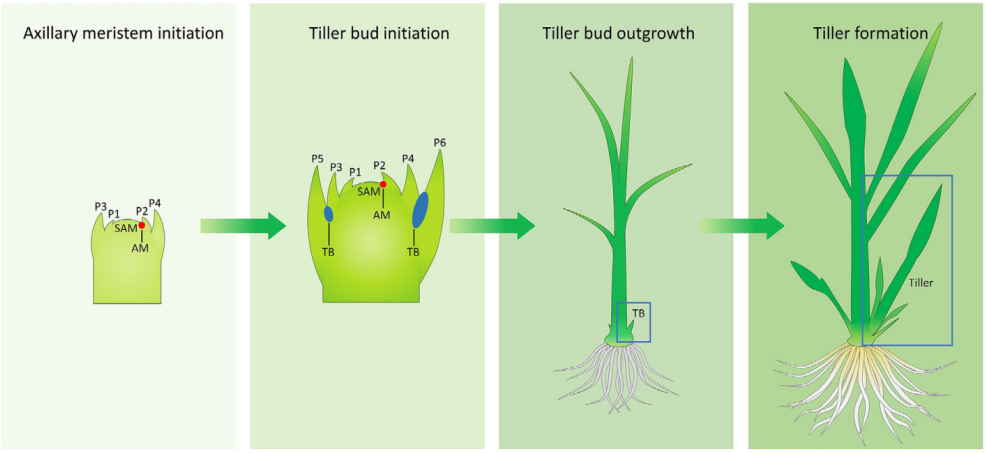

Figure 2. The formation process of a rice tiller (adapted from Yan et al., 2023). During the tillering stage, AM cells detach from the primary SAM and establish within the leaf axil. Once established, the AM functions as a new SAM for secondary growth, differentiating into a tiller bud with leaf sheath and leaf primordia. After forming its second leaf primordium, the tiller bud undergoes rapid elongation to develop into a tiller bearing several leaves. In the illustration, red dots indicate axillary meristems, while blue regions represent tiller buds. (Abbreviations: AM, axillary meristem; TB, tiller bud; SAM, shoot apical meristem; P1–P6, leaf primordia).

Despite the progress of rice, the mechanisms underlying tillering in wheat, which is a major staple food for one-third of the human race (Dong et al., 2023), are just beginning Tillering in wheat is influenced by endogenous and environmental factors (Cao et al., 2020a) in the environmental cues, population density, temperature, salinity, and more impact tillering. For the genetic control of tillering, several genes controlling tillering have been identified and characterized by homologous cloning. *TaMOC1*, a key regulator of AM initiation of rice, regulates AM formation for panicles. *TaMOC1* expression has been detected in the epidermal cells of leaf primordia and is subsequently expressed in axillary buds, SAM, and young leaves (Dixon et al., 2018). TaTB1 interacts with FLOWERING LOCUS T1 (FT1), modulating the strength of the flowering signal received by the developing inflorescence. This interaction helps coordinate the formation of reproductive branches, known as spikelets (Dixon et al., 2018). *TaD27* genes in wheat, orthologs of rice *D27* encoding an enzyme involved in SLs biosynthesis, play critical roles in regulating wheat tiller number by participating in SLs biosynthesis (Zhao et al., 2020a). Collectively, broad conservation of gene function exists between wheat and rice, while the distinct effects of these genes on development and phenotypic traits are species-specific.

To conduct more in-depth research on wheat tillering, it is imperative to identify and characterize more novel genes. Several quantitative trait loci (QTL) have been mapped to respective chromosomes. For example, the *tiller inhibiter 2* (*tin2*) gene was isolated to chromosome 2A (Peng et al., 1998), while the *tin3* gene was mapped to the distal region of chromosome 3A of diploid wheat (*Triticum monococcum*) (Kuraparthy et al., 2007). The *fertile tiller inhibition* (*ftin*) gene gene regulates fertile tiller development was mapped to chromosome 1A (Zhang et al., 2013). However, only two novel genes have been isolated via forward genetics thus far (Kebrom et al., 2012; Hyles et al., 2017; Dong et al., 2023). One example is the *tin1* gene, encoding a cellulose-synthase-like protein, which has been cloned through a map-based cloning approach (Hyles et al., 2017). Another is the *tiller number1* (*tn1*) gene, which encodes an ankyrin repeat protein with a transmembrane domain (ANK-TM) (Dong et al., 2023). Despite these advances, building a molecular network for wheat tillering, similar to that in rice, remains a distant goal.

Gene cloning in hexaploid wheat (*Triticum aestivum* L., AABBDD, 2n = 6x = 42) is challenging due to its complex genome, which is characterized by its large size (17 Gb) and the high proportion of repetitive sequences (IWGSC, 2014). With the advent of the next-generation resequencing technology and its ever-declining cost, various smart algorithms have been developed, such as MutMap (Abe et al., 2012) and Gradedpool (Wang et al., 2019b), for rice, *Arabidopsis*, and more. Nevertheless, these current strategies do not adequately address the intrinsic repetitive noise, which can cause reads to align to multiple genome positions. Our newly developed uni-BSA (uniquely aligned bulked segregant analysis) method resolves this issue primarily by significantly reducing read-alignment noise. This method also increases the accuracy of SNP calling. Utilizing uni-BSA, we successfully isolated the *LT1* (*Less Tiller 1*) gene controlling tillering in wheat. We anticipate that wider adoption of uni-BSA for cloning genes governing important traits will benefit molecular breeding efforts to ensure food security (this work has been published in the molecular breeding journal).

2. Objective

This research focuses on cloning the *LT1* gene through our innovative and highly effective method, uni-BSA, which has been specifically developed to overcome the longstanding challenges associated with wheat gene cloning. We then comprehensively characterize *LT1* through genetic and functional analyses.

Six specific objectives were pursued during this work and are summarized as follows:

(1) Identification tillering mutants in wheat: Screening a collection of mutants mutagenized by the Ethyl Methanesulfonate (EMS) mutagen to identify those with defects in tillering, which will take approximately one year;

(2) Generation of a segregating population for gene cloning: to develop an F_2 population by backcrossing and selfing, which will spend one and half years;

(3) Uni-BSA pipeline: to establish algorithms specifically designed to handle the large datasets generated by resequencing. These algorithms effectively reduce ambiguous

alignments, improve variant quality, and efficiently narrow down candidate gene regions;

(4) Functional validation of *LT1*: We will employ two approaches to validate the function of *LT1*: CRISPR/Cas9 mutagenesis and analysis of overexpression transformants;

(5) Pathway identification: We try to perform co-expression analysis to identify key regulatory pathways potentially involved in tillering regulation by *LT1*;

(6) *LT1* interaction partner screening: we intend to utilize the yeast two-hybrid system to identify candidate proteins directly interacting with *LT1*.

3. Research outline

The literature review presents the latest advancements in branching/tillering, with a primary focus on AM initiation and its outgrowth, which is crucial for determining the final branches or tillers. These reviews reveal complex mechanisms influenced by both external and internal cues (Chapter II). Moreover, we have examined the cutting-edge developments in forward genetics and proposed new directions for future efforts to clone wheat genes (Chapter II).

The research timeline for this thesis work is shown in Figure 3. We attempt to unveil the mechanism underlying tillering in wheat by identifying and characterizing the *LT1* gene. First, we selected a mutant displaying fewer tillers from an EMS- mutagenized pool. Then, we generated an F_2 segregating population and isolated this gene. This gene was verified by transformants showing *lt1* phenotypes. Biochemical analysis revealed that *LT1* can be polyubiquitinated, leading to its degradation. *LT1* interacts with heat shock protein 90s (Hsp90s), chaperone proteins involved in various developmental stages (Tichá et al., 2020). Co-expression analysis using three developmental stages, namely the 2-, 3-, and 4-leaf stages, uncovered the involvement of many pathways. For example, the ortholog of *LAX1* in rice (Komatsu et al., 2003b) regulating AM initiation is down-regulated. Sucrose levels, essential for tiller bud outgrowth (Barbier et al., 2015b), are decreased. Plant hormones like auxin, which is a key component in inhibiting tillering (Ferguson and Beveridge, 2009), and cytokinin, which plays an antagonistic role to auxin (Shimizu-Sato et al., 2009), are also influenced. Notably, we developed new pipelines, uni-BSA, deliberately reducing alignment noises (Chapter III). We believe that the wide adoption of uni-BSA will accelerate the steps of gene cloning in wheat.

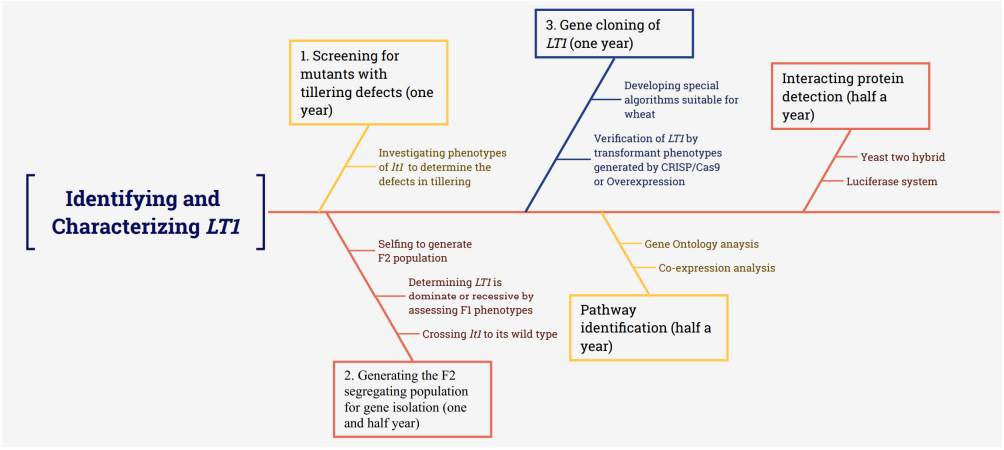

Figure 3. The timeline for identifying and characterizing *LT1*. This timeline indicates the research tasks that were carried out and the approximate durations for each task.

Chapter II

State of the art on branching/tillering and the developing methods for cloning wheat genes

Section I of this chapter is from Yuan Y, Du Y, Delaplace P (2024) Unraveling the molecular mechanisms governing axillary meristem initiation in plants. Planta 259 (5):101. Contributions: Yundong Yuan wrote the initial draft and revised the manuscript. Yan Fang Du and Pierre Delaplace thoroughly reviewed the manuscript and provided insightful feedback.

Section II of this chapter is from Yuan Y, Khourchi S, Li S, Du Y, Delaplace P (2023) Unlocking the multifaceted mechanisms of bud outgrowth: advances in understanding shoot branching. Plants-Basel 12 (20):3628-3652. Contributions: Yundong Yuan wrote the initial draft and revised the manuscript. Said Khourchi, Shujia Li, and Yanfang Du helped to enhance English writing. Pierre Delaplace Pierre Delaplace entirely reviewed the manuscript and provided insightful feedback.

I. Unraveling the molecular mechanisms governing axillary meristem initiation in plant

1. Introduction

Plants demonstrate remarkable shoot plasticity to ensure survival and propagation, particularly in the face of challenging external and internal conditions. In seed plants, the architecture of shoots is predominantly influenced by factors such as the number, position, orientation, and size of shoot branches. The precise regulation of shoot branching represents a crucial adaptive strategy orchestrated by a complex regulatory network.

The development of the primary shoot axis originates from the activity of the SAM, a group of mitotic cells formed during embryogenesis. Subsequently, derivatives of this meristem generate all above-ground portions of plants (Bowman and Eshed, 2000). The SAM continuously produces aerial organs by adding growth units called phytomere, which typically consist of an internode, a leaf, and an AM which is initiated in the leaf axil (Wang et al., 2018a). The AM, acting as a new SAM of the secondary growth axis, differentiates into, such as in rice, tiller bud, leaf sheath primordium, and leaf primordium (Wang, 2021; Yan et al., 2023). Therefore, the scalable branching across multiple levels is enabled by the specification and activity of AMs, leading to the generation of diverse architectural forms. Notably, AM activity has long been a target of breeding selection due to its significant contribution to crop yield through effects on tiller/branch number, panicle number, and panicle branches (Wang and Li, 2008; Springer, 2010; Wang and Li, 2011; Shao et al., 2019).

Numerous studies over several decades have sought to elucidate AM initiation mechanisms. The prevailing model proposes that main endogenous and developmental cues interact to regulate this process. For instance, the *LAS*, *REV*, and *CUC* genes (Aida et al., 1999; Otsuga et al., 2001; Greb et al., 2003a; Hibara et al., 2006) can function together in a regulatory cascade controlling AM initiation. An Auxin minimum niche is required to sustain *SHOOT MERISTEMLESS* (*STM*) expression in the boundary zone, thereby maintaining AM identity (Guo et al., 2015). Subsequently, *WUS* follows the expression of STM and then activates the expression of *CLV3* (Shi et al., 2016; Cao and Jiao, 2020; Cao et al., 2020b), forming a *WUS-CLV3* loop in the center of leaf axils to maintain stem cell activity (Xin et al., 2017), indicating the completion of AM initiation and *de novo* formation of new stem cell niches in the leaf axils (Yang et al., 2023). Following the auxin minimum, a cytokinin pulse occurs in the leaf axil during AM formation (Wang et al., 2014). In addition to auxin and cytokinin, many other phytohormones (e.g., strigolactones, brassinosteroids, gibberellins, and abscisic acid) participate and interplay in shoot AM formation (Napoli and Ruehle, 1996; Beveridge et al., 1997; Beveridge, 2000; Chatfield et al., 2000; Foo et al., 2001; Morris et al., 2001; Turnbull et al., 2002; Sorefan, 2003; Beveridge, 2006; Reddy et al., 2013; Zhang et al., 2020b). These factors intricately interact and regulate AM initiation through shared or distinct mechanisms.

In this section, we focus on explaining, unifying, and differentiating the intertwined mechanisms of AM development in several plant species, including *Arabidopsis* (*Arabidopsis thaliana*) and rice (*Oryza sativa*). Additionally, we discuss the challenges of identifying more genes that are specially involved in AM formation and propose available methodologies suitable for resolving these problems. This work summarized AM initiation and was published (Yuan et al., 2024a). All the genes mentioned in this review are listed in Table 1.

1. Origin of AM: Detached or *de novo*?

A distinctive characteristic of plants is their remarkable ability for reiterative growth and continuous organogenesis over their lifetimes. Analogous to the SAM, AMs play a pivotal role in initiating the development of lateral organs. This precisely regulated developmental process can result in AM formation, subsequently giving rise to the development of branches/tillers. In crops such as wheat and rice, the branches or tillers originating from AMs ultimately contribute to the formation of panicles, determining the overall grain yield (Figure 4). However, grain yield loss will occur if plants have defective AM formation (Figure 4).

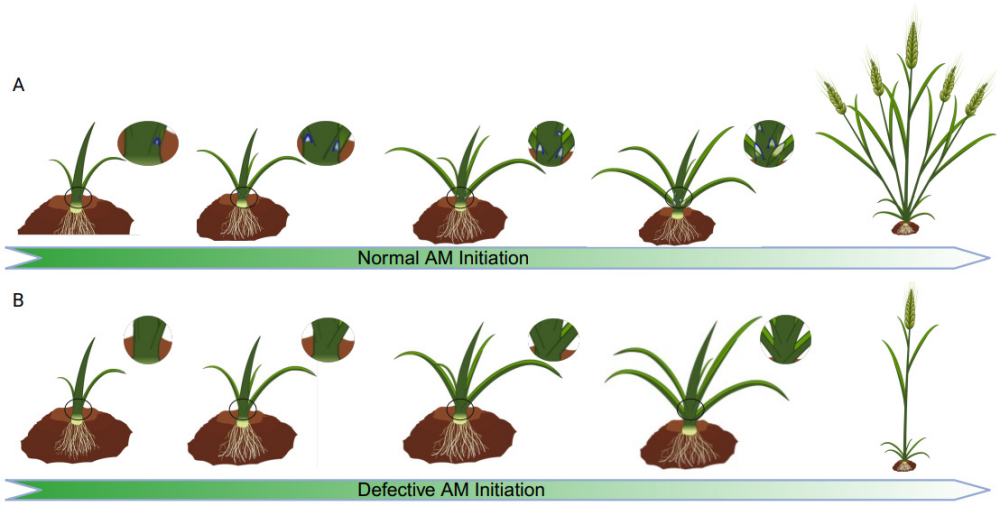

Figure 4. Illustrution of the dynamic developmental process of axillary meristem AM initiation of crops and effects comparisons between plants with normal ability to launch AM initiation or not. A in the up line depicts the plant with the normality to generate AMs, resulting in more tillers/branches (the top right) if AMs can outgrow subsequently. AMs arise from the leaf axil framed by black circles. The AMs boxed in the shoot base are closed up in the top right. B in the bottom line delineates the plant with severe defects in AM initiation, which can lead to monoculm phenotype with low grain yield (the bottom right). The leaf axils that generate AM are barren.

Two alternative models for AM formation have been proposed: the 'detached meristem' model and the '*de novo* induction' model (Figure 5). The 'detached meristem' model posits that AMs form from pluripotent stem cells that bud off from the primary SAM and maintain meristematic potential in the leaf axils as leaf primordia develop (Steeves and Sussex, 1989). This is supported by evidence showing that leaf axil cells remain undifferentiated and express the meristem marker STM (Grbic and Bleecker, 2000; Long and Barton, 2000). Laser ablation experiments have also shown that AMs originate from cells with STM mRNA persistence (Shi et al., 2016). In addition, studies reveal that AM progenitor cells are set aside early in SAM development (Burian et al., 2016).

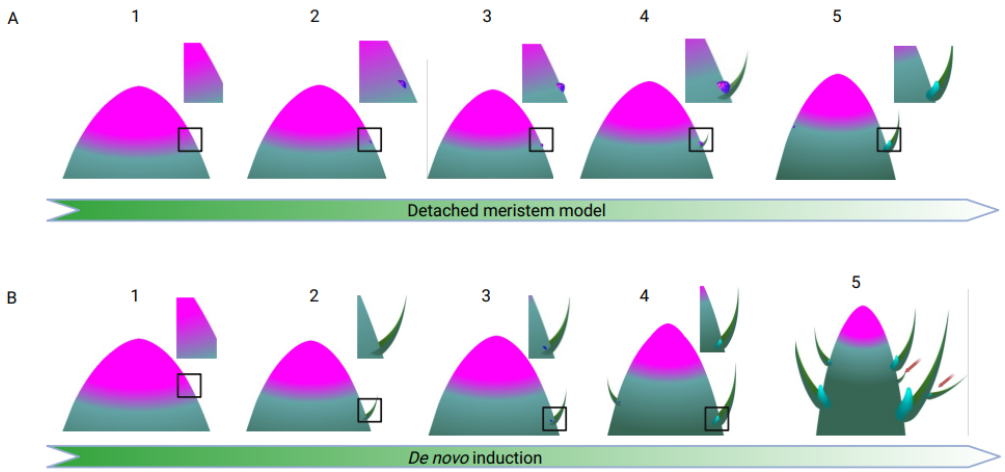

Figure 5. Dynamic illustration of distinct origins of AM. (A) The detached meristem model: one axillary meristem emanates from the shoot apical meristem (SAM). This process is represented successively from a group of cells first derived from the SAM, which then grow up to one tiller bud. (B) The *de novo* induction model: In some cases, the tiller forms directly from determinate cells, without derivation from the SAM. When essential genes are disrupted, the tiller can even form far from the leaf axil (indicated by arrow). The enlarged pictures (top right) represent the region framed by boxes.

Alternatively, the '*de novo* induction' model proposes that a set of differentiated cells equivalent to their neighbors can form an AM given an appropriate localized signal (Long and Barton, 2000). Evidence for this includes AMs arising on the underside of leaves in *Arabidopsis phabulosa-1d* (*phb-1d*) mutants. (McConnell and Barton, 1998). Additionally, ectopic AM occurs in stm mutants that lost SAM characteristics (Endrizzi et al., 1996). Adventitious SAMs can arise from the axils of cotyledons and cultured root explants in pinhead mutants (McConnell and Barton, 1995). Ectopic expression of the AM regulator, *Super determinant 1A* (*SDE1*), which is confined in leaf axils and regulates AM development, leads to ectopic meristem formation at the distal leaflets even in the shoot away from leaf axils (López et al.,

2021). Together, these observations lend credence to the '*de novo* induction' hypothesis, whereby differentiated cells can acquire meristematic identity.

The ongoing debate between the "detached meristem" and "*de novo* induction" theories in AM initiation is complex. Moreover, the expression of STM in the interprimordial regions between SAM and leaf axils (Shuai et al., 2002; Greb et al., 2003a) complicates this debate, as it challenges clear differentiation between these two concepts. We propose investigating a range of mutants, specifically those with distinct AM initiation but no SAM defects as a strategy, and the second one that knocks out the AM-specific expressed genes by high-through CRISPR/Cas9 system, to bridge the understanding between these two mechanisms. This approach is promising because the genes associated with these mutants might exclusively influence AM or interact with SAM-related genes to trigger AM initiation.

Table 1. Gene list involved in AM initiation

Gene Names	Accession Numbers	Species	Functional Annotation	References
MOC1	Os06g0610350	Rice	A GRAS protein	(Li et al., 2003)
LAS	AT1G55580	*Arabidopsis*	A GRAS protein	(Greb et al., 2003a)
STM	AT1G62360	*Arabidopsis*	A class I knotted-like homeodomain protein	(Long et al., 1996)
REV	AT5G60690	*Arabidopsis*	A small homeodomain-leucine zipper family	(Otsuga et al., 2001)
SPS	AT1G16410	*Arabidopsis*	A member of CYP79F proteins	(Tantikanjana et al., 2001)
CLV3	AT2G27250	*Arabidopsis*	CLAVATA3/ESR -related	(Otsuga et al., 2001)

ATH1	AT3G47730	*Arabidopsis*	A BEL1-like homeodomain (BLH) type three-amino-acid loop extension (TALE) class homeodomain protein	(Cao et al., 2020b)
WUS	AT2G17950	*Arabidopsis*	A homeodomain transcription factor	(Wang et al., 2017)
MOC3	Os04g0663600	Rice	A homeodomain transcription factor	(Lu et al., 2015b)
PHV	AT1G30490	*Arabidopsis*	Belonging to HD-Zip family	(Shi et al., 2016)
RAX1	AT4G23100	*Arabidopsis*	Belonging to the class R2R3 MYB genes	(Wang et al., 2017)
RAX2	AT2G36890	*Arabidopsis*	Belonging to the class R2R3 MYB genes	(Guo et al., 2015)
RAX3	AT3G49690	*Arabidopsis*	Belonging to the class R2R3 MYB genes	(Guo et al., 2015)
ARR1	AT3G16857	*Arabidopsis*	An *Arabidopsis* response regulator (ARR) protein	(Zheng and Chen, 2011)
AXR1	AT1G05180	*Arabidopsis*	Encoding a subunit of the RUB1 activating	(Stirnberg et al., 1999)

			enzyme	
PINHEAD	AT5G43810	*Arabidopsis*	A member of the EIF2C class of proteins	(Zhang et al., 2020a)
CUC1	AT3G15170	*Arabidopsis*	A transcription factor	(Hibara et al., 2006)
CUC2	AT5G53950	*Arabidopsis*	A transcription factor	(Hibara et al., 2006)
CUC3	AT1G76420	*Arabidopsis*	A transcription factor	(Hibara et al., 2006)
DA1	AT1G19270	*Arabidopsis*	A ubiquitin-activated peptidase	(Li et al., 2020)
UBP15	AT1G17110	*Arabidopsis*	A ubiquitin-specific protease	(Li et al., 2020)
DPA4	AT5G06250	*Arabidopsis*	Transcription repressor of CUC2/CUC3	(Li et al., 2020)
SOD7	AT3G11580	*Arabidopsis*	Encoding nuclear localized B3 DNA binding domain	(Li et al., 2020)
DRN	AT1G12980	*Arabidopsis*	Encoding an AP2/ERF protein	(Tian et al., 2014)
drnl	AT1G24590	*Arabidopsis*	Encoding an AP2/ERF protein	(Tian et al., 2014)

miR164A	AT2G47585	*Arabidopsis*	A microRNA that targets several genes containing NAC domains	(Rhoades et al., 2002)
miR164C	AT5G27807	*Arabidopsis*	A microRNA that targets several genes containing NAC domains	(Wang et al., 2004)
miR164B	AT5G01747	*Arabidopsis*	A microRNA that targets several genes containing NAC domains	(Bonnet et al., 2004)
LFY	AT5G61850	*Arabidopsis*	A transcriptional regulator	(Chahtane et al., 2013)
LOF1	AT1G26780	*Arabidopsis*	A MYB-domain transcription factor	(Lee et al., 2009)
EXB1	AT1G29860	*Arabidopsis*	A member of WRKY Transcription Factor	(Guo et al., 2015)
ROX	AT5G01305	*Arabidopsis*	Encoding a bHLH protein	(Yang et al., 2012)
LAX1	Os01g0831000	Rice	Encoding a bHLH protein	(Komatsu et al., 2003a)
BA1	Zm00001d042988	Maize	Encoding a bHLH protein	(Gallavotti et al., 2004)
PIN1	AT1G73590	*Arabidopsis*	Encoding Encodes an auxin	(Wang et al.,

			efflux carrier	2014)
TAA1	AT1G70560	*Arabidopsis*	The auxin biosynthesis gene	(Guo et al., 2015)
PIN5	AT5G16530	*Arabidopsis*	A functional auxin transporter	(Guo et al., 2015)
PID	AT2G34650	*Arabidopsis*	A protein serine/threonine kinase	(Michniewicz et al., 2007)
MP	AT1G19850	*Arabidopsis*	a transcription factor (IAA24)	(Guo et al., 2020)
BZR1	AT1G75080	*Arabidopsis*	a positive regulator of the (BR) signaling pathway	(Van De Velde et al., 2017)
SPL9	AT2G42200	*Arabidopsis*	A putative transcriptional regulator	(Zhang et al., 2020b)
GA2ox4	AT1G47990	*Arabidopsis*	A gibberellin 2-oxidase	(Zhang et al., 2020b)
LOB	AT5G63090	*Arabidopsis*	Involved in lateral organ development	(Gendron et al., 2012)
BAS1	AT2G26710	*Arabidopsis*	A member of the cytochrome p450 family	(Bell et al., 2012)
DSP	Os02g0594300	Rice	APETALA2/ethylene-responsive element binding	(Yu et al., 2023)

			protein	
AHK2	AT5G35750	*Arabidopsis*	A histidine kinase	(Wang et al., 2014)

2. The genetic and epigenetic factors regulate AM initiation

Understanding the molecular mechanisms that govern shoot branching heavily relies on characterizing genes responsible for AM initiation. To this end, multiple genes, including the key genes like rice *MOC1* and its orthologues *LS* and *LAS* in tomato and *Arabidopsis*, *STM*, and *REV* in *Arabidopsis* (Long et al., 1996; Schumacher et al., 1999; Otsuga et al., 2001; Greb et al., 2003a; Li et al., 2003), have been identified and thoroughly studied.

Generally, current mutants related to AM development exhibit morphological defects that can be categorized into two classes. The first type comprises mutations affecting AM initiation, leading to a lack of AM formation, as the las mutant exemplifies. The second type enhances AM formation, resulting in a bush phenotype, as observed in *supershoot* (*sps*) mutants (Tantikanjana et al., 2001). In the following discussion, we summarize genes associated with AM initiation, accompanied by a critical analysis of their hierarchical relationships, where applicable (Figure 6).

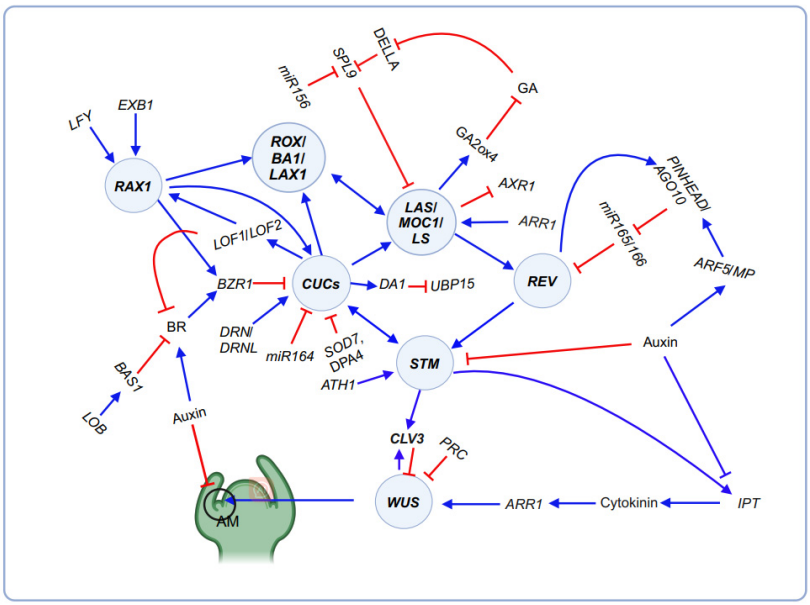

Figure 6. Summary diagram of components and phytohormones in AM initiation. Blue arrows indicate promotion and red-flat ended lines depict inhibition. The AM is circled. The genes acting with several genes are framed framed with circles with blue background. In this model, many factors function at different stages, including key hub genes (e.g., *CUCs* and *LAS* genes) and phytohormones (e.g., auxin, BR, and CK, etc.). Abbreviations: *LFY*, *LEAFY*;*EXB1*, *EXCESSIVE BRANCHES1*; *LOF1/LOF2*, LATERAL ORGAN FUSION1/2; RAX1, *REGULATOR OF AXILLARY MERISTEM1*; BR, Brassinosteroid; BZR1, *BRASSINAZOLE-RESISTANT1*; *BAS1*, *PHYB ACTIVATION TAGGED SUPPRESSOR1*; *LOB*, *LATERAL ORGAN BOUNDARIES*; DRN, DORNROSCHEN; CUCs, *CUP-SHAPED-COTYLRDON genes*; *BRC1*, *BRANCHED1*; *STM*, *SHOOT MERISTEMLESS*; *LAS*, *LATERAL SUPPRESSOR*; *SPL9*, *SQUAMOSA PROMOTER BINDIN PROTEIN-LIKE9*; GA, Gibberellin; *ATH1*: *ARABIDOPSIS THALIANA HOMEOBOX GENE1*; *GA2ox4*, *gibberellin 2- oxidase4*; *DELLA*, *aspartic acid–glutamic acid–leucine–leucine–alanine*; *AXR1*, *Arabidopsis auxin-resistant 1*; *ROX, REGULATOR OF AXILLARY MERISTEM FORMATION*; *REV*, *REVOLUTA*; *PAGO10*, *ARGONAUTE10*; BR, Brassinosteroid; *MP*, *monopteros*; *ARF5*, *Auxin Response Factor 5*; *IPT*, *ADENYLATE ISOPENTENYLTRANSFERASE*; *ARR1*, *Arabidopsis response regulator1*; *WUS*, *WUSCHEL*; *CLV3*: *CLAVATA3*; *PRC*, *Polycomb repressive complex*; *DPA4*, *NGATHA-LIKE transcription factors DEVELOPMENT-RELATED PcG TARGET IN THE APEX4*; *SOD7*, SUPPRESSOR OF DA1-1 7; UBP15, *UBIQUITIN-SPECIFIC PROTEASE15*; *B-ARR*, *type-B Arabidopsis response regulator*.

Given that AMs function as new SAMs, generating vital plant structures such as tillers/branches, leaves, flowers, etc., it is getting essential to explore whether the genes instrumental in SAM development also play a role in the initiation of AMs. It

is hypothesized that these sorts of genes are involved in the fundamental processes of meristem initiation. This involvement is direct unless the formation of AMs is an indirect result of these mutations. To understand this relationship, we examine key genes like *STM*, *CUCs*, *REV*, *PINHEAD*, *CLV3*, and more, aiming to elucidate their functions and the potential hierarchical interactions among them. For example, the sustained expression of *STM*, a number of the *KNOTTED* class of homeodomain genes essential for SAM formation, suggests the presence of cells in an indeterminate state (Long et al., 1996). Overexpression of *STM* can lead to many ectopic SAMs in tobacco plants, indicating a cell fate switch from determinacy to indeterminacy in cell fate (Sinha et al., 1993). Notably, despite the role of *STM* in SAM, ablation of most cells within the *STM*-expressing region prevents AM initiation (Shi et al., 2016). These findings indicate that AM and SAM share a comparable molecular regulatory mechanism, with *STM* also playing a crucial role in AM initiation. However, it is noteworthy that ectopic *STM* expression is inadequate to activate AM formation from leaf axil cells that have lost *STM* expression (Shi et al., 2016), suggesting some cells undergo irreversible fate change or require special triggers to reverse them to indeterminate states. As *STM* has been proposed as an early marker of AM initiation (Long and Barton, 2000), a small group of stem cells in the boundaries between the SAM and the emerging leaf primordium will develop into AM expressing *STM*, suggesting an involvement of *STM* in AM initiation (Keller et al., 2006). Furthermore, Wang et al. proposed a two-stage model for cell division during AM initiation, associating each stage with distinct *STM* expression levels (Wang and Jiao, 2018b). In this model, maintaining low *STM* expression is required but insufficient for AM initiation. A subsequent increase in *STM* induces AM initiation and bulging (Shi et al., 2016). The early low levels of *STM* expression are presumably needed for stem cell competence, although these cells lack *CLV3* or *WUS* expression, which are also essential for AM formation (Shi et al., 2016). It has been shown that the *ARABIDOPSIS THALIANA HOMEOBOX GENE1* (*ATH1*), encoding a *BEL1-like homeodomain* (*BLH*) *type three-amino-acid loop extension* (*TALE*) class homeodomain protein, maintains *STM* expression, thus preserving the meristemic cell fate (Gómez-Mena and Sablowski, 2008).

The *WUS* gene, a homeodomain transcription factor expressed in the SAM of the organing center, defines the stem cell niche (Wang et al., 2017). Despite its role in embryonic SAM formation, *WUS* in *Arabidopsis* and its rice ortholog *MONOCULM 3* (*MOC3*) are required to initiate AM (Lu et al., 2015b; Tanaka et al., 2015; Wang et al., 2017; Xin et al., 2017). Moreover, *WUS* expression is repressed by a polypeptide signal encoded by *CLV3*, acting as a stem cell marker (Schoof et al., 2000). Interestingly, *WUS* and *CLV3* have a feedback relationship during AM initiation (Figure 6). *CLV3* is undetectable in leaf axils in wus mutants, suggesting that *WUS* can activate *CLV3* (Xin et al., 2017). However, *WUS* expression is highly elevated in the *clv3-2* leaf axils, where the AM primordium is larger than that in the wide-type, suggesting *CLV3* signaling already restricted *WUS* expression to enable proper AM size determination in early developmental stages (Xin et al., 2017). *WUS* expression precedes AM initiation after *STM* expression in the initial phases (Guo et al., 2020)

(Figure 6). Moreover, Wang et al. 2021 considered that AM initiation concurs with the expression of *WUS* and *CLV3* between leaf primordium 11 (P11) and P13 in *Arabidopsis* (Wang, 2021). This finding is underscored by a similar observation in rice, where AM initiation can be detected at the P3 stage, as evidenced by the expression of *MOC3*, a rice ortholog of *WUS* (Lu et al., 2015b; Shao et al., 2019).

The *REV* gene, encoding an HD-ZIPIII transcription factor, is indispensable for forming all lateral meristems in addition to its role in SAM development (Otsuga et al., 2001). Indeed, loss-of-function of *REV* mutants leads to the absence of AMs (Talbert et al., 1995; Tian et al., 2014). *REV* can bind to the *STM* promoter region, indicating the *STM* requirement of *REV* during AM formation (Tian et al., 2014). The ectopic expression of the *REV* homolog *PHAVULOTA* (*PHV*) maintains and further activates ectopic *STM* expression on the abaxial leaf side, leading to ectopic AM formation (Shi et al., 2016). Histological analysis revealed that *REV* expression precedes *WUS*, which indicates that *REV* activity is epistatic to *WUS* (Otsuga et al., 2001). Regarding *STM* expression, no difference exists between wus mutants and wild-type, consistent with *STM* acting epistatically to *WUS* (Wang et al., 2017).

The redundant *CUC* genes (*CUC1*, *CUC2*, and *CUC3*) in *Arabidopsis* encode NAC transcription factors that significantly contribute to embryonic shoot meristem formation and shoot organ boundary specification. *CUC2* and *CUC3*, but not *CUC1*, influence AM formation. Further analysis indicates that *CUC3* plays a more significant role in regulating shoot branching than *CUC2*, but the effect is most prominent when *CUC2* and *CUC3* are combined (Hibara et al., 2006). *CUC2* and *CUC3* can directly activate the expression of *DA1*, encoding a ubiquitin-dependent peptidase, while mutations of the *DA1* substrate in *UBIQUITIN-SPECIFIC PROTEASE15* (*UBP15*) lead to repression of AM initiation (Li et al., 2020). Two transcription factors, the *NGATHA-LIKE* transcription factors *DEVELOPMENT-RELATED PcG TARGET IN THE APEX4* (*DPA4*) and *SUPPRESSOR OF DA1-1 7* (*SOD7*) redundantly repress *CUC* expression in the leaf axil and *dpa4-2 sod7-2* double mutants display delayed AM initiation (Nicolas et al., 2022). *DRN* and its homolog *DRNL*, which encode AP2-type transcription factor family proteins, are required for AM initiation by directly activating *CUC2*. Large portions of the leaf axils in single or double *dornroschen* (*drn*) and drnlike (*drnl*) mutants are barren (Tian et al., 2014).

Recessive mutations in the *PINHEAD* locus of *Arabidopsis* disrupt the primary shoot meristem and AM formation, resulting in a single leaf or a slender pin-like organ and reduced lateral buds both in axils of cauline and rosette leaves (McConnell and Barton, 1995; Ratcliffe et al., 1999; Zhang et al., 2020a). *PINHEAD* expression coincides in the leaf axil where AMs form and its overexpression occasionally produces more than one AMs per leaf axil (Zhang et al., 2020a), indicating its role in controlling AM formation. *STM* and *REV* are both down-regulated and up-regulated in the pinhead and its overexpression mutants, respectively (Figure 6), suggestive of an epistatic role of *PINHEAD* to *REV* and *STM* (Zhang et al., 2020a).

Plant microRNAs are endogenous, single-stranded, and nontranslated RNA molecules that are highly complementary to their target mRNAs, mediating post-transcriptional gene silencing through mRNA cleavage (Bartel and Bartel, 2003). The

miR164 genes, comprising *miR164A*, *miR164B*, and *miR164C* (Rhoades et al., 2002; Bonnet et al., 2004; Wang et al., 2004), post-transcriptionally regulate *CUC* genes (Schwab et al., 2005; Raman et al., 2008). Constitutive overexpression of *miR164* phenocopies the branching habits of *cuc1 cuc2* double mutants by downregulating *CUC1* and *CUC2* transcripts (Mallory et al., 2004; Nemhauser et al., 2004). Conversely, the loss of function of *miR164* genes can produce more accessory buds (Raman et al., 2008). In contrast, overexpression of *miR164* in the *cuc3-2* mutant abolishes AMs, indicating that *miR164*, *CUC1*, *CUC2*, and *CUC3* play a pivotal role in AM initiation (Raman et al., 2008). Besides *miR164*, the rescue of AM defects in *Arabidopsis argonaute 10* (*ago10*, also known as pinhead) by sequestering *miR165/166* (Zhu et al., 2011), which targets *REV*, suggests *AGO10/PINHEAD* acts upstream of the *REV* and *STM* through the sequestration of *miR165/166*.

Collectively, these genes, such as *STM*, *CUCs* and *REV*, are integral in orchestrating the development of both the SAM and AMs. Yet, the precise timing and spatial dynamics governing the initiation of AM development remain topics for further exploration. We hypothesize that these genes depend on additional genes, specifically expressed in the leaf axils, to function as triggers for the initiation of AMs. This activation might occur either preceding or following their expression. Notably, a particular category of genes, known to manifest AM defects by specially regulating AM initiation, includes *Arabidopsis LAS* and *Regulator of Axillary Meristem* (*RAX*) (Keller et al., 2006), as well as rice *MOC1* (Li et al., 2003) and *LAX1* (Komatsu et al., 2003b). Disruption of *MOC1* in rice or its orthologous genes (e.g., *LS* in tomato and *LAS* in *Arabidopsis*), transcription factors of the *GRAS* family, results in the shortage of AMs and, consequently, fewer branches or tillers. *MOC1* and *LAS* are expressed explicitly in the AM initiation zone (Schumacher et al., 1999; Greb et al., 2003a; Li et al., 2003). These studies suggest a conserved function of these genes in both monocot and dicot. It is worth noticing that, in *Arabidopsis*, *STM* is focused on a group of small and densely cytoplasmic cells near the adaxial center of the primordium border, where it is required for AM initiation. However, these cells fail to develop into a new AM without *STM* expression in *las* mutants, suggesting that focused *STM* expressing denoting meristem organization onset relies on *LAS* function (Greb et al., 2003a). This coincides with our hypothesis that SAM-regulating genes necessitate precise mediation to initiate AM development at an appropriate location. Furthermore, *LAS* has been proven to be a hub gene that integrates inputs from many upstream genes, as indicated by a leaf axil-enriched gene regulatory network analysis (Tian et al., 2014).

Despite the similarities in expression patterns between *LAS* and *MOC1*, notable differences exist. *LAS* expression regions extend to several layers of SAM beyond the AM, compared with *MOC1*, which remains undetectable in SAM (Greb et al., 2003a; Li et al., 2003). Furthermore, the inflorescence meristems generated by AM were not affected in las mutants but otherwise in *moc1* mutants (Greb et al., 2003a; Zhang et al., 2021b; Chun et al., 2022). These variations underscore the evolutionary divergence between monocotyledonous and dicotyledonous plants, highlighting distinct regulatory mechanisms in plant architecture development. Likewise, *ROX* is

an *Arabidopsis* gene encoding bHLH protein, orthologous to the branching regulators *LAX1* in rice and *BARREN STALK1* (*BA1*) in maize (Komatsu et al., 2003b; Yang et al., 2012; Matthes et al., 2019). Loss-of-function of *ROX* caused compromised AM formation. Its expression extended to the SAM and the AM, unlike *LAX1*'s AM location (Oikawa and Kyozuka, 2009). In contrast to *LAX1* and *BA1*, flower development was uninfluenced in *rox* mutant (Yang et al., 2012), further supporting the hypothesis of evolutionary distinctions between monocotyledons and dicotyledons. However, this inference should be pitched out with caution as the differences between *las* and *rox* mutants of *Arabidopsis* and their corresponding wild types became more pronounced when studied under short-day conditions (Greb et al., 2003a; Yang et al., 2012). Because *Arabidopsis*, native to Europe and central Asia, has spread in the temperate climate zones of the five continents and, therefore, originally adapted to long-day conditions (Hsu et al., 2019).

In addition to the reliance of *STM* on *LAS*, other SAM-related genes are also affected in las. For example, *WUS* is undetectable in *las* mutants, indicating that *WUS* acts downstream of *LAS* (Wang et al., 2017). Enriched *REV* expression in leaf axils relies on *LAS*, a member of the *GRAS* family controlling AM initiation (Greb et al., 2003a). Substantial upregulation of *LAS* accumulation was observed in mir164 triple mutants, indicating that *miR164* can negatively regulate *LAS* (Raman et al., 2008). Thus, AM initiation is inhibited by *miR164* through restricting *CUC1/2* accumulation, which in turn regulates *LAS* expression (Raman et al., 2008). This is further substantiated by the observed down-regulation of LAS in the double mutant *cuc1 cuc2*, placing *LAS* downstream of *CUC1* and *CUC2* (Hibara et al., 2006) (Figure 6). The regulation of the AM-specific gene *LAS* by *CUC* genes suggests that specific signals from the SAM are necessary to trigger AM initiation. This mechanism underscores the intricate interplay between SAM and AM development.

In addition to *LAS*, other genes specially expressed in or near AM also mediate SAM-related genes. For example, *Regulator of axillary meristem 1* (*RAX1*), a homolog of *Blind* (*BL*) encoding an *MYB* family gene, promotes AM initiation by specifying the location of the stem cell niche. *RAX1* functions redundantly with *RAX2* and *RAX3* to regulate AM initiation (Keller et al., 2006). *RAX1* is initially detectable in a subregion along the boundary between the meristem and leaf primordia, similar to *LAS* (Keller et al., 2006). *LEAFY* (*LFY*), a master regulator of the transition of the reproductive stage, directly activates *RAX1* to promote AM initiation (Chahtane et al., 2013). *RAX1* also directly enhances *CUC2* expression in vivo and in *vitro* (Tian et al., 2014). *LATERAL ORGAN FUSION1* (*LOF1*) is also an *MYB* domain gene, which is expressed in the boundary domain of the SAM and leaf primordia. Loss-of-function if *lof1* mutants lack AMs and STM expression in the corresponding boundary domain (Lee et al., 2009). Further gene expression analysis in *lof1* mutants positioned *LOF1* upstream of the *RAX1*, *STM*, *LAS*, and *CUC* genes (Shuai et al., 2002). However, Gendron et al. (2012) suggested that *CUC* genes may positively regulate *LOF1* and *LOF* genes (Gendron et al., 2012), a way similar to that of *LAS* and *CUC*s. The *EXCESSIVE BRANCHES1* (*EXB1*) gene, encoding the *WRKY* transcription factor *WRKY71*, affects AM initiation. *EXB1* disruption results in reduced branching, while

overexpression of *EXB1* in *exb1-D* gain-of-function mutants leads to severe bushy and dwarf phenotypes (Guo et al., 2015). *EXB1* is shown to control AM initiation by positively regulating the transcription of *RAX1*, *RAX2*, and *RAX3* (Guo et al., 2015). Consistently, *wrky-b* mutants in tomato exhibited reduced lateral branches, while the *WRKY-B* overexpression lines produced many more lateral branches (Yang et al., 2024). Interestingly, overexpression of rice *WRKY72* in *Arabidopsis* also increases shoot branches (Song et al., 2010), implying evolutionary conservation of *EXB1/WRKY71* function in AM formation between monocots and dicots (Guo et al., 2015).

In addition to the precise regulation of SAM-related genes by genes expressed in AMs, epigenetic modulation allows nuanced expression of the same gene in distinct cell types and developmental contexts. Such epigenetic control allows for the diverse expression patterns of genes, which may be broadly expressed yet exhibit distinct functions in various cellular environments and stages of plant development. Thus, epigenetic control is intrinsically involved in all developmental processes, including AM initiation. DNA methylation plays a critical role in gene imprinting, genome stability, development, and response to the environment. For example, the disruption of DNA methylation gene *SlCMT4* can lead to increased lateral buds (Guo et al., 2022). Since many genes mediating *WUS* and *STM*, such as *REV* and *ARABIDOPSIS RESPONSE REGULATOR 1* (*ARR1*) which is a type-B ARR transcription factor, are not exclusively expressed in leaf axils. Therefore, precisely expressing the genes needed for particular stages is imperative for AM control. The *Polycomb Repressive Complex 2* (*PRC2*) establishes the *histone methylation* (*H3K27me3*) mark in plants and animals, providing a docking site for *PRC1* to impose repressive chromatin (Zheng and Chen, 2011). In mature leaves, where cells are fully differentiated, both *WUS* and *STM* exhibit high levels of *H3K27me3*, indicative of a low abundance of *WUS* and *STM* mRNA. In contrast, these two genes have a low concentration of *H3K27me3* and a high concentration of *H3K4me2/3*, a mark associated with active chromatin, in tissues containing the leaf axil *STM*-expressing cells (Shi et al., 2016; Cao and Jiao, 2020). Accordingly, *STM* and *WUS* are elevated in *prc* mutant (Shi et al., 2016; Wang et al., 2017). Moreover, applying histone deacetylation inhibitor trichostatin A induces ectopic *WUS* expression (Xin et al., 2017). Wang et al. showed that epigenetics contributes to the dynamic of WUS, with expression terminated in leaf axils and then reactivated *de novo* (Xin et al., 2017). A large abundant *H3K27me3* represses the *WUS* expression in the leaf axil, while histone *H3/H4* acetylation (*H3/4Ac*) is depleted. Before *WUS* activation, the levels of the *H3K27me3* repressive mark decrease, while the levels of the active *H3/4Ac* mark increase (Wang et al., 2017; Cao and Jiao, 2020).

In conclusion, the intricate web of genetic and epigenetic factors orchestrates the initiation of axillary meristems in plants. The collaborative action of genes such as *CUC*, *WUS*, *STM*, *REV*, *LAS*, and others, along with the regulatory influence of miRNAs, creates a finely tuned molecular symphony.

3. How do phytohormones precisely control AM initiation?

Phytohormones regulate diverse developmental processes throughout the plant life cycle. For example, auxin orchestrates developmental responses such as gravitropism and apical dominance, which depend on forming auxin gradients in plant tissues (Leyser, 2018; Casanova-Sáez et al., 2021). Cytokinins (CKs) influence agricultural processes, including growth, nutrient responses, and biotic/abiotic stress responses (Kieber and Schaller, 2018). Gibberellins (GAs) promote growth by regulating seed germination, root/shoot elongation, flowering, and fruit patterning (Binenbaum et al., 2018). Brassinosteroids (BRs) also stimulate plant growth and development by controlling cell division, elongation, and differentiation (Planas-Riverola et al., 2019). Given multiple roles of phytohormones in plant development, regulatory mechanisms must exist to precisely control AM initiation. Based on current understanding, we synthesize the hormonal control of AM initiation as follows:

Lines of evidence that auxin is involved in AM initiation have been reported. For example, the AGC III kinase *PINOID* (*PID*) modulates polar auxin transport by regulating *PIN1* localization within the cell (Michniewicz et al., 2007). Severe homozygous pid mutants resemble pin1 mutants and fail to form AMs compared to wide-type plants (Wang et al., 2014). This highlights the importance of directional auxin transport for AM initiation. In the dominant mutant *exb-1D*, which displays excessive branching, the auxin biosynthesis gene *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1*) and the auxin transport genes (e.g., *PIN5*), are repressed by *EXB1* induction (Guo et al., 2015), suggesting the importance of auxin homeostasis to control shoot branching. Additionally, as in *auxin resistance 1* (*axr1*) mutants, reduced auxin sensitivity enhances AM formation (Stirnberg et al., 1999), implying the significance of auxin in mediating AM initiation. The dynamic requirement of auxin was further investigated. Namely, the auxin minimum is one prerequisite for AM initiation, exemplified by that: *PIN1* mediates auxin flow in the adaxial domain away from the leaf axil toward the tip of the leaf primordium, thus establishing the auxin minimum in the leaf axil (Bayer et al., 2009; Wang et al., 2014). The shoot meristem marker *STM* is activated during AM formation (Long and Barton, 2000; Greb et al., 2003a). Conversely, ectopically expressing an auxin biosynthesis gene, indicating higher auxin levels in the leaf axil, decreases *STM* expression (Wang et al., 2014). This auxin minimum niche sustaining *STM* expression in the boundary zone to maintain the AM identity was evidenced by Guo et al. 2015 (Guo et al., 2015). In contrast, an elevated auxin concentration in the stem cell maintenance stage, driven by a specially located leaf axil gene promoter, perturbs the AM initiation (Wang et al., 2014). Consistent with this, *pin-formed 1* (*pin1*)-like phenotypes are observed in mutants lacking *PID* function. Because the *PID* disruption presumably maintains unidirectional auxin apical-basal transport, consequently increasing auxin to inhibit AM formation (Friml et al., 2004). These lines of evidence show that the auxin minimum is the precondition for AM initiation. However, maize auxin biosynthesis mutants display defects in vegetative AM formation (Matthes et al., 2019), implying that auxin is still required at some points during AM initiation. The requirement of auxin during AM initiation was demonstrated by the monopteros

(*mp*) mutant. *MP*, also known as *ARF5*, is an auxin response factor that activates downstream signaling in response to auxin. *MP* expresses in the youngest leaf primordia and ceases its expression at the later AM formation stage when AM starts bulging (Guan et al., 2017; Guo et al., 2020), indicating the existence of a high concentrate auxin in the later AM bulging stage and a positive role for auxin in AM initiation (Zhang et al., 2020a). Furthermore, *MP* may activate the expression of PINHEAD, whose protein sequesters miR165/166 to release *REV* and *STM*, thereby promoting AM initiation (Zhang et al., 2020a). Moreover, rescued AM defects in mpΔ mutants by deleting the *ARF5*-binding site in the *AGO10/PINHEAD* promoter indicated auxin signaling is required in late AM initiation stages (Zhang et al., 2020a). In summary, auxin is essential for AM initiation, playing dynamic roles during this process. In the early stages of AM formation, an auxin minimum is required for meristematic cell maintenance, while in the later stages, higher auxin levels promote their activation. Further dissection of auxin synthesis, transport and signaling dynamics will provide deeper insights into its complex regulation of AM development.

Genetic analysis has shown that CK perception and signaling are essential for AM initiation. Several instances have favored the requirement of CK. For instance, AM initiation deficiency of *rax1* mutants can be partially rescued by either CK production in leaf axil or exogenous CK treatment (Wang et al., 2014). Additionally, CK receptors and the signaling detector in the leaf axils are upregulated prior to and during AM initiation, indicating cytokinin's role in AM initiation (Wang et al., 2014). Mutants defective in CK receptors, such as *Arabidopsis* histidine kinase 2 (*ahk2*), *ahk3*, and *ahk4*, with compromised CK perception and the corresponding double mutants exhibit defects in AM initiation. Moreover, *B-type ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) transcription factors, which act downstream of the CK signaling pathway, are also required for AM initiation (Wang et al., 2014). Furthermore, the SAM-related genes and CKs were mutually regulated to guarantee AM initiation. For example, during AM initiation, *STM* activates CK biosynthesis in leaf axils (Guo et al., 2015). CK signaling then activates *de novo WUS* expression in leaf axils (Wang et al., 2017; Guo et al., 2020). *Type-BRRs,* transcriptional activators in CK signaling, especially *ARR1*, directly bind to the *WUS* promoter to activate its expression (Cao and Jiao, 2020). *ARR1* also directly binds to the *LAS* promoter to activate its expression (Tian et al., 2014). In addition to the low auxin condition, a subsequent pulse of CK occurs prior to AM initiation (Wang et al., 2014). No CK signal can be detectable without an auxin minimum in the leaf axil (Wang et al., 2014), demonstrating the dependence of the leaf axil CK pulse on the auxin minimum. Supporting this, *sps* mutants, which show a bushy phenotype due to enhanced AM formation and lateral bud release, have elevated levels of CK but decreased levels of auxin (Tantikanjana et al., 2001). Furthermore, in mutants such as *las*, *rax*, and *rev* with compromised AM initiation, the leaf axils lack CK signaling pulse (Wang et al., 2017), indicating that CK is required for AM initiation. Overall, the evidence demonstrates that CK signaling is a key step following the establishment of the auxin minimum niche. CK perception and downstream transcriptional activation of *WUS*,

LAS and other AM regulators promote the activation and bulging of meristematic cells to initialize AM development.

GAs are growth-promoting hormones that mediate various plant developmental processes throughout the plant life cycle (Yamaguchi, 2008). However, exogenous GA application decreases AM formation. Leaf axils ectopically expressing a GA biosynthesis gene showed significantly lower AM formation (Zhang et al., 2020b). Conversely, the GA-deficient mutant ga1-3 displays more AMs, indicating a negative role of GA in AM formation. However, how GA precisely regulates AM initiation, especially regulating AM-specific or SAM-related genes, deserves investigation due to the importance of AMs. DELLA proteins, master repressors of GA signaling, participate in various physiological processes by interacting with various transcription factors, including BRASSINAZOLE-RESISTANT1 (BZR1), ARR1, and ARF6 (Van De Velde et al., 2017). A della pentuple mutant shows defects in AM formation, suggesting that DELLAs play a role in this process. DELLAs interact with SPL9, thus attenuating its repression of *LAS*. This promotes AM initiation, with *LAS* then inducing GA deactivation enzyme *Gibberellin 2-beta-dioxygenase 4* (*GA2ox4*) to form a low-GA condition in leaf axils (Zhang et al., 2020b). Thus, the crosstalk and balance between GA metabolism and LAS precisely modulate AM formation spatiotemporally.

In addition to auxin and CKs, BR-responsive genes are highly enriched in organ boundary cells, suggesting these sites are novel centers of BR activity (Tian et al., 2014). BR is an essential plant steroid hormone regulating cell division and expansion (Gendron et al., 2012). As low cell division rates are required in the boundary zone to maintain AM competence, BR accumulation is negatively regulated in leaf axils by *LATERAL ORGAN BOUNDARIES* (*LOB*), a key boundary-specific transcription factor. *LOB* directly upregulates *PHYB ACTIVATION TAGGED SUPPRESSOR1* (*BAS1*), a cytochrome P450 enzyme that inactivates BRs through C-26 hydroxylation, thereby reducing BR levels to decrease cell division and expansion in the boundary zone (Bell et al., 2012; Gendron et al., 2012). Furthermore, the SAM-developmental gene *CUC3* was inhibited by the BR-activated gene *BRASSINAZOLE-RESISTANT1* (*BZR1*) by directly binding to the promoter of *CUC3*, indicating low BR levels in boundary zones are required to activate AM initiation. In summary, *LOB* restricts BR accumulation in leaf axil boundary zones through *BAS1* induction. Low BR levels inhibit cell division and expansion while also alleviating *BZR1* repression of *CUC3*. Fine-tuned crosstalk between BR and key AM regulators like *LOB* and *CUC3* allows proper AM initiation.

While plant hormones, such as auxin, CKs, BRs, and GAs, participate in various development throughout the entire plant's life, precisely where and when they act is the key point for development. Likewise, AM initiation is associated with phytohormones, which must be involved at the right time and location.

4. Genes regulating AM formation affect grain yield

For crop species, AMs are essential for producing tillers bearing grains, determining the number of seed spikes per plant and the number of seeds per spike - all key factors

influencing overall crop yield (Wang and Jiao, 2018a), such as in rice, maize, and wheat. These factors are directly determined by branching ability during vegetable and reproductive growth stages. Namely, AM essentially harbors a niche with a group of meristematic cells to influence branching in tilers and panicles. For instance, the *LAX1* and *MOC1* genes in rice are involved in the formation of both tillers and panicle branches. Mutations in either *MOC1* or *LAX1* resulted in a reduced number of both tillers and panicle branches (Wang and Li, 2011). Further exploring indicated that *LAX1* is regulated indirectly by the gene defective stigma and panicle (*DSP*), determining tiller primordium formation and synergistically regulating panicle primordium development (Yu et al., 2023). Likewise, in Helianthus annuus, a dicotyledonous species, the mutated *REGULATOR OF AXILLARY MERISTEM FORMATION-LIKE* (*Ha-ROXL*), akin to *LAX1*, affects both AM initiation and *Floral meristems* (*FMs*) (Basile et al., 2019), which suggested a shared role of LAX1 and its orthologs in influencing grain yield across dicots and monocots. However, disruption of *ROX* in *Arabidopsis*, an ortholog of *LAX1*, displays compromised AM formation during the vegetative phase, particularly noticeable under short-day photoperiods (Yang et al., 2012). Again, we must be cautious about drawing definitive conclusions regarding this abnormality observed in *Arabidopsis*, since *Arabidopsis* originated in and is generally grown under long-day conditions (Hsu et al., 2019). Furthermore, in *las* and *rax* mutants, the AM initiation defects are more easily recognized under short-day conditions than that under long-day conditions (Greb et al., 2003a; Keller et al., 2006), concurring with our perspective in making critical sense of AM formation in *Arabidopsis*. Collectively, further research on AM initiation genes is needed to elucidate the genetic mechanisms underlying tiller and panicle branching.

Another crucial aspect of AM that impacts grain yield is its developmental fate, which determines whether it differentiates into an inflorescence or a vegetative shoot. During the vegetative stage, the SAM generates shoots and leaves. After transitioning to the reproductive stage, it produces flowers (Benlloch et al., 2007). This process is regulated by the balance between two homologous genes: *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER 1* (*TFL1*). Both belong to the *FT/TFL1* gene family and encode proteins similar to phosphatidylethanolamine-binding proteins (PEBP) (Wickland and Hanzawa, 2015). In *Arabidopsis*, the transcription factor CONSTANS (CO) activates *FT* in the leaves, where the gene is transcribed and translated. The FT protein is then transported via the phloem to the vegetative apex, where it activates genes associated with floral meristem identity (Abe et al., 2005). Interestingly, overexpression of *TaCol-B5*, a CONSTANS-like protein in common wheat, promotes tillering and increases spikelet number (Zhang et al., 2022c). Tomato *self-pruning* (*SP*) is an ortholog of *Arabidopsis TFL1*. Two mutations of *Suppressor of SP* (*SSP*) have been identified to suppress the bushy growth habit of field tomatoes (Wang et al., 2018a). In contrast, *TFL1* regulates inflorescence meristem identity by delaying the transition of the SAM to the reproductive phase (Zhu et al., 2020). In rice, the knock-down of *RICE CENTRORADIALISs* (*RCNs*), the rice homologs of *TFL1*, reduces panicle size and panicle branching (Liu et al., 2013). Similarly, a mutation in the *TFL1* homolog of barley results in fewer spikelets and tillers (Bi et al., 2019). In wheat,

tatfl1-5 mutations decrease tiller numbers during the vegetative stage, reduce effective tillers per plant, and lower spikelet numbers per spike at maturity (Sun et al., 2023). The balance between *FT* and *TFL1* is essential for defining the plant's growth habit as either indeterminate or determinate by modulating the formation of vegetative and reproductive structures in the apical and axillary meristems. Specifically, a high *FT/TFL1* ratio promotes early flowering and results in short-statured plants, as the apical meristem converts into a terminal flower. In contrast, a lower *FT/TFL1* ratio enhances vegetative identity, leading to fewer flowers (Moraes et al., 2019).

5. Challenges and opportunities regarding isolation genes involved in AM formation

While essential for plant development and agriculture, the molecular mechanisms underlying AM formation stay elusive. The study of AM initiation has been hurdled, mainly due to the shortage of mutants, which especially affect AM development in plants like rice and *Arabidopsis*. This implies that many unknown AM initiation regulators demand to be identified. Notably, alternative methodologies developed recently could be employed to resolve this problem. For example, Yang et al. suggested the utilization of genetic backgrounds with reduced apical dominance to identify more AM initiation regulators (Yang et al., 2023). Genome editing technologies drive significant advances in life sciences due to precise modifications at target genomic loci (Doudna and Charpentier, 2014; Xing et al., 2023). CRISPR/Cas9 systems have been broadly adopted as a targeted genetic manipulation tool that has been applied to many species, such as rice, wheat, tomato, and more (Doudna and Charpentier, 2014). This routine technology can also be used to identify and validate new genes that act specifically in AM interactions. The utility of this technology is not limited to model plants, but can further be extended to cultivated crop plants and their wild progenitors, which often have very different architectures. Utilizing this technology allows for discovering AM-specific genes across a diverse range of plant species. Emerging yet thriving omics may also help distinguish new genes involved in AM initiation regulation. For example, single-cell omics technologies reveal the intracellular dynamics of different individual cells and answer biological questions with high-dimensional catalogs of millions of cells, including transcriptomics, genomics, chromatin accessibility, epigenomics, and proteomics data across species (Mo and Jiao, 2022). Initially applied in animals, single-cell RNA sequencing (sc-RNA) technologies have been embraced by the field of plants. In *Arabidopsis*, Zhang et al. carried out Sc-RNA to define the cellular taxonomy of the *Arabidopsis* vegetative shoot apex at the transcriptome level and found that the shoot apex is composed of highly heterogeneous cells (Zhang et al., 2021a); in maize, sc-RNA analyzed single cells from developing maize ears, helping to identify candidate genes associate crops yield traits (Xu et al., 2021); in rice, analysis of root tips using Sc-RNA provided insight into the transcriptomic landscape of major cell types of rice root tip at single-cell resolution (Wang et al., 2021). In addition, a gene regulatory network-based investigation of trichoblast differentiation in *Arabidopsis* revealed novel transcription factors and previously unknown feedback loops/mechanisms by

harnessing trajectory inference, one algorithm used in Sc-RNA analysis (Denyer et al., 2019). Despite Sc-RNA, single-cell level chromatin has also been practiced in plants, which is essential in AM initiation. For example, scATAC-seq has been applied to profile root tip cells in *Arabidopsis*, along with sc-RNA data, suggesting a connection between chromatin accessibility and expression dynamics (Farmer et al., 2021). Together, since the efficiency of the omics-based approaches, these techniques are more commonly used to investigate cell identity and fate changes, which also occur in AM initiation. As cell identity and fate changes occur during AM initiation, applying single-cell omics techniques could reveal new genes and networks controlling this process. These emerging approaches may expedite research on AM initiation mechanisms. Combined with clever genetics, single-cell omics technologies provide promising avenues to elucidate the molecular control of AM development.

6. Concluding remarks

This review has covered significant recent advances in elucidating the intricate molecular mechanisms governing AM initiation. Research over decades has revealed that AM development relies on coordinated regulation by transcriptional, hormonal, and epigenetic factors. Key regulators like *LAS*, *RAX1*, *STM*, *REV*, *WUS*, and *CUC2* converge to control gene expression programs activating meristematic fate in leaf axil cells. Intricate crosstalk between auxin, CKs, GAs, and other hormones establishes a niche conducive for AM formation. Moreover, dynamic changes in chromatin modifications facilitate spatiotemporal patterns of AM gene expression. Despite progress, questions remain regarding the developmental origin of AM progenitor cells, limitations for identifying more AM-specific, and integration of the various pathways regulating AM initiation. Key next steps include: (1) Elucidating the developmental relationship between the shoot apical meristem and AM progenitor cells and reconciling detached versus *de novo* origins during AM initiation; (2) Identifying additional novel regulators and networks of AM formation, combing omics-sequencing and cellular resolution imaging techniques; (3) Exploring divergence and specialization of AM developmental programs between plant species; (4) Leveraging knowledge of AM formation mechanisms to improve crop architecture and yield. Collectively, unraveling the AM initiation process remains an exciting frontier in plant development biology. Translation of these fundamental findings to crop species holds immense promise for agricultural enhancement. We anticipate the next decades would witness transformative discoveries illuminating how plants elaborate their axillary meristems to elaborately branch out their forms.

II. Unlocking the multifaceted mechanisms of bud outgrowth: advances in understanding shoot branching

1. Introduction

The plasticity exhibited by plants in their shoot development is remarkable, as it allows them to adapt to various harmful external and internal conditions in order to survive and thrive. Shoot architecture in seed plants is primarily determined by factors such as the number, position, orientation, and size of shoot branches. The regulation of shoot branching/tillering constitutes a critical survival and propagation strategy governed by a complex, sophisticated regulatory network.

Initiation of the primary shoot axis can be traced back to the SAM, a group of mitotic cells that forms during embryogenesis. Subsequently, the derivatives of this meristem give rise to all above-ground parts of plants (Bowman and Eshed, 2000). The SAM produces aerial organs by continuously adding growth units called phytomers, generally comprising three parts: an internode, a leaf, and an AM that emerges at the leaf axil (Wang et al., 2018a).

AMs are new stem cell niches derived from the SAM during post-embryonic development. AM activity plays a vital role in generating the intricate branching patterns that contribute to a plant's fractal architecture. Given its significant influence on shoot branching/tillering and panicle branching, the AM has been a focal point in breeding selection for improving crop production and management (Wang and Li, 2008; Jiao et al., 2010; Springer, 2010). For the convenience of readers to understand the influence of crop yield, please refer to Figure 7, which dynamically indicates the process of AMs' outgrowth and their impact on grain yield.

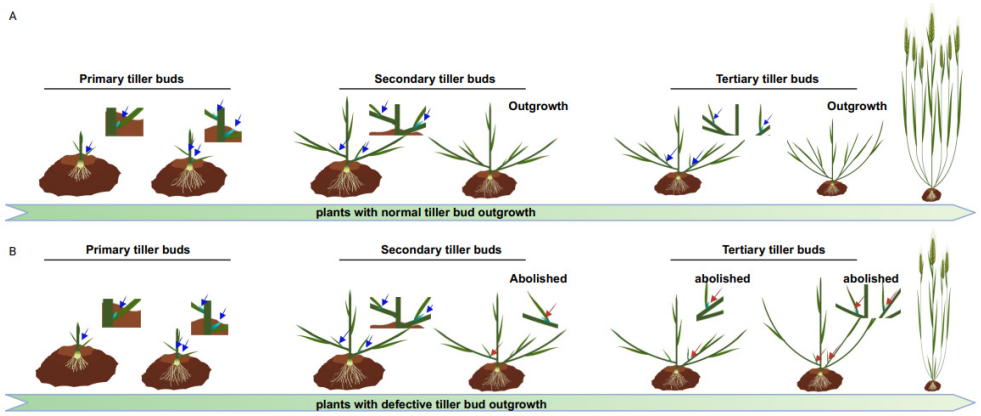

Figure 7. Illustration of the dynamic process of tiller formation and comparisons between plants with normal and defective tiller bud outgrowth. A portrays the process of the tiller bud formation successively and their outgrowth to generate more panicles than that in B. The primary tiller buds (arrows indicated) arise from the leaf axils of the main stem. Secondary tiller buds occur from leaf axils of the primary tillers and so on for subsequent higher-level tillers. We highlight with red arrows the abolished tiller buds that cannot grow to form tillers in B.

Numerous studies conducted over the past decades have aimed to unravel the mechanisms underlying shoot branching. The prevailing understanding is that various inputs, such as endogenous factors, developmental cues, and environmental signals, interlock to regulate shoot branching. For instance, among multiple essential genes controlling shoot branching in plants, *TB1* acts locally in buds to inhibit bud outgrowth and is considered to be an integrator of diverse phytohormonal, trophic, and environmental signaling networks (Figure 8) (Aguilar-Martínez et al., 2007; Wang et al., 2019c). In addition, the signals defined by phytohormones, nitrogen, light, and sugars have been shown to significantly affect shoot branching (Figure 7) (Helliwell et al., 2001; Dharmasiri et al., 2005a; Lo et al., 2008; Bayer et al., 2009; Lin et al., 2009; Finlayson et al., 2010; Wang et al., 2013; Mason et al., 2014; Tegeder, 2014; Tian et al., 2014; Wang et al., 2014; González-Grandío et al., 2017; Zhang et al., 2020b).

Significant advances have been made in our understanding of shoot branching, and numerous essential genes described in the literature have been demonstrated to influence shoot branching. However, the underlying mechanisms involving these genes are complicated, such as the effect of plant resistance genes on branching/tillering (in addition to resistance) when disrupted (Igari et al., 2008; Pan et al., 2022). This review aims to provide a comprehensive summary of recent advances in our understanding of shoot branching, with a primary focus on *Arabidopsis* and rice. Elucidating and differentiating the complex mechanisms underlying shoot branching will contribute to the field of crop breeding, as shoot branching/tillering crucially determines plant architecture, directly influencing yield

and overall productivity. This section has been published (Yuan et al., 2023b) as a review.

2. Mechanisms regulating shoot branching

Once a branch/tiller bud is formed, the plant is confronted with a critical decision to stimulate bud sprouting, giving rise to a branch/tiller, or to maintain bud dormancy. Bud outgrowth typically progresses through three discernible stages: dormancy, transition, and sustained growth (Stafstrom and Sussex, 1992; Devitt and Stafstrom, 1995; Dun et al., 2006). The fate of buds in the transition stage is influenced by the complex interplay of environmental and endogenous cues, ultimately determining whether buds return to dormancy or enter a sustained growth phase (Waldie et al., 2010). Consequently, the final count of branches/tillers is not solely determined by the number of axillary buds but is also influenced by the potential of buds to undergo growth (Wang and Li, 2011). In the following sections, we primarily focus on elucidating shoot branching through the lens of endogenous cues and environmental signals. For a visual representation of the interplay among various components, please refer to the conceptual model of bud outgrowth shown in Figure 8. The genes mentioned in this section are referred to in Table 2.

2.1 Internal inputs determine bud outgrowth

2.1.1 *TB1/BRC1* acts as a key integrator of branching

The expression of *TB1*, encoding a non-canonical basic helix–loop–helix (bHLH) transcription factor of the TCP family, is negatively correlated with bud growth (Doebley et al., 1997; Cubas et al., 1999). This TCP protein family is represented by four founding members: *TB1*, *CYCLOIDEA* (*CYC*), *PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR1* (*PCF1*), and *PCF2*. These family members were identified by their functions in plant development or their DNA binding capacity (Doebley et al., 1995; Luo et al., 1996; Doebley et al., 1997; Kosugi and Ohashi, 1997; Doebley, 2004). The maize (*Zea mays*) *tb1* mutant exhibits an uncontrolled proliferation of tillers, resulting in a bushy architecture reminiscent of its ancestor, teosinte (Hubbard et al., 2002). The inhibitory effect of *TB1* on bud outgrowth is spatially restricted to axillary buds as soon as they become visible (Hubbard et al., 2002). By contrast, in teosinte, *TB1* is not expressed in axillary buds, allowing axillary bud outgrowth (Hubbard et al., 2002). The role of *TB1* in suppressing axillary bud outgrowth is conserved in rice, as ectopic overexpression of its ortholog *OsTB1* under the control of the actin promoter leads to reduced tillering (Takeda et al., 2003). Conversely, loss-of-function mutants of *OsTB1*, such as *fin*e culm 1 (*fc1*), show increased tillering (Takeda et al., 2003). *TB1* and its orthologs (e.g., *OsTB1* or *FC1* in rice, *BRANCHED1* (*BRC1*) in *Arabidopsis*, *PsBRC1* in pea (*Pisum sativum*), and *SlBRC* in tomato) operate in conjunction with other vital genes and plant hormones to regulate bud outgrowth (Aguilar-Martínez et al., 2007; Minakuchi et al., 2010; Martín-Trillo et al., 2011; Braun et al., 2012). These genes and phytohormones are discussed in subsequent sections. The coordinated action of *TB1* with these factors has earned it the title “branching integrator” (Figure 8).

It is worth noting that several studies have indicated that inhibition of bud outgrowth can occur independently of *TB1* and its orthologs (Braun et al., 2012; González-Grandío et al., 2013; Seale et al., 2017; Wang et al., 2018a). Hence, *TB1* may condition bud activation potential, thus contributing to the regulation of branching.

Table 2. Genes involved in bud outgrowth

Gene Names	Accession Numbers	Reported Species (Homolog)	Functional Annotation	References
OsTB1	Os03g0706500	*Arabidopsis*, rice, maize, pea, tomato	Transcription factor TCP family	(Hubbard et al., 2002; Aguilar-Martínez et al., 2007; Martín-Trillo et al., 2011; Braun et al., 2012)
OsSPL14 (*IPA1*), *OsSPL15*	Os08g050960, Os08g0513700	Rice, *Arabidopsis*	SQUAMOSA promoter binding protein-like transcription factors	(Schwarz et al., 2008; Jiao et al., 2010; Rameau et al., 2015)
AXR1	AT1G05180	*Arabidopsis*	A subunit of the RUB1 activating enzyme	(Lincoln et al., 1990)
YUCCA	AT4G32540	*Arabidopsis*	A flavin monooxygenase-like enzyme, auxin biosynthesis	(Zhao et al., 2001)
PIN1	Os02g0743400	Rice	An auxin transporter	(Xu et al., 2005)
OsPIN5b	Os09g0505400	Rice	An auxin transporter	(Lu et al., 2015a)
MKK7	AT1G18350	*Arabidopsis*	MAP kinase kinase7	(Dai et al., 2006)

MKK6	AT5G56580	*Arabidopsis*	MAP kinase kinase 6	(Jia et al., 2016)
TIR1	Os05g0150500, AT1G72930	Rice, *Arabidopsis*	Auxin receptor	(Dharmasiri et al., 2005a; Kepinski and Leyser, 2005)
IAA12	AT1G04550	*Arabidopsis*	An auxin-responsive gene	(Dharmasiri et al., 2005b)
AFB2	Os04g0395600	Rice	Auxin signaling f-box 2	(Xia et al., 2012)
RUB1	AT1G31340	*Arabidopsis*	A ubiquitin-related protein	(Dharmasiri et al., 2005a)
D27	Os11g0587000	Rice	An iron-containing protein	(Lin et al., 2009; Alder et al., 2012)
CCD7/MAX 3	AT2G44990, Os04g0550600	*Arabidopsis*, rice	Carotenoid cleavage dioxygenases	(Booker et al., 2004)
CCD8/MAX 4	AT4G32810, Os01g0746400	*Arabidopsis*, rice	Carotenoid cleavage dioxygenases	(Sorefan, 2003)
MAX1	AT2G26170	*Arabidopsis*	Belonging to the CYP711A cytochrome P450 family	(Booker et al., 2005)
MAX2/D3	AT2G42620, Os06g0154200	*Arabidopsis*, rice	Belonging to a member of the F-box leucine-rich repeat family	(Booker et al., 2005)
D14	Os03g0203200, AT3G03990	Rice, *Arabidopsis*	An alpha/beta hydrolase	(Smith and Li, 2014)

D53	Os11g0104300	Rice	The substrate of SCF-D3 ubiquitin complex	(Wang et al., 2015)
SMXL6, *SMXL7*, *SMXL8*	AT1G07200, AT2G29970, AT2G40130	*Arabidopsis*	D53-like proteins	(Soundappan et al., 2015; Wang et al., 2015)
IPT	AT3G23630	*Arabidopsis*	An isopentenyl transferase	(Medford et al., 1989)
SPS	AT1G16410	*Arabidopsis*	Belonging to a member of CYP79F	(Tantikanjana et al., 2001)
AMP1	AT3G54720	*Arabidopsis*	A glutamate carboxypeptidase	(Helliwell et al., 2001)
PsCKX2	LOC127082854	Pea	Cytokinin dehydrogenase 6-like	(Shimizu-Sato et al., 2009)
NCED3	AT3G14440	*Arabidopsis*	A 9-cis-epoxycarotenoid dioxygenase	(Reddy et al., 2013)
ABA2	AT1G52340	*Arabidopsis*	A cytosolic short-chain dehydrogenase	(Yao and Finlayson, 2015)
HB21, *HB40*, *HB53*	AT2G02540, AT4G36740, AT5G66700	*Arabidopsis*	Homeobox proteins	(González-Grandío et al., 2017)
SLR1	Os03g0707600	Rice	A DELLA protein	(Rameau et al., 2015)
BES1	AT1G19350	*Arabidopsis*	A transcription factor	(Yin et al., 2005)

MOC2	Os01g0866400	Rice	A cytosolic fructose 1,6-bisphosphatase	(Koumoto et al., 2013)
OsNPF7.7	Os10g0579600	Rice	One nitrate transporter	(Huang et al., 2018)
TaNAC2-5A	AY625683	Wheat	A transcription factor	(He et al., 2015)
OsMADS57	Os02g0731200	Rice	A MADS transcription factor 57	(Guo et al., 2013)
OsBZR1, *BES1*	Os07g0580500, AT1G19350	Rice, *Arabidopsis*	A key transcription factor involved in brassinosteroid (BS) signaling	(Bai et al., 2007)
DLT	Os06g0127800	Rice	A GRAS protein	(Tong et al., 2009)
GSK2	Os05g0207500	Rice	A conserved glycogen synthase kinase 3-like kinase	(Tong et al., 2009)
RLA1	Os05g0389000	Rice	An APETALA2 (AP2) DNA binding domain protein	(Qiao et al., 2017)
BRI1/D61	Os01g0718300	Rice	A BR receptor	(Tong et al., 2009)

2.1.2. SQUAMOSA binding proteins inhibit bud outgrowth

SQUAMOSA promoter binding protein-like (*SPL*) transcription factors, which are specific to plants, mediate various aspects of plant development, including branching (Rameau et al., 2015). Different members of the *SPL* gene family in *Arabidopsis* are post-transcriptionally regulated by *miR156* (Rhoades et al., 2002). Overaccumulation of *miR156* leads to a considerably bushy phenotype (Schwab et al., 2005; Wei et al.,

2012). Notably, double mutants of the *Arabidopsis* paralogs *SPL9* and *SPL15* exhibit an increased branching phenotype, highlighting the crucial role of miR156-targeted *SPL* genes in regulating shoot branching (Schwarz et al., 2008) (Figure 8). Accumulation of the *SPL9* and *SPL15* ortholog *OsSPL14* results in fewer tillers and increased yield in rice (Jiao et al., 2010). Likewise, *miR156* can target several *SPL* genes, impacting the formation of lateral branches in tomato (Zhang et al., 2011; Cui et al., 2020). These findings highlight the roles of SPL proteins in inhibiting branching. Additionally, *OsSPL14*, whose encoding transcripts are targeted by *miR156*, directly activates *OsTB1* expression (Jiao et al., 2010).

2.1.3. Auxin indirectly inhibits sustained bud outgrowth

A principal function of auxin, as observed in various studies, is mediating apical dominance, as lateral bud outgrowth is inhibited by auxin. For instance, the *auxin-resistant 1* (*axr1*) mutant of *Arabidopsis* exhibits a lower sensitivity to auxin than wild-type plants, resulting in weak apical dominance and an increased number of branches due to the release of axillary buds, highlighting the role of auxin in inhibiting axillary bud outgrowth (Lincoln et al., 1990). In addition, the transcription factor *SlTCP26* influences auxin pathways to diminish apical dominance and activate lateral bud dormancy, thus enhancing the growth of lateral branches (Wei et al., 2021). Conversely, auxin-overproducing mutants with elevated levels of free auxin, such as the *Arabidopsis* yucca mutants, exhibit stronger apical dominance than wild-type plants (Zhao et al., 2001).

Moreover, apical dominance largely depends on polar auxin transport (PAT) mediated by *PIN-FORMED* (*PIN*) proteins in the stem (Matthes et al., 2019). Studies involving RNA interference (RNAi)-mediated knockdown and overexpression of *OsPIN1* have demonstrated the negative effect of *OsPIN1* on tillering in rice (Xu et al., 2005). In tomato, WRKY-B can directly bind the promoters of *PIN4*, thus regulating lateral bud outgrowth (Yang et al., 2024).

In addition, overexpression and knockdown experiments with *OsPIN5B* revealed the influence of this gene on tiller numbers (Lu et al., 2015a). Mitogen-activated protein kinase (MAPK) cascades are essential for transducing external and internal cues into adaptive and programmed responses. The MKK7 (MAPK KINASE 7)-MPK6 signaling pathway regulates PAT by phosphorylating the specific substrate PIN1, thereby modifying shoot branching in *Arabidopsis* (Dai et al., 2006; Jia et al., 2016).

In addition to the crucial role of PAT in regulating branching/tillering, the auxin signaling pathway also influences shoot branching/tillering via SKP1-CULLIN1-F-box (SCF)-mediated protein degradation. Auxin receptors, including TRANSPORT INHIBITOR RESPONSE 1 (TIR1) and closely related family numbers AUXIN SIGNALING F-BOX (AFB) (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005), bind to auxin to stabilize the interactions between TIR1/AFBs and members of the Aux/IAA (Auxin/INDOLE-3-ACETIC ACID INDUCIBLE) family of transcriptional repressors (Tan et al., 2007). Their interaction with TIR1/AFBs leads to the degradation of Aux/IAA, permitting auxin-mediated

upregulation of transcription (Gray et al., 2001; Tiwari et al., 2001; Zenser et al., 2001). By contrast, loss of function of *IAA12* in *Arabidopsis* leads to auxin-resistant stabilization of the SCF complex and, thus, constitutive suppression of target auxin-upregulated genes (Dharmasiri et al., 2005b), resulting in a bushy phenotype. Overexpressing *OsMIR393*, whose mature miRNA product *OsmiR393* targets and downregulates the transcripts of the auxin receptor genes *OsTIR1* and *OsAFB2*, leads to increased tiller production (Xia et al., 2012).

Moreover, many Aux/IAA genes are rapidly transcriptionally induced by auxin in a $SCF^{TIR/AFB}$-dependent manner (Park et al., 2002). *TIR1/AFB* genes encode F-box proteins that interact with the cullin CUL1 and the SKP1-like proteins ASK1 or ASK2 to form an SCF ubiquitin protein ligase (E3). *TIR1/AFB* genes function as transcription factors that bind to auxin-response elements (AuxREs) located in the upstream regions of auxin-inducible genes (Ulmasov et al., 1997). Auxin resistant 1 (AXR1) is required for proper SCF function as it facilitates conjugation of the ubiquitin-like protein RELATED TO UBIQUITIN 1 (RUB1) to the cullin subunit (Wu et al., 2000; Del Pozo et al., 2002; Dharmasiri et al., 2005a). Correspondingly, mutations in *AXR1* result in changes in the expression of $SCF^{TIR1/AFB}$-dependent auxin-responsive genes, leading to defects in downstream auxin responses (Dharmasiri et al., 2005a) (Figure 8).

It should be noted that the effect of auxin on branching/tillering is indirect, as apically derived auxin cannot enter buds (Prasad et al., 1993). Two primary models have been proposed to explain this phenomenon: the canalization model and the second-messenger-based model. According to the canalization model, axillary buds are activated when the amount of auxin initially flowing out of the bud is sufficient to trigger the establishment of polar auxin transport connected to the auxin stream in the stem, thereby promoting bud outgrowth (Li and Bangerth, 1999; Domagalska and Leyser, 2011). Conversely, the continuous flow of auxin in the stem originating from the apex restricts the export of auxin from the axillary buds on the same axis, thereby maintaining apical dominance (Barbier et al., 2015a). The establishment of auxin transport involves a positive regulatory feedback loop between the polarization of auxin efflux-facilitating PINs at the plasma membrane in the direction of the initial flow and the directional flow (Bennett et al., 2014). In addition, SLs act upstream of auxin by stimulating the removal of PIN1 from the plasma membrane, thereby reducing the ability of the bud to create its own polar auxin transport (Shinohara et al., 2013). According to the second-messenger-based model, auxin flow in the main stem negatively modulates CK biosynthesis (Shimizu-Sato et al., 2009) and positively regulates SL levels (Brewer et al., 2009), with these two phytohormones acting antagonistically on buds by inducing and inhibiting their outgrowth, respectively (Hayward et al., 2009; Dun et al., 2012). Furthermore, the antagonistic effects of Cks and SLs in buds are integrated by *BRC1*, the *Arabidopsis* homolog of *TB1* that is mainly expressed in dormant axillary buds (Aguilar-Martínez et al., 2007; Braun et al., 2012; Dun et al., 2012).

The transition of a bud from dormancy or quasi-dormancy to more active outgrowth is associated with increased expression of genes involved in the cell cycle (Devitt and

Stafstrom, 1995; Shimizu and Mori, 1998; Kebrom et al., 2009). Expression of cell cycle-related genes is repressed by auxin biosynthesized in the shoot apex, leading to suspension of cell division and bud dormancy (Devitt and Stafstrom, 1995).

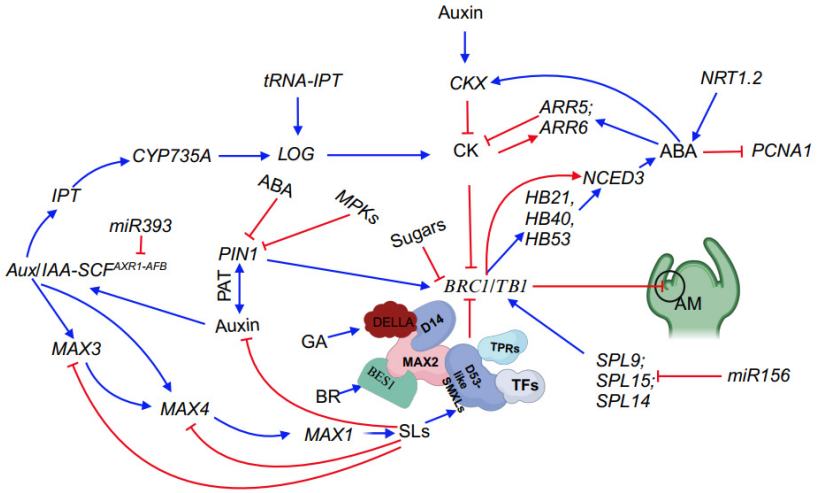

Figure 8. Summary of phytohormones and key genes involved in shoot branching. Blue arrows represent promotion, whilst red flat-ended lines denote inhibition. In this model, *BRC1/TB1* acts as an integrator to interact with other genes, such as SPL genes and phytohormones, to mediate shoot branching. Abbreviations: SL, Strigolactone; ABA, Abscisic acid; CK, cytokinin; GA, Gibberellin; PIN1, PIN-FORMED1; SCF, Skp-Cullin-F-box; Aux/IAA, Auxin/Indole-3-Acetic Acid; IPT, ADENYLATE ISOPENTENYLTRANSFERASE; CYP735A, cytochrome P450 monooxygenase 735A; PAT, polar auxin transport; *LOG*, *LONELY GUY*; *tRNA-IPT*, *transfer RNA isopentenyltransferase*; *CKX*, *cytokinin oxidase*; *ARR5* and *ARR6*, *RESPONSE REGULATOR5* and *6*; *NRT1.2*, *nitrate transporter 1.2*; *PCNA1*, *PROLIFERATING CELL NUCLEAR ANTIGEN1*; *SPL9*, *SPL14* and *SPL15*, *SQUAMOSA PROMOTER BINDIN PROTEIN-LIKE9*,*14* and *15*; *BRC1/TB1*, *BRANCHED1/ TEOSINTE BRANCH 1*; NCED3, *9-CIS-EPOXYCAROTENOID DIOXYGENASE3*; *HB21*, *HOMEOBOX PROTEIN 21*; *HB40*, *HOMEOBOX PROTEIN 40*; *HB53*, *HOMEOBOX PROTEIN 53*; R:FR, Red:Far-red light ratio; *DELLA*, *aspartic acid–glutamic acid–leucine–leucine–alanine*; *D14*, *DWARF 14*; *MAX3*, *2*, *1* and *4*, *more axillary growth 3*, *2*, *1* and *4*; TPRs, *TOPLESS-RELATED PROTEINs*; *TFs*, *transcription factors*; D53-like SMXLs, *DWARF53-LIKE SMAX1-LIKEs*; *BES1*, *bri1-EMS-suppressor 1*; AM, axillary meristem.

2.1.4. Strigolactones have an inhibitory effect on bud outgrowth

SLs are a collection of carotenoid-derived lactones secreted by plants. These phytohormones are primarily known for their roles as rhizosphere signals used by root-parasitic plants to detect their hosts **(**Cook et al., 1966**)** and as cues for mycorrhizal fungi to form symbiotic associations **(**Akiyama et al., 2005**)**. Importantly, SLs also inhibit the outgrowth of axillary buds. This inhibitory effect was initially

observed in *ramosus* (*rms*) mutants in pea, which exhibit excessive branching, as well as *decreased apical dominance* (*dad*) mutants in petunia (Petunia hybrida) and *more axillary growth* (*max*) mutants in *Arabidopsis* (Napoli and Ruehle, 1996; Beveridge et al., 1997; Beveridge, 2000; Foo et al., 2001; Morris et al., 2001; Turnbull et al., 2002; Sorefan, 2003; Beveridge, 2006).

SL biosynthesis involves several enzymes. *DWARF27* (*D27*) is responsible for isomerizing all-trans-β-carotene at the C-9 position to form 9-cis-carotene (Lin et al., 2009; Alder et al., 2012). *Carotenoid cleavage dioxygenase 7* (*CCD7*, also named *MAX3*) and *CCD8* (also named *MAX4*) then cleave 9-cis-carotene to produce carlactone, a key endogenous SL precursor (Sorefan, 2003; Booker et al., 2004). The conversion of carlactone to SLs is catalyzed by *MAX1*, an *Arabidopsis* cytochrome P450 that acts downstream of *MAX4* and *MAX3* (Booker et al., 2005). *MAX1* converts carlactone to carlactonoic acid, which is further converted to methyl carlactonoate, an SL-like compound (Figure 8) (Abe et al., 2014).

SLs are perceived by MAX2, an ortholog of D3 from rice, which plays a crucial role in regulating plant branching. Disruption of MAX2 leads to a bushy phenotype in *Arabidopsis* (Booker et al., 2005). Moreover, the perception and signaling roles of SLs in rice require their interaction with D14, a putative SL receptor. D14 interacts with D3, an F-box protein of the SCF E3 ubiquitin ligase complex, to form an SL-induced D14-D3 complex (Smith and Li, 2014). This complex targets proteins for ubiquitination and degradation, resulting in changes in plant branching/tillering (Wang et al., 2015) (Figure 8).

The dominant *d53* mutant in rice is characterized by a high-tillering and dwarf phenotype and is resistant to the exogenous application of GR24, a synthetic SL. D53 is targeted for SL-dependent degradation by the SCF^{D3} ubiquitination complex (Jiang et al., 2013). In addition, SL treatment results in D53 degradation via the ubiquitin–proteasome system in a D14- and D3-dependent manner (Wang et al., 2015). D53 interacts with members of the TOPLESS-RELATED PROTEIN (TPR) family of transcriptional co-repressors, which may suppress the activities of their downstream transcription factors (Figure 8) (Smith and Li, 2014; Xiong et al., 2014).

In *Arabidopsis*, D53-like proteins, including SMAX1-LIKE6 (SMXL6), SMXL7, and SMXL8, are targeted for proteolysis by MAX2-mediated SL signaling (Soundappan et al., 2015; Wang et al., 2015). Their overaccumulation in SL signaling mutants (such as max2) leads to increased branching and constitutively low BRC1 expression in buds. Conversely, disruption of SMXL6, SMXL7, and SMXL8 completely restores branching of max2 to wild-type levels and leads to very high levels of BRC1 transcript in inhibited buds (Soundappan et al., 2015; Wang et al., 2015). This effect highlights the role of SLs in inhibiting bud outgrowth by upregulating BRC1 transcription. Additionally, these SMXL proteins can form a complex with TPR2 and function as transcriptional repressors. However, D14 interacts with SMXLs and MAX2 in an SL-dependent manner, thereby inhibiting axillary bud outgrowth in *Arabidopsis* (Wang et al., 2015).

The relationship between SLs and *BRC1* and its corresponding orthologs suggests that the SL pathway acts upstream of *FC1*/*OsTB1* in rice. For example, the *fc1* mutant

does not respond to application of GR24, and the phenotype of the *fc1 d17* double mutant is similar to that of the SL-deficient mutant *d17*, suggesting the involvement of SLs in regulating *FC1/OsTB1* expression in rice (Minakuchi et al., 2010). In line with this notion, *PsBRC1* expression levels are significantly lower in SL-deficient mutants (*rms1*, *rms2*, and *rms4*) than in wild-type pea plants (Braun et al., 2012) but are upregulated in *rms1* and *rms2* after GR24 application, further supporting the idea that SLs act upstream of *BRC1*. Specifically, *BRC1* is repressed in non-elongated *max2* and *max3* axillary buds but induced in the *smxl6 smxl7 smxl8*, *max2 smxl6 smxl7 smxl8*, and *max3-9 smxl6 smxl7 smxl8* mutants of *Arabidopsis*, suggesting that *SMXL6*, *SMXL7*, and *SMXL8* inhibit *BRC1* expression (Wang et al., 2015).

2.1.5. Other phytohormones regulate tillering/branching

The inhibitory role of auxin in bud outgrowth is exerted indirectly within buds, as apically derived auxin is not transported into buds (Morris, 1977) and exogenous auxin directly supplied to buds fails to prevent their growth (CLINE, 1996). CKs are believed to be crucial in relaying the auxin signal into buds and promoting axillary branching. CKs are an important class of phytohormones that participate in various aspects of plant development, including organ formation, apical dominance, and leaf senescence (El-Showk et al., 2013). Therefore, CKs facilitate the growth and development of axillary buds by serving as a second messenger for the auxin signal.

Several studies have demonstrated that CKs can promote the outgrowth of buds that would otherwise remain inhibited and that CK levels in or near the bud are well correlated with bud fate (Medford et al., 1989; Tanaka et al., 2006). For example, in chickpea (*Cicer arietinum* L.) plants, CK levels dramatically increased in axillary buds within 24 h of shoot decapitation (Turnbull et al., 1997). Elevated levels of CKs, as achieved by overexpressing *ISOPENTENYL TRANSFERASE* (*IPT*) (encoding a key enzyme in CK biosynthesis), lead to reduced apical dominance (Figure 8) (Medford et al., 1989). Continuous treatment of pea plants with synthetic CKs overcomes the inhibition of lateral bud release, turning these into dominant organs (Li and Bangerth, 1992). In *Arabidopsis*, basally applied CKs suppress the inhibitory effects of apically supplied auxin in isolated nodes (Chatfield et al., 2000). Conversely, low local CK levels can limit bud outgrowth, even in auxin- and strigolactone-deficient plants (Ferguson and Beveridge, 2009). The *supershoot* (*sps*) mutant of *Arabidopsis* displays overproliferating branching due to cytokinin-promoted bud initiation as well as bud outgrowth, as endogenous cytokinin levels are elevated in this mutant (Tantikanjana et al., 2001). The *Arabidopsis* altered *meristem program 1* (*amp1*) mutant, with increased cytokinin levels, shows enhanced AM formation and bud elongation to generate branches (Helliwell et al., 2001). Interestingly, in this mutant, *BRC1* is slightly downregulated, suggesting that CKs downregulate *BRC1* expression (Aguilar-Martínez et al., 2007).

In addition to their biosynthesis, the metabolism of CKs also determines endogenous cytokinin levels. Cytokinin oxidase (CKX) is the enzyme responsible for inactivating CKs by irreversibly degrading active CKs, thereby regulating endogenous levels of active CKs (Jones and Schreiber, 1997; Werner et al., 2001).

Various CKXs, such as PsCKX2 in pea, predominantly regulate CK levels in the stem (Shimizu-Sato et al., 2009). Notably, the expression pattern of *PsCKX2* in the stem is opposite to that of CK levels before and after decapitation, suggesting that PsCKX2 induces a decrease in CK levels in the stem. Overall, these findings support the notion that CKs promote bud outgrowth.

While abscisic acid (ABA) is predominantly recognized for its roles in seed dormancy, growth inhibition, and stress responses, emerging evidence indicates that this phytohormone also influences plant branching/tillering. Mutations in key genes involved in ABA biosynthesis, such as *9-CIS-EPOXYCAROTENOID DIOXYGENASE3* (*NCED3*) and *ABA DEFICIENT2* (*ABA2*), enhance bud outgrowth (Reddy et al., 2013; Yao and Finlayson, 2015). Notably, ABA levels are elevated in buds with delayed outgrowth but are reduced in elongated buds (Yao and Finlayson, 2015). Exogenous application of ABA partially suppresses branch elongation, suggesting that ABA functions downstream or independently of genes responsible for bud growth (Yao and Finlayson, 2015). Unlike the IAA biosynthesis gene *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1*) and the auxin transporter gene *PIN1*, exogenously supplied ABA does not affect *BRC1* expression, confirming the downstream role of ABA in regulating *BRC1*. Furthermore, BRC1 binds to the promoter of and positively regulates the transcription of three genes encoding related homeodomain leucine zipper proteins (HD-ZIP): HOMEOBOX PROTEIN 21 (HB21), HB40, and HB53. Together with BRC1, these three proteins promote NCED3 expression, resulting in ABA accumulation and triggering a phytohormonal response, thereby suppressing bud development (González-Grandío et al., 2017) (Figure 8). Finally, ABA treatment represses the expression of the cell cycle-related gene *PROLIFERATING CELL NUCLEAR ANTIGEN1* (*PCNA1*), suggesting that ABA modulates bud outgrowth by regulating cell cycle-related gene expression (Yao and Finlayson, 2015).

GAs are renowned for their ability to regulate internode elongation. Dwarfism is often associated with an increase in shoot branching. GA-deficient mutants in *Arabidopsis*, rice, and pea exhibit higher levels of branching/tillering than their wild-type counterparts (Silverstone et al., 1997; Lo et al., 2008). Likewise, overexpressing GA catabolism genes decreases GA levels, resulting in increased branching/tillering (Lo et al., 2008). Moreover, mutants with disruption of DELLA proteins, the major negative regulators of GA signaling, display reduced shoot branching and/or altered branching patterns (Rameau et al., 2015). Additionally, in rice, DELLA SLENDER RICE 1 (SLR1) was found to interact with the SL receptor D14 in an SL-dependent manner (Rameau et al., 2015) (Figure 8).

BRs are important plant hormones that regulate various developmental processes, including stem elongation, leaf development, senescence, and branching (Yin et al., 2002). A series of BR-deficient mutants have been used to elucidate the function of BR. For instance, reduced expression of *Brassinazole Resistant 1* (*OsBZR1*) in rice leads to a dwarf phenotype with erected leaves and reduced BR sensitivity (Bai et al., 2007). *Dwarf and Low Tillering* (*DLT*), encoding a GRAS family protein, is another BR-related gene. Disruption of *DLT* results in a semi-dwarf mutant with fewer tillers

and decreased BR responses. The promoter of *DLT* can be targeted by OsBZR1 (Tong et al., 2009). *GLYCOGEN SYNTHASE KINASE 2* (*GSK2*) encodes a conserved glycogen synthase kinase 3-like kinase. Gain-of-function mutations within the *GSK2* coding sequencing or its overexpression suppress BR signaling, leading to plants with phenotypes resembling BR-deficient mutants (Li and Nam, 2002). The *reduced leaf angle 1* (*rla1*) mutant encodes a transcription factor containing an *APETALA2* (*AP2*) DNA binding domain that is required for OsBZR1 function (Qiao et al., 2017). RLA also can interact with GSK2 (Qiao et al., 2017).

A BR-defective rice mutant displays reduced branching like *dlt* (Tong et al., 2009), while in *Arabidopsis*, the *bri1-EMS-suppressor 1* (*bes1*) mutant displays a highly branched phenotype. In contrast, *BES1-RNAi* lines have fewer branches than the wild type (Yin et al., 2002; Yin et al., 2005). Interestingly, the dominant *bes1-D* mutant does not respond to GR24 treatment, and BES1 can interact with MAX2 and act as its substrate for degradation, which is regulated by SLs (Wang et al., 2013). Therefore, SL and BR signaling pathways converge on the same transcription factor *BES1*, an *Arabidopsis* homolog of *OsBZR1*, to control branching (Wang et al., 2013). However, other components upstream of *AtBES1* in the BR signaling pathway do not alter branching in *Arabidopsis* (Wang et al., 2013). In rice, BRs bind to the receptor *Brassinosteroid insensitive 1* (*BRI1*), activating the receptor complex and inhibiting the ability of OsGSK2 (Li and Nam, 2002) to phosphorylate members of the downstream transcriptional module, including OsBZR1, DLT, and RLA1, and thereby regulate their stability (Qiao et al., 2017). Furthermore, BRs strongly enhance tillering by promoting bud outgrowth in rice by regulating the stability of D53 and/or the OsBRZ1-RLA1-DLT module, a transcriptional complex in the BR signaling pathway (Wang et al., 2013). In addition, D53 interacts with OsBZR1 to inhibit the expression of *OsTB1*. This interaction depends on direct DNA binding by OsBZR1, which recruits D53 to the *OsTB1* promoter in axillary buds (Wang et al., 2013).

2.1.6. Phytohormones interact influencing bud outgrowth

The regulation of axillary bud outgrowth involves a complex network of phytohormones. Plant hormones, including auxin, CKs, and SLs, play central roles in bud outgrowth. Furthermore, plant hormones can mutually affect each other, ultimately regulating branching/tillering.

Auxin is an indispensable player in plant architecture, particularly branching, and a central component of interacting networks that regulate branching. Auxin exerts its role indirectly in buds (Prasad et al., 1993), whereas SLs are direct components that act on buds via auxin to inhibit bud outgrowth. Auxin inhibits bud outgrowth by regulating SL biosynthesis, as observed in *Arabidopsis* and rice (Zou et al., 2006; Arite et al., 2007; Hayward et al., 2009). For example, auxin that originates in the shoot apex modulates SL levels in pea by maintaining *RMS1* and *RMS5* transcript abundance (Foo et al., 2005; Johnson et al., 2006). The *iaa12* mutant exhibits increased numbers of branches and shows reduced expression of the SL biosynthesis genes *MAX3* and *MAX4* (Hayward et al., 2009), while *MAX3* and *MAX4* transcription is mediated by auxin (Bainbridge et al., 2005). Conversely, *Arabidopsis* mutants

defective in SL biosynthesis (such as *max4*) exhibit increased branching and resistance to auxin (Sorefan, 2003). Additionally, branching is inhibited by SL treatment in auxin-response mutants such as *axr1* and the *tir1 afb1 afb2 afb3* quadruple mutant (Beveridge et al., 2009), providing compelling evidence that loss of auxin signaling promotes bud outgrowth via SL depletion. Moreover, both auxin biosynthetic and auxin signaling mutants respond to SL treatment, indicating that SLs function downstream of auxin (Brewer et al., 2009).

SLs also inversely affect polar auxin transport by limiting the accumulation of the auxin efflux carrier PIN1 in cells involved in polar auxin transport (Domagalska and Leyser, 2011). The *Arabidopsis* max mutants, with defects in the SL pathway, show enhanced polar PIN accumulation and auxin transport (Bennett and Leyser, 2006; Prusinkiewicz et al., 2009; Crawford et al., 2010). Similarly, the rice *d27* mutant (characterized by low SL concentrations) displays enhanced auxin transport (Lin et al., 2009). Therefore, auxin and SLs interact via interconnected feedback loops, where each phytohormone regulates the level of the other.

Auxin and CKs play antagonistic roles in regulating bud outgrowth. Auxin inhibits AM outgrowth, whereas CKs counteract auxin activity in *Arabidopsis* by promoting bud activation (Wang et al., 2014). The inhibitory effect of auxin is probably mediated, at least in part, by its ability to reduce both CK export from roots and CK biosynthesis locally at the node (Bangerth, 1994; Nordström et al., 2004). Stem girdling in pea, which prevents polar auxin transport via a mechanism similar to decapitation, increases the expression of CK biosynthesis genes and promotes the growth of buds below the girdling site (Ferguson and Beveridge, 2009). Decapitated bean (*Phaseolus vulgaris* L.) plants (with a decrease or loss of polar auxin transport) have higher CK concentrations in xylem exudates than control plants. However, applying auxin to the shoots of decapitated plants eliminates the effect of shoot-tip removal on CK concentration, further supporting the antagonistic relationship between auxin and CKs (Bangerth, 1994). Auxin inhibits expression of the CK biosynthesis gene IPT in the stem (Bangerth, 1994; Li et al., 1995; Tanaka et al., 2006; Ferguson and Beveridge, 2009). In *Arabidopsis*, auxin-mediated suppression of CK biosynthesis is dependent on *AXR1* (Nordström et al., 2004). However, in addition to repression by auxin, CKs also enhance auxin production and promote downward auxin transport out of growing buds. This, in turn, suppresses the production of CKs lower within the stem, limiting their accessibility to other buds (Bangerth et al., 2000; Tanaka et al., 2006; Shimizu-Sato et al., 2009).

In addition to the inhibitory roles of auxin on CKs, ABA represses CK signaling by inducing the expression of type-A response regulator genes, such as *RESPONSE REGULATOR5* (*ARR*5) and *ARR6*, encoding negative regulators of CK signaling (To et al., 2004) (Figure 8). Moreover, ABA inhibits the accumulation of CKs in the roots and shoots of wheat and promotes CKX activity, contributing to the decrease in CK levels (Vysotskaya et al., 2009). Conversely, overexpression of IPT results in reduced ABA abundance in petunia flowers (Chang et al., 2003). ABA suppresses auxin biosynthesis and auxin transport out of axillary buds (Yao and Finlayson, 2015).

2.1.7. Sugars play an essential role in bud release

Sugars such as sucrose serve not only as a carbon source for plant metabolism but also as essential signaling compounds (Rolland et al., 2006; Smeekens et al., 2010; Wind et al., 2010). Bud outgrowth responds to limiting nutrients and resources, and thus sugars have been attracting increasing interest. Axillary buds are often maintained in a dormant state or their growth is suppressed by the growing shoot apex long after their initial formation. Intriguingly, changes in sugar availability can facilitate the first visible growth of buds, a phenomenon known as axillary bud release (Mason et al., 2014). The term "apical dominance" is commonly used to describe shoot branching, referring to the role of the shoot tip in preventing the growth of the axillary buds below it (Barbier et al., 2015b). However, in several plant species, auxin supplementation to the decapitated stump, even at high levels, fails to fully restore apical dominance (CLINE, 1996; Morris et al., 2005). Therefore, it is unlikely that auxin is the first component inhibiting axillary bud outgrowth. Rather, after decapitation, sugars are rapidly redistributed over a significant distance and accumulate in axillary buds, coinciding with the timing of bud release, before auxin depletion occurs in the corresponding axillary buds (Renton et al., 2012; Mason et al., 2014). This observation suggests that sugars have the potential to promote bud release.

Enhancing the sugar supply alone is sufficient for bud release. Plants employ various mechanisms to maintain apical dominance, one of which is limiting the sugar supply to axillary buds (Mason et al., 2014). This effect can be observed in the wheat *tiller inhibition* (*tin*) mutant, whose reduced tillering is associated with a decreased sucrose content in axillary buds (Kebrom et al., 2012). Likewise, reduced tiller formation in the rice *monoculm 2* (*moc2*) mutant is attributed to a disruption in fructose-1,6-bisphosphatase, an enzyme involved in sucrose biosynthesis, resulting in a decline in the sucrose supply (Koumoto et al., 2013). The requirement for sugars for bud outgrowth has been demonstrated in rose (*Rosa hybrida*), where sugar is required for triggering bud outgrowth in single nodes cultivated *in vitro* (Rabot et al., 2012; Barbier et al., 2015a). Sucrose can also modulate the dynamics of bud outgrowth in a concentration-dependent manner, especially during the transition phase between bud release and sustained bud elongation (Barbier et al., 2015a). Additionally, removal of competing sugar sources or sinks within buds through defoliation further supports the role of sugars in bud release (Mason et al., 2014; Kebrom and Mullet, 2015). Collectively, these lines of evidence highlight the trophic role of sugars in bud release.

Besides the roles of sugars as nutrients, an effect of sugars on phytohormone homeostasis has been demonstrated in single nodes of *R. hybrida* (Barbier et al., 2015a). For instance, sucrose stimulates CK biosynthesis in bud-bearing stem segments by upregulating the expression of two CK biosynthesis-related genes (Barbier et al., 2015a). Sucrose can also modulate auxin metabolism, as treatment with sucrose or its non-metabolizable analogs increases auxin levels in *R. hybrida* buds in a concentration-dependent manner (Barbier et al., 2015a). Furthermore, elevated sucrose levels in buds promote the export of auxin from the bud to the stem, which is favorable for bud outgrowth according to the auxin canalization model (Barbier et al., 2015a). When exogenously supplied to rose, sucrose reduces the expression of *MAX2*,

a gene involved in SL signaling (Barbier et al., 2015a). A dose-dependent inhibitory effect by sucrose has also been detected for *RhBRC1* expression, which is decisive in preventing bud outgrowth (Braun et al., 2012; Brewer et al., 2013). Notably, palatinose, a non-metabolizable sucrose analog, can trigger bud outgrowth (Rabot et al., 2012). These findings collectively demonstrate the crucial role of sugar signaling in regulating bud release.

From a trophic perspective, axillary buds are sink organs that require imported sugars to fulfill their metabolic demands and support their growth. In the context of apical dominance, the supply of sugars to lateral buds is the first signal that releases bud dormancy, preceding detectable auxin depletion. Thus, the growth potential of a bud can be determined by its sink strength, representing its ability to acquire and utilize sugars. The interplay in the demand for sugar between the apical bud and lateral buds is thus crucial for the systemic regulation of shoot branching, encompassing both nutritional support and signaling mechanisms.

2.2 Effects of environmental inputs on bud outgrowth

Bud outgrowth and the transition to dormancy are tightly regulated by various environmental factors, including photoperiod, light intensity, nutrient availability, and stress conditions (Dun et al., 2006; Ongaro and Leyser, 2007). Notably, ABA, a pivotal phytohormone involved in regulating bud outgrowth, strongly accumulates under stressful conditions such as osmotic stress (Yoshida et al., 2014). Interestingly, ABA levels are elevated in buds with delayed outgrowth but are reduced in elongated buds (Yao and Finlayson, 2015). This dynamic modulation of ABA levels underscores the remarkable ability of plants to adjust their branching capability in response to diverse environmental cues. Such adaptability represents a successful evolutionary trait that has evolved to accommodate the sessile nature of plants. Here, we focus on environmental cues reported so far. The genes mentioned in this section are referred to in Table 3.

Table 3. Genes related to lateral bud outgrowth response to environmental inputs

Gene Names	Accession Numbers	Reported Species (Homolog)	Functional Annotation	References
PHYB	LOC8081072	Sorghum bicolor	Phytochrome B	(Kebrom et al., 2006)
SbTB1	LOC8062930	Sorghum bicolor	Belonging to transcription factor of the TCP family	(Kebrom et al., 2006)

NCED3	AT3G14440	*Arabidopsis*	A 9-cis-epoxycarotenoid dioxygenase	(Reddy et al., 2013)
ABA2	AT1G52340	*Arabidopsis*	A cytosolic short-chain dehydrogenase/reductase	(Reddy et al., 2013)
OsNPF7.7	Os10g0579600	Rice	Belonging to the peptide transporter (PTR) gene family	(Huang et al., 2018)
OsNR2	Os02g0770800	Rice	NADH/NADPH-dependent $NO3^-$ reductase 2	(Gao et al., 2019)
NGR5	Os05g0389000	Rice	One APETALA2-domain transcription factor	(Zhao et al., 2020a)
PRC2	Os03g0108700	Rice	A polycomb repressive complex 2-associated coiled-coil protein	(Zhao et al., 2020a)
D14	Os03g0203200	Rice	A strigolactone receptor	(Zhao et al., 2020a)
SPL14	Os08g0509600	Rice	A squamosa promoter-binding-like transcription activator	(Zhao et al., 2020a)
OsDEP1	Os09g0441900	Rice	One unknown phosphatidylethanolamine-binding protein (PEBP)-like domain protein	(Sun et al., 2014)

OsAFB2	Os04g0395600	Rice	An auxin receptor	(Li et al., 2016)
OsTIR1	Os05g0150500	Rice	A F-Box auxin receptor protein	(Li et al., 2016)
OsTCP19	Os06g0226700	Rice	A class-I TCP transcription factor	(Liu et al., 2021b)
TaNAC2-5A	LOC606326	Wheat	NAC domain-containing protein 2	(He et al., 2015)
OsMADS57	Os02g0731200	Rice	A MADS-box transcription factor	(Guo et al., 2013)
OsPHR2	Os07g0438800	Rice	A MYB-CC family protein	(Fioreze et al., 2012)
NSP1	Os03g0408600	Rice	A GRAS-domain transcription factor	(Li et al., 2022)
NSP2	Os03g0263300	Rice	A GRAS-domain transcription factor	(Li et al., 2022)
OsHAK5	Os01g0930400	Rice	A potassium transporter	(Chérel, 2004)
OsABCB14	Os04g0459000	Rice	An auxin transport	(Xu et al., 2014)
WOX11	Os07g0684900	Rice	A WUSCHEL-related homeobox protein	(Chen et al., 2015)
OsHAK16	Os03g0575200	Rice	A high-affinity potassium transporter	(Chen et al., 2015)

OsAUX1	Os01g0856500	Rice	An auxin transporter	(Xia et al., 2012)
LRK2	Os02g0154000	Rice	A leucine-rich repeat receptor-like kinase	(Kang et al., 2017)
GHD7	Os07g0261200	Rice	A CCT(CONSTANS, CONSTANS-LIKE, and TIMING OF CHLOROPHYLL A/B BINDING1) domain protein	(Weng et al., 2014)
OsRAN1	Os01g0611100	Rice	A small GTPase	(Xu and Cai, 2014)

2.2.1. Light plays a critical role in bud outgrowth

Light intensity is pivotal for regulating bud outgrowth across numerous plant species. For instance, low-intensity light inhibits tillering in wheat (Evers et al., 2006). By contrast, high-intensity light stimulates branching, as observed in Rosa species (Girault et al., 2008).

Photoperiod also has a significant influence on the distribution of bud outgrowth along the plant stem. For example, under short-day conditions, the formation of basal branches is enhanced in pea. Bud outgrowth in the upper nodes often coincides with the onset of flowering and may also be controlled by photoperiod (Stirnberg et al., 2002; Beveridge et al., 2003).

At high density, shade also regulates bud dormancy in cultivated plants (Kebrom and Brutnell, 2007). When plants intercept incident light, the light intensity decreases, preferentially in the red part of the light spectrum. Shade is, therefore, characterized by a reduction in the red (R) to far-red (FR) light ratio (R:FR) due to R light absorption and FR reflection by leaves (Ballaré et al., 1990). This decrease in R:FR serves as a signal of shade or competition for light, prompting plants to respond by inhibiting axillary bud outgrowth, elongating their stature, and accelerating flowering to evade the detrimental consequences of shading. This suite of responses, known as shade avoidance syndrome, is mediated by the R- and FR-absorbing photoreceptor phytochrome B (PHYB) (Smith and Whitelam, 1997). In densely grown sorghum (*Sorghum bicolor*) plants experiencing shade, inhibition of bud outgrowth due to an enriched FR-light environment is associated with activation of the *TB1*-like gene *SbTB1* in buds (Kebrom et al., 2006; Finlayson et al., 2010). Conversely, in the absence of shade, a higher proportion of phyB in the active form induces bud outgrowth by downregulating *SbTB1*. Shade signals with their low R:FR ratios

decrease the proportion of the active form of phyB, thereby enhancing the expression of *SbTB1* and promoting bud dormancy (Figure 9). Interestingly, ABA also controls bud growth in response to R:FR shifts (Reddy et al., 2013). Furthermore, branching in response to a low R:FR ratio is defective in the ABA-deficient mutant *aba2-1* and the ABA biosynthetic mutant *nced3-2* (Reddy et al., 2013).

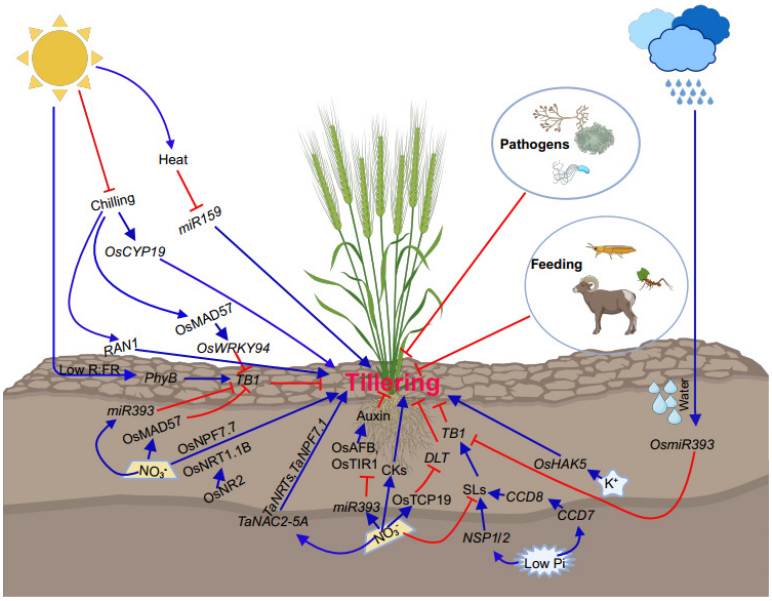

Figure 9. Effects of environmental inputs on bud outgrowth. The roles of various enviromental factors are indicated and genes involved are depicted. Blue arrows represent promotion while red-flat-ended lines denote inhibition. In this model, the abiotic factor like nutritents, light, temperature and biotic stresses imapct tillering significantly. Abbreviations: *OsCYP19*, *Oryza sativa cytochrome P450 family 19*; *OsMAD57*, *Oryza sativa MADS-box protein 57*; *miR159*, microRNA 159; PhyB, phytochrome B; *TB1*, *TEOSINTE BRANCHED 1*; *OsNPF7.7*, *peptide transporters family 7.7*; *OsNRT1.1B*, *Oryza sativa Nitrate Transporter 1.1B*; *OsNR2*, *Oryza sativa NADH/NADPH-DEPENDENT NITRATE REDUCTASE 2*; *OsWRKY94*, *Oryza sativa WRKY transcription factor 94*; *TaNAC2-5A, Triticum aestivum NAC transcription factor 2-5A*; *miR393*, *microRNA 393*; CK, cytokinin; SL, strigolactone; *OsAFB*, *Oryza sativa AUXIN SIGNALING F-BOX 2*; *OsTIR1*, *Oryza sativa TRANSPORT INHIBITOR RESPONSE*; OsTCP19, *TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR 19*; *DLT*, *DWARF AND LOW-TILLERING*; *NSP1/2*, *Nodulation Signaling Pathway 1/2*; Pi, Phosphorus; *OsHAK5*, *Oryza sativa High-Affinity K+ Transporter 5*; K+, Potassium; *CCD7/8*, *Carotenoid cleavage dioxygenase 7/8*.

2.2.2. Impacts of nutrients on bud outgrowth

Nitrogen (N) is an essential macronutrient that dominates plant growth and plant productivity (Luo et al., 2020; Luo et al., 2021). N limitation can significantly limit the tiller numbers in rice (Luo et al., 2020). Furthermore, high branching ability is positively correlated with the capacity for N uptake. Several N transporters have been identified that regulate shoot branching in rice. One such transporter is *peptide transporters family 7.7* (*OsNPF7.7*), whose increased abundance facilitates the influx of NO_3^- and NH_4^+, thereby promoting the outgrowth of axillary buds (Huang et al., 2018). Indica *NADH/NADPH-DEPENDENT NITRATE REDUCTASE 2* (*OsNR2*) promotes NO_3^- uptake through interaction with *OsNRT1.1B*, a low-affinity NO_3^- transport gene, and increases effective tillers in japonica rice. Similarly, the japonica allele of *OsNR2* also promotes tillering but not to the extent observed in indica *OsNR2*-overexpression lines (Gao et al., 2019; Gao et al., 2022). The rice APETALA2-domain transcription factor encoded by a *NITROGEN MEDIATED TILLER GROWTH RESPONSE 5* (*NGR5*) allele is upregulated under conditions of increased nitrogen availability. *NGR5* interacts with a component of the *polycomb repressive complex 2* (*PRC2*) to regulate the expression of *D14* and *OsSPL14* by mediating levels of *histone methylation* (*H3K27me3*) modification, thereby regulating rice tillering (Zhao et al., 2020a; Gao et al., 2022). Overexpression of *DENSE AND ERECT PANICLE1* (*OsDEP1*) can increase tiller numbers under high N supply (Sun et al., 2014). *OsSPL14* can directly activate the expression of *OsDEP1* and *OsTB1* to regulate tiller bud outgrowth (Duan et al., 2019). A microRNA, miR393, can target and repress the expression of *OsTB1* and the two auxin receptors *OsAFB2* and OsTIR1 under NO_3^- conditions, which influences the transport of auxin and eventually regulates tillering (Li et al., 2016). The rice *TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR 19* (*OsTCP19*) transcription factor can directly bind and repress the activity of the tiller-promoting *DLT* gene, thereby negatively regulating tillering in the presence of nitrogen (N). Further investigation revealed a 29-bp insertion/deletion polymorphism in the *OsTCP19* promoter that confers differential transcriptional response to N among rice varieties (Liu et al., 2021b). In wheat, the NO_3^--inducible *CEREAL-SPECIFIC NAM, ATAF*, and *CUC* (*NAC*) transcription factor *TaNAC2-5A*, whose gene expression is induced by a limited NO_3^- supply, directly binds to the promoters of genes encoding NO_3^- transporters and glutamine synthetase, thereby enhancing N acquisition and assimilation. Notably, *TaNAC2-5A* overexpression leads to enhanced tiller numbers (He et al., 2015). OsMADS57, a NO_3^--inducible MADS-box transcription factor, interacts with OsTB1 and targets D14 to control the outgrowth of axillary buds in rice (Guo et al., 2013) (Figure 9).

Following decapitation, the primary forms of available and transported N are amino acids through the phloem, which might be easy for buds to obtain (Tegeder, 2014). This notion is supported by the finding that the levels of three key amino acids, aspartate, asparagine, and glutamine, increase in axillary buds after decapitation, coinciding with the initiation of bud outgrowth. Interestingly, sucrose fails to trigger

bud outgrowth in excised rose nodes in the absence of asparagine in the growth medium (Eveland and Jackson, 2012).

Given that N availability influences CK and SL levels, it is plausible that N functions as a second messenger to mediate branching/tillering (Yoneyama et al., 2012; Kamada-Nobusada et al., 2013; Drummond et al., 2015). In several species, limited N availability promotes SL production, subsequently inhibiting branching/tillering. N limitation also leads to a reduction in CK production. N fertilization can suppress the expression of SL biosynthesis genes (Yoneyama et al., 2013; Luo et al., 2021). Collectively, these findings highlight the important role of N (as with sugars) in influencing branching.

Phosphorus (Pi) is an essential macronutrient for plant growth and metabolism. However, the availability of Pi in soils is often low due to chemical fixation and poor diffusion, resulting in low-Pi environments that can limit plant development and processes like tillering (Paz-Ares et al., 2022). For example, Pi deficiency has been shown to reduce tiller numbers in rice (Yuan et al., 2023a), with the transcription factor *Oryza sativa PHOSPHATE STARVATION RESPONSE2* (*OsPHR2*) implicated in the repression of tillering under low Pi conditions (Fioreze et al., 2012). Pi deficiency also induced SL biosynthesis by increasing transcription of SL biosynthetic genes like the β-carotene cis-trans isomerase *DWARF27* (*D27*), the *carotenoid cleavage dioxygenase 7* (*CCD7*)/*D17*, and *CCD8/D10* (Umehara et al., 2010; Wang et al., 2018a; Yuan et al., 2023a), which play a key role in regulating tillering, as described above. Additionally, *nodulation signaling pathway 1* (*NSP1*) and *NSP2*, two GRAS family transcription factors, have been shown to promote SL production in rice under low-Pi conditions by directly binding to SL biosynthetic gene promoters as a complex (Li et al., 2022). Moreover, Pi deficiency represses tiller numbers by promoting the degradation of *D53* and the expression of *OsTB1*. Further examination of the mechanisms of genes in response to Pi availability will provide a deeper understanding of nutrient-mediated tillering.

Potassium (K^+) is the most abundant cation in plants and an essential macronutrient (Chérel, 2004; Yang et al., 2020). Adequate K^+ availability can increase tillering in plants, as evidenced by enhanced tillering in rice overexpressing *Oryza sativa High-Affinity K^+ Transporter 5* (*OsHAK5*). In contrast, knockout of *OsHAK5* reduces tillers in rice (Chérel, 2004), producing a phenotype resembling loss-of-function mutants of the auxin transporter *OsABCB14* (Xu et al., 2014), implying a potential interaction between K^+ and auxin. Furthermore, driving expression of the WUSCHEL-related homeobox transcription factor gene *WOX11* using the promoter of *OsHAK16*, which encodes a low K^+-induced K^+ transporter, leads to increased effective tiller numbers in rice (Chen et al., 2015). Together, these findings indicate that K^+ availability modulates tillering at least through effects on K^+ transporters like *OsHAK5* and associated transcriptional networks (Chen et al., 2015). However, the specific molecular mechanisms by which K^+ influences tiller development remain unclear. Further research is needed to elucidate the signaling pathways and gene regulatory networks through which K^+ is perceived and transduced in axillary buds, promoting bud outgrowth.

2.2.3. Water availability influences bud outgrowth

Tillering/branching processes are susceptible to drought stress, one of the most limiting factors affecting agricultural yields. Optimal water availability is critical for normal plant growth and development, including tillering. Tolerance to this stress is multigenic and complex in nature. Drought stress triggers specific alterations of gene-expression patterns in plant tissues (Panda et al., 2021). For instance, the drought-inducible microRNA *miR393* was shown to be upregulated in *Arabidopsis* (Sunkar and Zhu, 2004), and *miR393* also regulates tiller number increases in rice by modulating auxin signaling through auxin receptor genes like *OsAUX1* and *OsTIR1*. In addition, overexpression of *OsmiR393* downregulates the rice tillering inhibitor *OsTB1*, leading to increased tiller numbers (Xia et al., 2012) (Figure 9). Overexpression of *LRK2*, which encodes a leucine-rich receptor-like kinase gene, increases drought tolerance and tiller numbers in rice (Kang et al., 2017). *Grain number*, *plant height*, and *heading date7* (*GHD7*) encoding a CCT (CONSTANS, CONSTANS-LIKE, and TIMING OF CHLOROPHYLLA/B BINDING1) domain protein regulates the rice flowering pathway and also contributes to rice yield potential. Overexpression of *GHD7* increases drought sensitivity, while knock-down of *GHD7* raises drought tolerance. Moreover, *GHD7* also regulates the plasticity of tillering by mediating the *PHYTOCHROME B-TEOSINTE BRANCHED1* pathway (Weng et al., 2014). In summary, water availability or drought stress regulates tillering/branching in plants through effects on gene expression, microRNA levels, and modulation of hormonal signaling pathways like auxin signaling.

2.2.4. Effects of temperature on tillering

Temperature is a critical factor influencing tillering in crops (Prasanth et al., 2017). High temperatures caused by extreme weather events can reduce tiller numbers, as evidenced in B. *distachyon*. Tiller numbers declined linearly in B. *distachyon* from 24 to 36°C at a rate of approximately one tiller for every 1.7°C increase in temperature (Harsant et al., 2013). Genes involved in heat stress play an important role in tillering. For instance, in rice, miR159 is downregulated by heat stress, and its overexpression increases heat sensitivity and significantly reduces tillering (Wang et al., 2012). At the other extreme, chilling also detrimentally impacts tillering. Chilling tolerance is a complex agronomic trait governed by intricate genetic networks and signal transduction cascades. Mechanistic insights into cold-stress effects on tillering are emerging. For example, overexpression of *OsMADS57* maintains rice tiller growth under chilling stress. *OsMADS57* directly binds and activates the defense gene *OsWRKY94* for cold-stress responses while suppressing its activity under normal temperatures (Chen et al., 2018). Additionally, *OsWRKY94* is directly targeted and repressed by the tillering inhibitor *OsTB1* during chilling. *D14* transcription was directly promoted by *OsMADS57* for suppressing tillering under chilling treatment, whereas *D14* was repressed for enhancing tillering under normal conditions (Chen et al., 2018) (Figure 9). Likewise, overexpression of *OsCYP19-4* results in cold-resistance phenotypes with significantly increased tiller numbers (Yoon et al., 2016). Ran is a small GTPase that involves various developments like nuclear assembly and cell-cycle control (Clarke and Zhang, 2008). The levels of mRNA encoding OsRAN1

were greatly increased by chilling, and *OsRAN1* overexpression in *Arabidopsis* increased tiller numbers (Xu and Cai, 2014). Elevated expression of *miR393* also improves chilling tolerance and tillering (Liu et al., 2017). In summary, modulation of chilling-tolerance genes may benefit crop-breeding efforts to sustain tiller development under temperature extremes caused by climate change.

2.2.5. Biotic stresses impact tiller bud outgrowth

Biotic stress affects plant development, including tillering, fundamentally disrupting and depriving plants of the nutrients they rely on for survival. More specifically, biotic stresses caused by plant pathogens, insect pests, and parasitic organisms can impair growth and developmental processes such as tillering and branching (Figure 9). These biotic agents injure plant tissues both directly through feeding/infection and indirectly by inhibiting the uptake and utilization of water, nutrients, and photoassimilates required for plant growth. Pathogens, insects, and parasites disrupt key physiological processes like metabolism, resource allocation, and energy balance, ultimately reducing the plant's capacity for producing new tillers or branches. Here, we take some examples to elucidate these processes. For instance, Striga is an obligate parasitic plant that can attach to host roots to deplete them of nutrients (Holbrook-Smith et al., 2016). The rice cultivar *Azucena*, belonging to the japonica subspecies, exudes high SL levels and induces high germination of the root-parasitic plant Striga hermonthica. In contrast, Bala, an indica cultivar, is a low-SL producer, stimulates less Striga germination, and is highly tillered (Cardoso et al., 2014). Plants relocate resources while fighting against pathogens and exhibit reduced tillering/branching. For example, the *UNI* gene encodes a coiled-coil nucleotide-binding leucine-rich repeat protein that belongs to the disease-resistance (R) protein family involved in pathogen recognition. The *uni-1D* mutation induces the upregulation of the pathogenesis-related gene while evoking some morphological defects like increased branches (Igari et al., 2008). Further research into mitigation strategies against prevalent biotic agents would benefit efforts to secure plant growth and crop yields.

3. Concluding remarks

Shoot branching is a highly intricate regulatory developmental program that involves the complex interplay of multiple genes, plant hormones, and environmental cues. A thorough understanding of the underlying mechanisms governing shoot branching/tillering is crucial for crop breeding and improving productivity(Figure 7). In this review, we provide a comprehensive overview of current advances in understanding the intricate regulatory mechanisms of shoot branching. We have highlighted key genes such as *TB1*, several phytohormones such as auxin, and environmental and internal inputs such as N, Pi, K^+, light, water availability, and biotic stresses, underscoring the interplay of these components.

However, the complex regulation of shoot branching/tillering requires further investigation. The interplay of the various underlying internal and external cues is still not entirely understood. For example, further research is needed to fully understand how sugars collaborate with other factors to regulate shoot branching/tillering.

Notably, plants with strong resistance to some pathogens often display fewer branches/tillers, but the exact mechanisms at play require further study. Importantly, AMs, which give rise to shoot branches/tillers, can also influence the patterns of panicles, hence affecting crop yields, an important factor in crop production and management (Wang and Li, 2008; Jiao et al., 2010; Springer, 2010). With new achievements in understanding shoot branching, a comprehensive landscape of factors controlling shoot branching will be established. A better understanding of shoot branching will ultimately facilitate the control of the process.

III. Advanced Strategies for Gene Cloning in Forward Genetics: Overcoming Challenges in Wheat Gene Isolation

1. Introduction

Natural and artificial genetic variations contribute to inheritable differences in plant traits. Deciphering the causal relationships between phenotypes and genotypes is crucial for enhancing our understanding of plant growth and development and facilitating marker-assisted selection in crop breeding programs. Two primary methods can be used to establish a connection between the sequence and function of a specific gene: forward and reverse genetics. Reverse genetics strategies rely on known sequence information. Researchers typically begin by selecting a sequence of interest and attempt to gain insights into its underlying function by disrupting the sequence and observing the resulting effects. Reverse genetics techniques, such as tagging with endogenous or exogenous transposable elements or T-DNA constructs, have provided opportunities for gene discovery. However, these tagging approaches are primarily limited to a restricted number of model plant species (Parinov and Sundaresan, 2000; Bouché and Bouchez, 2001). While tagging methods can identify a particular gene by unveiling a specific phenotype, they may not be suitable when a specific function is controlled by multiple redundant genes, necessitating a step-by-step analysis (Meissner et al., 1999), such as using CRISPR-Cas9 to disrupt the candidate genes one by one (Ma et al., 2016). Other reverse genetics strategies, including antisense, cosuppression, and RNA interference, are less targeted compared to tagging approaches like T-DNA (Matzke et al., 2001). Although these techniques aim at particular functions, they risk simultaneously disrupting sets of related genes, potentially obscuring the contribution of each individual gene to the observed phenotype. Constructs targeting a single gene can be valuable when working with species where targeted systems are not feasible (Peters et al., 2003). Notably, a more broadly applicable and promising species-independent targeting method is TILLING (Targeting Induced Local Lesions in Genomes) (McCallum et al., 2000).

In contrast to reverse genetics, forward genetics excels in identifying novel genes with unknown functions. By inducing mutations or leveraging existing genetic variations in plants and observing the resulting phenotypes, scientists can embark on a detective mission to uncover the gene responsible for a particular trait. Mutants created by chemical mutagenesis or radiation exposure offer several advantages: 1) mutagenesis can be applied to any species of interest; 2) the spectrum of induced mutants is broader than with tagging approaches; 3) mutagenesis is generally more efficient. Notably, map-based cloning (MBC) is a powerful and feasible method for cloning genes in forward genetics. A comprehensive study revealed that 86.1% of 364 genes were cloned using MBC (Liang et al., 2021), underscoring its significance in gene cloning. Based on our analysis of a random selection of 20 critical genes regulating a broad range of traits (e.g., domestication, adaptation, resistance, plant architecture), the candidate gene intervals delineated by MBC vary from 0.04 kb to

1010 kb, with a median of approximately 10 kb (Fridman et al., 2000; Yano et al., 2000; Takahashi et al., 2001; Wang et al., 2006; Song et al., 2007; Yu et al., 2007; Long et al., 2008; Fujii and Toriyama, 2009; Itabashi et al., 2011; Li et al., 2011; Shibaya et al., 2016; Xie et al., 2017; Tian et al., 2019; Wang et al., 2019a; Zhang et al., 2019b; Zhang et al., 2019c; Cheng et al., 2020; Ruan et al., 2020; Wang et al., 2020). These tiny candidate gene regions, which contain several (Long et al., 2008; Shibaya et al., 2016; Xie et al., 2017) or even a single gene (Song et al., 2007; Li et al., 2011; Wang et al., 2019a; Ruan et al., 2020), increase confidence in subsequently characterizing gene function.

MBC, while effective, is a labor-intensive and time-consuming process. This method utilizes molecular makers linked to genes of interest to narrow down the candidate interval. While determining the initial linkage region is relatively straightforward, fine-mapping the final 100 kb or smaller region presents significant challenges. This stage often needs screening a large number of recombinant individuals and employing more polymorphic markers. For example, cloning the *Thousand Grain Weight 2* (*TGW2*) gene involved screening 5,892 individuals to narrow the candidate gene interval to 7.6 kb, containing a single gene (Ruan et al., 2020). The cloned *ELS3* gene, which controls leaf senescence, required screening 10,133 individuals and spanned several years (Xie et al., 2023).

The shortage of molecular markers is another significant challenge for fine-mapping the gene of interest. While advancements in MBC, such as the availability of saturating marker systems (Table 4), have improved the accessibility for smaller candidate regions, the screening processes for suitable markers remain laborious. Thus, technologies that can detect all polymorphisms with genome-wide accessibility are required for MBC. Despite the ever-declining costs of next-generation sequencing, multiple approaches have been developed to obtain comprehensive polymorphisms and reduce the time of cloning genes, such as MutMap (Abe et al., 2012), BSE-Seq (Dong et al., 2020), and others.

The integration of these new technologies is crucial for accelerating the isolation of genes of interest. Of course, the combination of gene cloning with next-generation resequencing technologies has already led to the identification and characterization of multiple genes in model plants such as rice and *Arabidopsis*. For example, a variety of genes controlling important traits in crops have been cloned using the MutMap method (Abe et al., 2012), including genes related to male sterility (Chen et al., 2014), salt resistance (Takagi et al., 2015), erect panicle architecture (Hu et al., 2016), early heading (Komura et al., 2024) and more.

Wheat is a crucial staple food crop, providing sustenance for one-third of the global population and contributing approximately 20% of daily caloric intake (Zörb et al., 2018). It accounts for about 30% of global cereal production (Murray and McIntosh, 2019). To meet the demands of a growing population, wheat yield needs to increase from the current 3 tons per hectare to 5 tons per hectare (Godfray et al., 2010). This underscores the critical importance of identifying more genes regulating grain yield in wheat. However, gene cloning in wheat has progressed more tardily compared to other species due to two main challenges: the high ratio of repetitive sequences and

the enormous genome size (IWGSC, 2014). These factors highlight the urgent need to develop an accurate and efficient workflow for gene isolation in wheat.

This review explores the significance of MBC and other bulked segregant analysis (BSA) methods, integrated with datasets generated from high-throughput next-generation sequencing platforms. Furthermore, we also detail the algorithms used to identify candidate genes from these BSA-based methods, highlighting their advantages and potential risks. Regarding wheat gene cloning, we examine the factors that have impeded the rate of gene isolation and discuss strategies to overcome these barriers.

2. The power of forward genetics: unearthing novel genes and pathways

Forward genetics is an unbiased approach that allows researchers to discover entirely novel genes and pathways without prior knowledge of their sequences or functions. Unlike reverse genetics, which relies on existing sequence information, forward genetics begins with observable phenotypic changes caused by mutations. By identifying and isolating these mutated genes, scientists could explore fundamental plant processes and gain insights into complex traits like disease resistance, flowering time, grain yield, and more. One prime example is the identification of the rice semi-dwarfing gene *SD-1*, a cornerstone of the "Green Revolution." Through MBC, researchers found that *SD-1* encodes a key enzyme (gibberellin 20-oxidase) in the gibberellin biosynthesis pathway, directly influencing plant height (Monna et al., 2002). This discovery not only shed light on the mechanisms regulating plant stature but also enabled breeders to utilize marker-assisted selection (MAS) to improve grain yield in rice breeding programs. Another notable example is the *IPA1* gene. A point mutation in *IPA1*, identified through MBC, creates an "ideal" rice plant with fewer tillers, improved lodging resistance, and higher yield (Jiao et al., 2010). This discovery sparked further research on *IPA1*'s role in grain yield (Song et al., 2017; Zhang et al., 2017; Jia et al., 2022), contributing significantly to our understanding of this complex trait. The *MOC1* gene is another prominent MBC example. *MOC1* regulates axillary meristem development, directly impacting tillering patterns and, ultimately, grain yield (Li et al., 2003). Subsequent research provided deeper insights into *MOC1*'s function (Xu et al., 2012; Zhang et al., 2021b).

Forward genetics is not limited to model organisms and can be applied to non-model species, making it a valuable tool for crop improvement and understanding the genetic basis of important traits in various plant species. Moreover, by combining forward genetics with modern technologies like next-generation sequencing and genome editing, researchers can accelerate the discovery of novel genes and pathways, ultimately advancing our knowledge of plant biology and driving sustainable agricultural practices.

In conclusion, forward genetics offers a powerful, unbiased approach to uncovering novel genes and pathways. Its ability to reveal the unknown makes it an invaluable tool for understanding fundamental plant processes, improving crop productivity, and driving sustainable agricultural practices. Forward genetics propels our understanding

of plant biology and complex traits, contributing significantly to crop improvement and food security.

3. The classic map-based cloning workflow: a well-defined strategy

Genetic mapping, the foundation of MBC, has been instrumental in identifying and understanding the genetic basis of traits. The concept of genetic maps, introduced by Alfred H. Sturtevant in 1913 (Sturtevant, 1913), laid the groundwork for a century of remarkable developments in theoretical understanding and technological breakthroughs.

Genetic maps are constructed based on recombination events between non-sister chromatids of homologous chromosomes during meiosis. The order of linked genetic markers is determined through genetic localization experiments, with distances measured in units such as centimorgans (cM) or percentage recombination. It's important to note that recombination frequencies can vary across different chromosomal regions, and genetic distances may differ depending on the parental lines used.

MBC leverages the principle that as the physical distance between a gene of interest and the analyzed marker decreases, the frequency of recombination between them also decreases. The classic MBC workflow for forward gene cloning in plants typically involves the following steps:

1. Mutagenesis and trait identification: Plants are exposed to mutagens like radiation or chemicals to induce random mutations, or natural variations are utilized. Plants exhibiting attractive or desirable new traits are selected for further analysis.
2. Mapping population development: A mapping population is created by outcrossing the mutant to a genetically diverse parental line or by backcrossing the mutant to the wild-type plants. Backcrossing is preferred when phenotypes are difficult to distinguish.
3. Linkage analysis by BSA: BSA is a genome-wide mapping strategy used to determine if the mutation is linked to a specific genetic region (Figure 10). This involves analyzing bulked individuals displaying the recessive phenotype within the segregating population.
4. Fine mapping: Once the gene of interest is flanked by at least two markers, fine mapping is performed to narrow down the region further (Figure 11). This step requires a large mapping population and numerous genetic markers to pinpoint a small number of candidate genes, ideally a single one. Confirming the absence of previously reported mutants with similar phenotypes in the identified region is crucial, especially in model organisms like *Arabidopsis*. This can be done by consulting sequence-based maps. Additionally, allelism test crosses could be performed to ensure the mutation is not an allelic variation of a known gene.

The classic MBC workflow has been a valuable tool for gene cloning in various plants, including important crops. However, it has limitations. Fine mapping can be

labor-intensive and time-consuming, especially for species with large genomes and limited marker availability. Creating high-quality mapping populations can also be challenging for species with long generation times, low fertility, or complex reproduction.

This classic MBC workflow (Figure 11) has proven to be a powerful and effective strategy for gene cloning in various plant species, including important crop plants. However, it has limitations. The success of MBC heavily relies on the ability to create and maintain high-quality mapping populations. This can be a significant challenge for certain plant species, particularly those with long generation times, low fertility, or complex reproductive biology. Additionally, the accuracy of genetic mapping is influenced by the genetic distance between parental lines used for population development. Fine mapping can be labor-intensive and time-consuming, especially for species with large genomes and limited marker availability. Despite these challenges, MBC remains a valuable approach, particularly for well-studied model organisms and major crop species. The advent of Next-Generation Sequencing (NGS) technologies like whole-genome sequencing integrated with the principle of forward genetics has significantly transformed gene cloning, offering more efficient and high-throughput strategies.

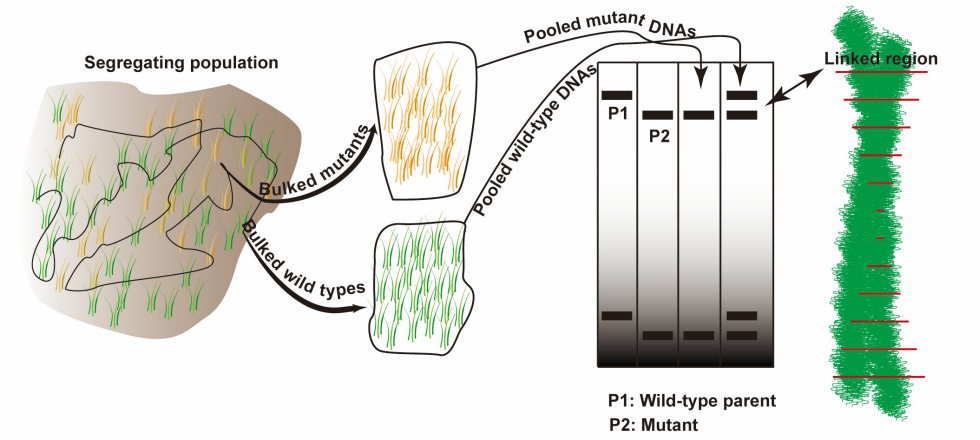

Figure 10. Schematic diagram indicating linkage analysis in BSA manner. In this image, the bulked mutants and the bulked wild types form a segregating population are used for DNA extraction in addition to their cross parents, the mutant (P2) and the crossing parent (P1). Only the makers show the same electrophoresis pattern between the P2 and the pooled mutant DNA, but not P1, can be recognized as a linkage event. The red lines indicate the positions of markers located in the chromosome.

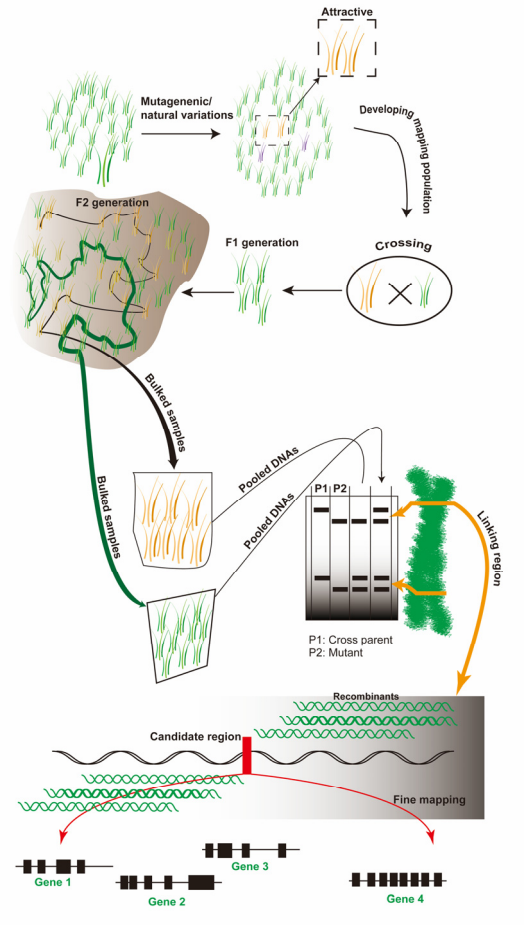

Figure 11. Workflow of MBC. This MBC workflow depicts how the genes are isolated from plant mutagenesis to fine mapping. Namely, how the mutants are selected, the corresponding mapping population is constructed, and BSA analysis and fine mapping processes are delineated.

Table 4. List of maker systems

Marker	Abbreviation	Refs
Restriction fragment length polymorphism	RFLP	(Botstein et al., 1980)
Variable number tandem repeat Minisatellites	VNTR	(Jeffreys et al., 1985)
Simple sequence length polymorphism	SSLP	(Bell and Ecker, 1994)
Simple sequence repeats	SSR	
Short tandem repeats	STR	
Inter-simple sequence repeat	ISSR	(Zietkiewicz et al., 1994)
Cleaved amplified polymorphic sequences	CAPS	(Konieczny and Ausubel, 1993)
Derived cleaved amplified polymorphic sequences	dCAPS	(Neff et al., 1998)
PCR amplification of specific alleles	PASA	(Gu et al., 1995)
Random amplified polymorphic DNA	RAPD	(Williams et al., 1990)
Amplified fragment length polymorphism	AFLP	(Vos et al., 1995)

4. The future of forward genetics: embracing new technologies and understanding their limitations

4.1 Advantages of NGS-related algorithms

The advent of NGS technologies and their continuously decreasing costs have revolutionized gene cloning, enabling swift and accurate identification of genetic determinants for traits of interest. This technological leap has spawned a diverse array of NGS-BSA-based strategies, each offering unique advantages for genetic research. These strategies include QTL-seq (Takagi et al., 2013a), GradedPool-seq (Wang et al., 2019b), QTG-seq (Zhang et al., 2019a), BSR-Seq (Liu et al., 2012), MutMap (Abe et al., 2012), MutMap+ (Fekih et al., 2013), MutMap-gap (Takagi et al., 2013b), MMAPPR (Hill et al., 2013), and BSE-Seq (Dong et al., 2020). These powerful and efficient approaches provide researchers with a diverse toolkit for identifying the genetic causes underlying traits of interest.

These strategies aim to determine the relationship between causal SNPs and traits of interest. The datasets from next-generation sequencing are typically derived from distinct bulked pools: mutants (Mus) and their parents (Cks), as well as the mutant-phenotype (MuB) bulk and the wild-type bulk (WtB) from the segregating population. The causal SNPs must be present homozygously in both Mus and MuB. The genotype of the causal SNP in Cks should differ from MuB, also in a homozygous status. Based on Mendelian inheritance laws, if the mutant phenotype is regulated by a recessive gene, the genotype of the causal SNP in WtB is likely to be heterozygous. These principles form the basis for various strategies. One such strategy is MutMap, introduced by Abe et al. (2012) (Abe et al., 2012), which pioneered the use of the SNP index algorithm for efficient gene mapping and has been widely applied in rice and other species (Yuan et al., 2017). The SNP index is calculated from Mub as the ratio of the read depth of the mutated SNP to the total read depth, including both mutant and wild-type genotypes. A SNP index value of 1 indicates that all SNPs are in the mutated format. By identifying SNPs with an index of 1 that also cause gene dysfunction, researchers can determine the likely cause of the mutant phenotype. MutMap+, a versatile extension of MutMap, facilitates gene identification from the M_3 generation derived from selfing of an M2 heterozygous individual. This approach is particularly suitable for identifying mutations that cause early developmental lethality, sterility, or generally hamper crossing (Fekih et al., 2013). MutMap+ introduced the δSNP index, which compares the SNP index values of wild-type and mutant bulks. This comparison is significant as it allows for the exclusion of false linkage sites, thereby improving the accuracy of causal mutation identification. MutMap-Gap combines MutMap with targeted *de novo* assembly of genomic gap regions to identify mutations located in areas containing significant structural variations compared to the reference genome (Takagi et al., 2013b). This method first uses MutMap to identify a rough interval of interest. It then employs *de novo* assembly using unmapped reads to construct scaffolds in gap regions. Consequently, mutations within these gaps can be identified through simple alignment of the newly assembled sequences. The majority of agronomically important traits are controlled by multiple

genes, each with a small effect (quantitative trait loci, QTLs). QTL-seq, a method using δindices similar to MutMap+, has successfully detected genes controlling traits such as rice blast disease resistance and seedling vigor (Takagi et al., 2013a). It is worth noting that this approach analyzes two pools from a segregating population that display extremely opposite values for a given phenotype. In addition to QTL-seq, GradedPool-seq (Wang et al., 2019b) is another QTL mapping method. GradedPool-seq distinguishes itself by implementing Ridit analysis on allelic frequencies from graded bulks of the segregating population. This approach calculates p-values for each SNP, facilitating the identification of candidate genes.

An alternative to whole-genome sequencing is RNA-seq, which can be more cost-friendly due to the smaller size of the transcriptome, allowing for greater read depth with the same number of reads (Liu et al., 2012; Hill et al., 2013). For example, MMAPPR (Hill et al., 2013) is designed to handle the significant noise inherent in RNA-seq data sets. It calculates allelic frequency using Euclidean distance (ED), focusing solely on the segregating population, which allows it to identify the mutation-containing region without parental information. The method also generates a list of potential coding region mutations within the linked genomic segment. ED's linear nature makes it less susceptible to errors in allelic frequency analysis and allows it to effectively subtract sequence-specific errors, which are artifacts of Illumina sequencing technology assumed to be equally present in both samples. These features make MMAPPR a robust tool for analyzing noisy RNA-seq data and identifying mutations. BSR-seq employs an empirical Bayesian approach to analyze RNA-seq data from the segregating population, enabling gene isolation (Liu et al., 2012). A key advantage of RNA-seq-based methods is their dual utility: they provide not only mapping data but also insights into how the mutation affects global gene expression patterns. This comprehensive view allows researchers to simultaneously identify the causal gene and understand its broader impact on the organism's transcriptome.

However, RNA-seq-based approaches have certain limitations. They rely on the gene of interest being actively expressed at the time of tissue collection and producing a functional effect on the protein amino acid sequence. If the gene of interest is silenced or does not result in a detectable level in the transcriptome, these methods may fail to identify the causal gene. In such cases, alternative approaches like MBC may be necessary. MBC does not depend on gene expression and can identify genes based on their genomic location, making it a valuable complementary technique when RNA-seq-based methods are ineffective.

To overcome the challenge of large genomes, like in wheat, exome capture technologies are employed. These technologies primarily enrich genes' coding regions (exons), significantly reducing the amount of sequence data that needs to be analyzed. This targeted approach particularly benefits species with large, repetitive genomes like wheat. BSE-Seq (Dong et al., 2020) is a strategy that combines BSA with the varBScore algorithm. This method integrates a whole exome capture panel to sequence bulked segregant pools from segregating populations with the robust varBScore algorithm. In large, complex genomes like wheat, sequencing-based methods for identifying causal mutations often face challenges due to high

background noise from numerous SNPs and sequencing/mapping errors. VarBScore addresses this issue by calculating the variance between observed and expected allele frequencies in a sliding window, significantly reducing noise during the process of mapping causative mutations.

In summary, the advent of NGS technologies and the development of NGS-BSA strategies have facilitated gene cloning in plants. These advancements have significantly increased the efficiency of gene identification while also reducing the time required to obtain results and enabling the study of complex genomes.

4.2 Mitigating potential risks in NGS-BSA Methods: strategies and recommendations

NGS-BSA methods offer significant advantages in speed and efficiency for gene cloning compared to traditional MBC, which relies on recombinants and their markers to identify candidate regions. However, these methods define candidate gene regions based on various statistical criteria, resulting in smaller but potentially less precise intervals. For instance, Abe et al. (2012) reported that the average interval determined by SNP indices greater than 0.9 in rice was approximately 2.1 Mb based on their statistical analyses. A stochastic selection of several genes cloned using MutMap-related methods revealed candidate intervals ranging from 0.6 Mb to 7 Mb, with a median of approximately 3 Mb (Chen et al., 2014; Takagi et al., 2015; Deng et al., 2017; Zou et al., 2017; Wang et al., 2018b; Cheng et al., 2019; Jiang et al., 2019; Song et al., 2019; Wu et al., 2020). A statistical survey in rice indicated that, on average, approximately 440 genes are present within a 3 Mb interval, representing the highest gene density. This high gene density within the candidate intervals can pose a significant challenge in identifying the causal gene responsible for the phenotype of interest, especially when multiple genes harbor mutations. There may also be a bias toward genes with known functions related to the trait, potentially leading to the oversight of novel genes that have not yet been assigned functions (Kong et al., 2020).

Additionally, relying solely on statistical cutoffs like p-values or Loess scores may be arbitrary due to potential sequencing noise or incorrect selection of individuals. For example, GradedPool-seq (Wang et al., 2019b) reported a candidate gene region of 0.4 Mb because the interval used for comparing p-values was set to 0.4 Mb. However, intervals with slightly lower p-value ratios may also contain the causal gene due to sequencing noise or incorrect individual selection. Similarly, BSE-seq (Dong et al., 2020) considers the interval they chose for calculating the mean values with the lowest varBScore as harboring the causal gene, but this may not always be accurate. In the MMAPPR method, peak regions are defined as regions where the Loess fitted values are bigger than three standard deviations above the genome-wide median (Hill et al., 2013). BSR-seq (Liu et al., 2012) scans the genome using sliding windows with a fixed number of SNPs and determines the regions with the highest "window linkage probability" as being closest to the causal genes. Collectively, the candidate gene regions provided by these NGS-BSA methods, which combine high-throughput sequencing data, should be interpreted with caution due to potential sequencing noise or biases. Namely, intervals near those highly suggested regions by these methods

may also harbor the causal gene. In this scenario, if the initial candidate gene cannot be confirmed, it is advisable to extend the candidate gene regions or revert to a step-by-step MBC approach to refine the location of the causal gene. In some cases, MBC has been employed to finalize fine-mapping after obtaining a large region using MutMap-related methods (Deng et al., 2017; Jiang et al., 2019; Song et al., 2019). Thus, a balanced approach that leverages the strengths of both NGS-BSA and MBC is likely the most effective strategy for accurate gene identification and characterization, especially in challenging genomes, like wheat.

While NGS-BSA-based methods offer significant advantages in terms of efficiency and speed, it is imperative to remain aware of their potential limitations and exercise caution when interpreting the results. A balanced approach, judiciously integrating the strengths of both NGS-BSA and traditional MBC techniques, may be necessary to ensure accurate identification, particularly in complex genomes with high levels of repetitive sequences or structural variations.

5. Overcoming the challenges in wheat gene cloning

Gene cloning is a crucial tool for identifying genes responsible for important traits in wheat, such as disease resistance and yield-related traits. However, wheat has lagged behind other species in gene cloning due to several challenges, particularly before the availability of a well-assembled wheat genome sequence. The major challenges in wheat gene cloning are as follows:

1. Marker development bottleneck: Prior to the release of the wheat genome sequence, marker development was a time-consuming process. This often limits the number of markers available for gene cloning if we employ MBC.

2. The complexity of MBC: Implementing MBC step-by-step in wheat can be impractical due to the large number of markers required for BSA analysis. For example, screening approximately 2,800 markers may be necessary if two markers are 6 M bps far, significantly more than in model organisms like *Arabidopsis* and rice (Figure 12). Identifying such a vast number of polymorphisms between cross-parents can be daunting, especially when the linkage interval remains elusive after first linkage scanning, necessitating the search for additional markers.

3. High repetitive genome content: The wheat genome comprises over 85% transposable elements (IWGSC, 2014), and coding regions have a repetitive content exceeding 90% (Dong et al., 2020). This highly repetitive nature poses challenges for accurate sequence alignment.

To overcome these barriers, the following strategies leveraging the advantages of NGS and related algorithms are proposed:

1. Leveraging the wheat genome sequence: The availability of the well-assembled wheat genome sequence (IWGSC, 2014) has accelerated gene cloning steps by providing a reference for marker development and sequence analysis.
2. Employing NGS-BSA-based methods: NGS and BSA-based methods, such as MutMap (Abe et al., 2012) and GradedPool-seq (Wang et al., 2019b), offer promising approaches for rapid gene isolation. However, these algorithms were initially developed for species with smaller genomes and may require adaptation to address the intricate noise arising from the highly repetitive wheat genome. Therefore, precise read alignment is crucial for gene cloning. Uni-BSA (Yuan et al., 2024b) primarily aimed to filter out ambiguous reads located in multiple regions and leveraged as many as possible reads, guaranteeing the accuracy of variant calling.

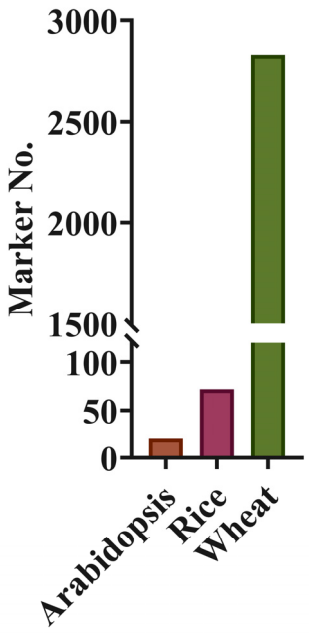

Figure 12. Comparisons of marker numbers between species used in the linkage analysis. Wheat uses more markers than *Arabidopsis* and rice if the average distance between the two makers is 6 Mb.

6. Conclusion

Forward genetics remains a powerful tool for uncovering the secrets of plant life. For example, it played a key role in identifying the genes behind grain yield in plants, which is crucial for global food security. The traditional MBC workflow, though labor-intensive, has been successful in model plants. However, recent advancements are revolutionizing gene cloning. NGS technologies and NGS-BSA-based methods like MutMap, GradedPool-seq, and BSE-Seq have greatly accelerated the process. These algorithms analyze massive amounts of sequencing data to rapidly pinpoint candidate regions containing the gene of interest. However, interpreting these results requires caution, as noise from sequencing and arbitrary cutoffs can lead to misidentification. Combining initial NGS-BSA mapping with fine mapping via traditional MBC can be a more accurate approach if isolating the gene becomes challenging.

For non-model crops like wheat, with their large and complex genomes, gene cloning has faced additional hurdles. The wheat genome's massive size and high repetitive content posed significant challenges. However, the release of the wheat reference genome and innovations like exome capture sequencing, which focuses on the gene-coding regions, are beginning to overcome these obstacles. As demonstrated by uni-BSA, strategies like analyzing only uniquely mapped reads and their mates in NGS-BSA methods can significantly improve accuracy (Yuan et al., 2024b). Currently, an integrative approach that leverages the strengths of both NGS-BSA methods and classic MBC is likely the key to unlocking wheat's full genetic potential for crop improvement.

Chapter III

A Novel Regulator of Wheat Tillering *LT1* Identified by Using an Upgraded BSA Method, uni-BSA

All the sections, except for 2.17, 2.18, 2.19, 2.20, 3.6, 3.7, and 4.3, are from Yuan Y, LB, Qi Juan, Liu Xin, Wang Yuanzhi, Delaplace Pierre, Du Yanfang (2024) A novel regulator of wheat tillering *LT1* identified by using an upgraded BSA method, uni-BSA. Mol. Breed 44 (7):47. Contributions: Yundong Yuan: conceptualization, investigation, developing uni-BSA, Writing the original draft, writing - review & editing. Bo Lyu: plant transformation. Juan Qi: Field investigation and CRISPR/Cas9 vector constructing. Yuanzhi Wang and Xin Liu: plant management. Pierre Delaplace: Supervision. Yanfang Du: Funding acquisition, Writing – review & editing, supervision.

1. Introduction

The tillering ability is a crucial agronomic trait that plays a vital role in determining the grain yield of cereal crops, such as it can generate productive tillers that bear grains (Kebrom et al., 2012; Wang et al., 2018a). Breeding programs often optimize tillering to improve crop performance and achieve high yields (Jiao et al., 2010). Wheat (*Triticum aestivum* L.) provides approximately one-fifth of human caloric intake worldwide, highlighting the importance of improving grain yield for global food security (Cao et al., 2020a). The mechanisms underlying tillering in wheat remain elusive relative to model species like rice and *Arabidopsis*.

Tillering is known to be regulated by external and internal factors. Tillering/branching involves the initiation of AMs and their outgrowth (Yuan et al., 2023b). Key genes regulating the process of AM initiation have been identified, such as *MOC1* (Li et al., 2003), *LAS* (Greb et al., 2003b), *ROX* and *LAX1* (Komatsu et al., 2003b; Yang et al., 2012). Bud outgrowth is inhibited in some species by the *TB1* transcription factor and its homologs, which integrate signals from various plant hormones, including strigolactones, auxin, and cytokinins (Takeda et al., 2003; Kepinski and Leyser, 2005; Tanaka et al., 2006; Shimizu-Sato et al., 2009; Alder et al., 2012; Smith and Li, 2014; Wang et al., 2018a; Matthes et al., 2019).

Emerging evidence highlights the importance of sugars in promoting bud outgrowth, acting as both nutrients and signaling molecules (Mason et al., 2014). For instance, reduced tillering in *wheat tiller inhibition* (*tin*) and rice *monoculm 2* (*moc2*) mutants is attributed to low sucrose levels (Kebrom et al., 2012; Koumoto et al., 2013). Notably, The necessity for sugars for bud outgrowth has been demonstrated in rose (*Rosa hybrida*), where sugar is required to trigger bud outgrowth in single nodes cultivated in vitro (Rabot et al., 2012; Barbier et al., 2015a). Sucrose can modulate bud outgrowth dynamics and influence phytohormone homeostasis by stimulating cytokinin biosynthesis and modulating auxin metabolism (Barbier et al., 2015a). According to the auxin canalization model, elevated sucrose levels within buds facilitate auxin export, promoting bud outgrowth (Mason et al., 2014; Kebrom and Mullet, 2015). These findings collectively demonstrate the crucial role of sugar signaling in regulating bud release.

The rapid development of sequencing technologies in recent years has accelerated the cloning of genes associated with important traits in crops. Traditional forward gene mapping methods, such as map-based cloning, are time-consuming and tedious, especially in wheat with a large and complex genome (17 G) (Consortium et al., 2018). For example, the recent research on the cloned gene *ELS3*, which controls leaf senescence, involved 10,133 individuals and spanned several years (Xie et al., 2023). Current breakthroughs using high-throughput sequencing techniques have accelerated the identification of genes linked to agronomic traits and made gene isolation more feasible and efficient. For instance, the adaptable method MutMap (Abe et al., 2012) has been widely used in identifying genes associated with a variety of traits, including but not limited to salt tolerance (Takagi et al., 2015), endosperm development (Wang et al., 2018c), flowering and seed size (Manchikatla et al., 2021), height and spikelet

(Huang et al., 2022) and more. MutMap-derived methods, such as MutMap+ (Fekih et al., 2013), MutMap-Gap (Takagi et al., 2013b), and QTL-seq (Takagi et al., 2013a), have also been developed to improve the efficiency and accuracy of gene mapping. However, the immense wheat genome remains cost-prohibitive for gene cloning using next-generation resequencing data (Consortium et al., 2018). To address the challenge of analyzing the complex wheat genome, the whole-exome resequencing method like BSE-seq has been developed (Dong et al., 2020). This method significantly reduces the amount of data analyzed by focusing on the exome, the protein-coding regions. However, a major hurdle remains: wheat is a hexaploid species (*Triticum aestivum* L., AABBDD, 2n = 6x = 42), and its genome has a remarkably high repetitive sequence content, exceeding 80% (Consortium et al., 2018). This repetitive nature causes short sequencing reads from next-generation sequencing platforms to map to multiple locations in the genome, posing significant challenges for accurately identifying genes. While current Bulk Segregant Analysis (BSA)-based methods, including BSE-seq, MutMap, and others, primarily focus on reducing noise generated by next-generation sequencing platforms, they lack algorithms specifically designed to tackle the noise generated by the inherent high repetitive sequence content of wheat.

Currently, gene cloning in wheat is facing significant challenges. To date, only two genes related to tillering, *TN1* (Dong et al., 2023) and the *tiller inhibit* gene (*tin*) (Spielmeyer and Richards, 2004; Hyles et al., 2017), have been successfully isolated using map-based cloning. Most research is focused on reverse genetics, targeted genes such as wheat *TaD27* (Zhao et al., 2020b), *TaD14* (Liu et al., 2021a), and *TaMOC1* (Zhang et al., 2015). This underscores the urgent need to develop efficient and precise gene cloning methods specifically tailored for wheat. In this study, we upgraded the bulked segregant analysis method called uniquely aligned bulked segregant analysis (uni-BSA), which focuses on reasonably filtering out the reads aligned to multiple positions to increase variant calling accuracy. Through this method, we successfully mapped the wheat '*LESS TILLER1*' (*LT1*) gene to the short arm of chromosome 2D. The candidate *LT1* gene, which encodes a nucleotide-binding domain protein, has been validated through the knockout of *LT1* by CRISPR/Cas9. *LT1* may play a role in regulating tillering through its involvement in auxin, cytokine, and sucrose levels. In summary, we have identified a novel wheat tillering regulator, *LT1*, using the advanced uni-BSA method, which is highly effective for cloning causal genes in wheat. Understanding the role of *LT1* will offer valuable perspectives for molecular breeding in wheat.

2. Materials and Methods

2.1. Plant materials and growth conditions

The *lt1* mutant is derived from a mutagenesis pool of a landrace Chang6878 (C6878) treated with 1% ethyl methanesulfonate (EMS) as follows:

To treat wheat seeds with EMS, prepare a 1% EMS solution and soak the seeds for 10 hours at room temperature, stirring gently. After treatment, rinse the seeds thoroughly with deionized water 3 to 4 times to remove residual EMS. For safe disposal, neutralize the EMS waste by mixing it with an equal volume of 10% sodium

thiosulfate ($Na_2S_2O_3$) solution and allow it to react for 24 hours. After neutralization, the waste should be collected and disposed of according to laboratory regulations. Once the seeds are dried, they are ready for planting to observe mutations.

Wheat plants are cultivated in the experimental field at Shandong Agriculture University, Tai'an, Shandong, China. The transgenetic plants are grown in a growth chamber maintained at 22/17°C day/night temperatures, 16-h photoperiod, and about 300 μmol m^{-2} s^{-1} photosynthetically active radiation at 45% humidity.

2.2. Common molecular experiments

The amplification of target fragments, restriction digestion, ligation, preparation of competent cells, transformation of ligation products, colony PCR, plasmid extraction, and restriction enzyme verification involved in the vector construction process of this thesis were all performed according to the "Molecular Cloning: A Laboratory Manual" (3rd edition). All plasmids were sequenced to ensure complete accuracy of the sequences.

In addition to conventional molecular cloning methods, this study also employed the In-fusion gene cloning method (In-Fusion® HD Cloning Kit, Clontech: 639650). This method primarily relies on Clontech's proprietary In-Fusion enzyme, which can recognize 15 bp homologous sequences at the ends of DNA fragments and linearized vectors, thereby efficiently and precisely fusing the DNA fragments and linearized vectors together. The specific operational steps were carried out according to the instructions provided by Clontech.

2.3. Wheat genomic DNA extraction

The extraction of wheat genomic DNA is performed using a modified CTAB method (Chatterjee et al., 2002) as follows:

Take approximately 0.1 g of young wheat leaves and place them in a 1.5 mL centrifuge tube. Snap-freeze the leaves with liquid nitrogen and grind them using a Tissue Crasher CK-1000 at a vibration frequency of 1000 rpm/min, with one 6 mm diameter steel bead added to each tube. Add 0.5 mL CTAB solution (Appendix 1) and mix well by shaking. Incubate in a 65°C water bath for 30 minutes. Add an equal volume of chloroform and mix thoroughly; Centrifuge at 10,000 rpm for 10 minutes; Transfer the supernatant to a new 1.5 mL centrifuge tube. Add 2 volumes of anhydrous ethanol. Centrifuge at 12,000 rpm for 10 minutes immediately. Discard the supernatant and allow the pellet to air dry. Add 100 μL of ddH2O to resuspend the DNA pellet. Store the extracted DNA at -20°C for future use.

2.4. Exome capture sequencing

Genomic DNAs were extracted from a minimum of 50 individuals with contrasting extreme phenotypes from an F_2 population, along with 10 *lt1* mutants and 10 C6878 plants serving as two control DNA pools, using the CTAB method mentioned previously. The mutant-type and wild-type DNA pools of the F_2 population were generated by bulking at least 50 genomic DNAs in an equal ratio. The *lt1* mutant and C6878 DNA pools were also generated in an equal ratio.

The datasets generated from Whole-Exome Sequencing (WES) for variation calling in this study were obtained from the Oebiotech company (Shanghai OE Biotech Co., Ltd). The capture probe sequences were primarily derived from RefSeq Annotation V1.0, encompassing 107,891 high-confidence (HC) and 50,000 low-confidence (LC) protein-coding genes, including their UTR and associated promoter sequences. The WES approach theoretically can generate 260 Mb of data per fold of the wheat genome. For each sample, we acquired 26 Gb of data, equivalent to a 100-fold coverage depth per gene, ensuring high-quality SNP calling.

2.5. The uni-BSA pipeline for rapid gene isolation

We developed an enhanced bulked segregant analysis pipeline called uni-BSA for rapid gene cloning in wheat (Figure. 14). This approach consists of the following steps. (1) Develop a segregating population from a backcross between the mutant and the wild-type parental line. (2) Extract and pool DNAs from the mutants, their wild types, and individuals with mutant and wild-type phenotypes of the F_2 population in equal proportions, forming four independent sample pools, respectively. (3) Subject the DNA pools to WES generating deep coverage data (100 folds). (4) Preprocess the raw reads with Fastp (v0.20.1) to remove adapters and low-quality reads (Zhang et al., 2018). Here is a code example: fastp -i ./sample-F.fq.gz -o sample_F.fq.gz -I ./sample-R.gz -O sample_R.fq.gz. (5) Align the clean reads to the IWGSC RefSeq v2.1 reference genome using BWA (v0.7.17) mem algorithm with default parameters (Li, 2013). Here is the code instance: bwa mem genome.ref sample_F.fq.gz sample_R.fq.gz > Sample.sam (6) Exclude unmapped, low quality and non-primary alignments with Samtools view (v1.7) (Li et al., 2009). Here is the code: samtools view -h -F 260 -S -q 20 Sample.sam -o Sample.1.Nopri.sam (7) Use custom Perl script (Filter.ambi.pl) we developed to process the primary filtered SAM files to retain unambiguous alignments. The main principle is to retain uniquely aligned reads along with their mate reads, even if they are multiply aligned. Here is the code: Filter.ambi.reads.pl Sample.1.Nopri.sam > Sample.2.uni.Nopri.sam. To provide a better understanding of this method, we have provided a schematic diagram (Figure 13) comparing different filtering approaches. (8) Remove PCR duplicates and sort the BAM files with Samtools. Here is the codes: Samtools view Sample.2.uni.Nopri.sam -o Sample.3.sort.uni.Nopri.bam; Samtools index Sample.3.sort.uni.Nopri.bam; samtools markdup -r Sample.3.sort.uni.Nopri.bam Sample.3.sort.uni.Nopri.dedup.bam (9) Use GATK (version 4.0) (McCormick et al., 2015) HaplotypeCaller to generate gVCF files, including all the variations. The combined gvcf format files are filtered with the suitable parameters, including QD <2, FS>60 and SQR>3.0 by using the gatk VariantFiltration function. Use GATK GenomicsDBImport and VariantsToTable to compile variants from all samples. Here is the codes: gatk HaplotypeCaller --reference wheat.fq --input Sample.3.sort.uni.Nopri.dedup.bam --output Sample.gvcf.gz --emit-ref-confidence GVCF; gatk GenomicsDBImport --genomicsdb-workspace-path my_database --sample-name-map sample_map.txt (containing all the gvcf format files) --intervals target_intervals.list (Including the areas you want to merge); gatk GenotypeGVCFs -R reference.fasta -V gendb:// enomicsdb-workspace-path -O combined_output.vcf;

gatk VariantFiltration -R wheat.fasta -V combined_output.vcf -O filtered_output.vcf --filter-expression "QD < 2.0 || FS > 60.0 || SOR > 3.0" --filter-name "LowQuality"; gatk VariantsToTable --variant filtered_output.vcf --fields CHROM -F POS -F REF -F ALT -F QUAL -F AC -GF GT -GF AD --output variant.table.

(10) Use the mean δ-index values (Abe et al., 2012) from 2 Mb sliding windows (0.1 Mb per slide) to define the candidate region. The term of δ-index is that the frequency of the variant of the mutant pool subtracts the frequency of the variant of the wild-type pool. The higher the δ-index, the stronger the linkage. The final linkage interval is the region framed by the positions whose corresponding mean δ-index values exceed the 95th percentile of the mean of all δ-index values. This is a computational process with a large workload that can be completed by uni-BSA's linkage analysis model by using the variant.table generated in the step 9 (11) Annotate variants using ANNOVAR (Wang et al., 2010) to determine functional effects in coding and non-coding regions. Here is the code: perl annovar_V2.pl --species wheat --file ./Sample.fine_merger.dat (produced by uni-BSA) --dir ./anno. The Sample.file_merger.dat is a special format file used for gene annotation that is automatically produced by the uni-BSA.

It is worth noting that steps five to eleven are integrated into uni-BSA, which is a comprehensive and easy-to-use software. What you should do with uni-BSA is to indicate species and population information. For more information, simply input uni-BSA --help to learn how to use it effectively. We also provide uni-BSA as an appendix in the bioinformatics section. Trying to decode uni-BSA requires that you have a certain level of expertise to understand Perl code. Here is an example as follows:

uni-BSA \

--Ck_1 C6878_R1.fastq.gz \ # the forward sequences of the wild type

--Ck_2 C6878_R2.fastq.gz \ #the mate sequences of the wild type

--Mu_1 LT1_R1.fastq.gz \ # the forward sequences of the mutant

--Mu_2 LT1_R2.fastq.gz \ #the mate sequences of the mutant

--MuB_1 MB_R1.fastq.gz \ # the forward sequences of the mutant bulk from the segregating population

--MuB_2 MB_R2.fastq.gz \ # the mate sequences of the mutant bulk from the segregating population

--MuW_1 WB_R1.fastq.gz \ # the forward sequences of the wild-type bulk from the segregating population

--MuW_2 WB_R2.fastq.gz \ # the mate sequences of the wild-type bulk from the segregating population

--T 20 \ # threads used by the server with Linux system, depending on the ability of your server.

--species wheat # You should specify the species, as uni-BSA can clone genes from various species, including but not limited to rice and *Arabidopsis*.

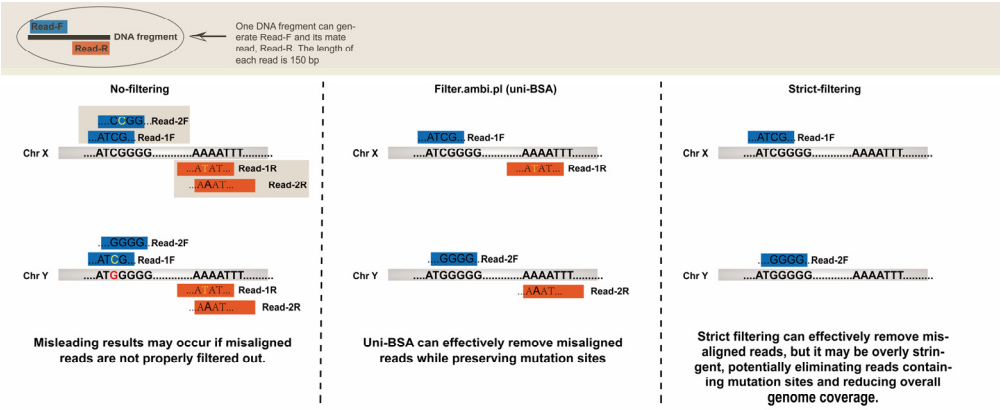

Figure 13. Comparisons between different filtering methods.

Consider a DNA segment with a single nucleotide difference ('C' to 'G,' highlighted in red) between Chromosomes X and Y. Due to the wheat's complex genome, a single read may align to multiple sites when no filtering is applied. This can potentially lead to errors in SNP calling, incorrectly identifying homozygous mutations as heterozygous. Filter.ambi.pl (integrated into uni-BSA) leverages the fact that paired-end reads (read-F and its mate read-R) originate from the same DNA fragment. If one read aligns uniquely to a position, its mate must be nearby. In contrast, the strict filtering method is highly stringent, eliminating all reads that map to multiple sites. This approach could result in several issues, including diminished genome coverage and the inadvertent elimination of reads containing important variants.

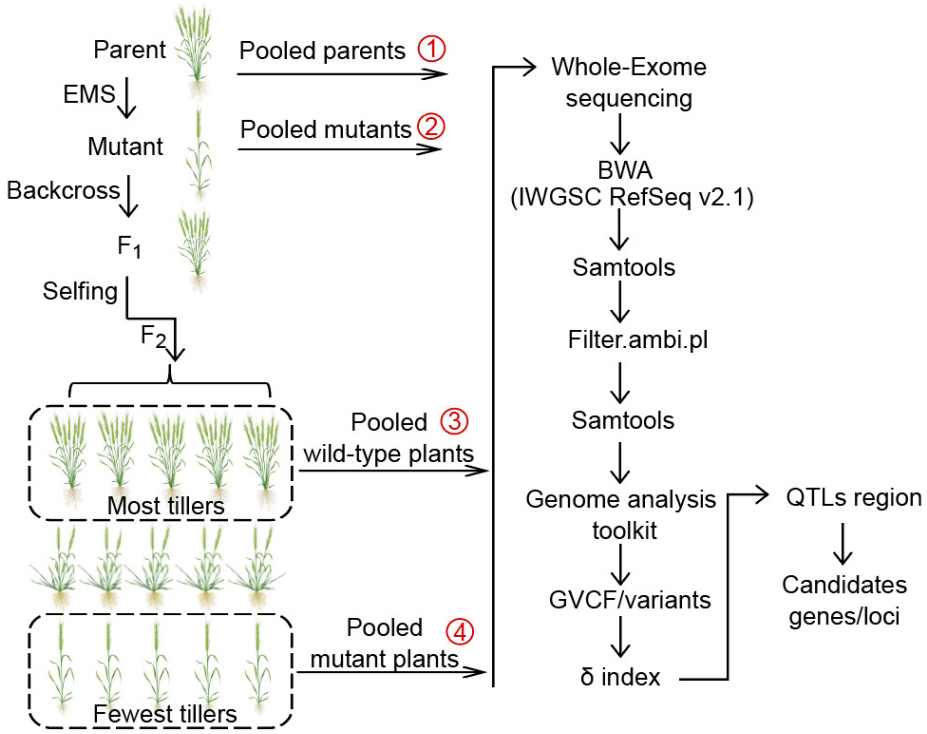

Figure 14. Pipeline of *LT1* cloning. In this flowchart, seeds of wheat C6878 were mutagenized by EMS. The resulting M_2 or higher generation mutant plant *lt1* was backcrossed to C6878 and self-pollinated to generate an F_2 segregating population. Four bulks indicated by red circled digits were subjected to WES. The big datasets were aligned against the Chinese Spring version 2.1. Then, the resulting sam files were primarily filtered and deep treated by the Perl script "Filter.ambi.pl" to leverage as many reads as possible. Sequentially, the GVCF files containing all variants were generated. Δindexes were calculated to define the candidate regions. The causal variants responsible for the mutant phenotype were identified in the candidate interval.

2.6. Transformation methods for *Escherichia coli* (*E.coli*) and *Agrobacterium tumefaciens*

Thaw 50 μL Trans5α chemically competent cells (Trans, Cat. No.: CD201-01) on ice, then add approximately 100 ng of target plasmid and mix gently. Incubate the mixture on ice for 30 minutes. Heat shock the cells at 42°C for 90 seconds, then immediately transfer to ice for 2 minutes without shaking. Add 500 μL of sterile LB medium to the tube and incubate at 37°C with shaking at 200 rpm for 1 hour. Spread 100 μL of the transformed cells onto an LB agar (Appendix 2) plate containing the

appropriate antibiotic. Invert the plate and incubate at 37°C overnight. Finally, screen for positive clones using PCR.

To perform electroporation of *Agrobacterium tumefaciens* using the Bio-Rad MicroPulser Electroporator, keep the *A.tumefaciens* competent cells (WEIDI, Cat. No.:GV3101) on ice and ensure that the electroporation cuvettes are pre-chilled on ice. Transfer 50 μL of competent cells into a pre-chilled 1.5 mL microcentrifuge tube, add 1-5 μL of plasmid DNA (about 100 ng) to the competent cells, gently mix by pipetting up and down a few times, and keep the mixture on ice for 1-2 minutes. Carefully transfer the cell-DNA mixture into a pre-chilled electroporation cuvette (ensure the mixture is settled at the bottom), and tap the cuvette gently to ensure the suspension is evenly distributed between the electrodes. Set the Bio-Rad MicroPulser Electroporator to the "*Agrobacterium*" setting (typically 2.2 kV, 5 ms pulse time), insert the cuvette into the holder of the MicroPulser, and press the pulse button to deliver the electric pulse. Immediately add 1 mL of sterile LB medium (at room temperature) into the cuvette after electroporation, transfer the contents of the cuvette into a sterile 1.5 mL microcentrifuge tube, and incubate the tube at 28°C with 200 rpm/min shaking for 1-2 hours to allow cell recovery. After recovery, plate 100-200 μL of the cells on LB agar plates containing the appropriate antibiotics for selection, and incubate the plates at 28°C for 2-3 days until colonies appear. Ensure all equipment, cuvettes, and reagents are sterile to avoid contamination and keep the cells on ice at all times before electroporation to maintain their competency.

2.7 Wheat genetic transformation

The wheat genetic transformation was modified based on the *Agrobacterium*-mediated method (Hiei et al., 1997). The culture media used are listed in Appendix 4. The specific steps are as follows:

1. Induction of callus from immature wheat embryos. Wash the wheat immature embryos with 70% (v/v) ethanol for 1 minute, rinse with sterile water 3 times, then sterilize with 2.5% (v/v) sodium hypochlorite solution for 45 minutes. After rinsing with sterile water 5 times, plate the embryos on NB medium to induce callus tissue. Subculture every two weeks until smooth, compact, and pale yellow embryogenic callus tissue is obtained for genetic transformation.

2. *Agrobacterium* culture. Use electroporation to transform the target binary vector into *Agrobacterium* strain GV3101. Plate *Agrobacterium* cells on YEP solid medium containing kanamycin (50 mg/L) and rifampicin (25 mg/L). After 2 days, positive clones are picked up and inoculated into the liquid YEP medium containing kanamycin and rifampicin. Shake culture at 28°C until OD_{600} reaches 0.8-1.0. Centrifuge at 5,000 rpm for 10 minutes to collect bacteria, then resuspend in *Agrobacterium* infection solution containing 100 μM acetosyringone (AS) to an OD_{600} of about 1.

3. *Agrobacterium* infection. Co-cultivate the well-grown callus tissue with *Agrobacterium* infection solution for 2 minutes, shaking occasionally. Then, blot dry excess bacterial solution with sterile filter paper, transfer to a Petri dish containing

multiple layers of sterile filter paper, and dark culture at 25°C for 2-3 days. Subsequently, transfer to NB medium containing hygromycin (50 mg/L) for selection.

4. Selection of resistant callus tissue and plant regeneration. Change the selection medium once a week. After about four rounds of continuous resistance screening, transfer the vigorously growing, compact resistant callus tissue to the differentiation medium. First, dark culture at 25°C for 3-5 days, then transfer to normal light conditions. After about 4 weeks of growth, seedlings will regenerate. Transfer to rooting medium for root and seedling strengthening. After about 20 days of growth, open the culture bottle for 2-3 days, then transplant to a greenhouse or field.

2.8. Construction of CRISPR/Cas9 vector and wheat transformation

To create the vector for gene editing via CRISPR/Cas9, two sgRNAs were designed in the region flanking the *LT1* mutation site by using the CRISPR MultiTargeter web tool (Prykhozhij et al., 2015), targeted on the $CDS^{692\text{-}710th}$ (target 1: AGTCATATAAACTACATGA) and the $CDS^{919\text{-}937th}$ (target 2: ATAGTGACAACAAGATCTG) of *LT1*, respectively, and no off-target effects are observed at other positions in the wheat genome. The resulting PCR fragment amplified from the intermediate vector pCBC-MT1T2 containing the targets and gRNA scaffold was inserted into the binary vector pUBE413 (Appendix 7, the plasmid map containing the target gene) using the "Golden Gate" method (Xing et al., 2014; Dong et al., 2023). The construction of the CRISPR/Cas9 system was carried out through simultaneous restriction digestion and ligation, as detailed below:

Components	Volume	Conditions
PCR fragment containing fragments of the vector pCBC-MT1T2 and the targets of *LT1* (100 ng/μL)	2 μL	5 hour at 37°C; 5 minutes at 50°C; 10 minutes at 80°C.
pUBE413 (500 ng/μL)	2 μL	
10× T4 DNA ligase Buffer (NEB)	1.5 μL	
10× BSA (100 μg/ml)	1.5 μL	
BsaI (Catalog #: R0535)	1 μL	
T4 DNA ligase (NEB)	1 μL	

ddH2O	6 μL

The reconstructed plasmid was introduced into *Agrobacterium* strain GV3101 and transformed into wheat cultivar "Fielder" as mentioned in the step 2.6. The *LT1* target region was PCR amplified and sequenced to identify mutations (Cr*LT1*-F: GGATTGGGCAAGTCCGAAGA, Cr*LT1*-R: ACCATCACACCTCCCCACTA). We used three T_2 plants for statistical analysis in every independent transformation event.

2.9. Quantification of sucrose content

The quantification of sucrose utilizes acid hydrolysis to break down sucrose into glucose and fructose. The fructose then reacts with phenol to form a colored product that can be detected at a 480-nanometer wavelength. Shoot base samples were harvested from 30 *lt1* mutants and wild-type C6878 plants at the developmental stages of two, three, and four leaves, with three technical replicates per genotype per stage. Approximately 50 mg of fresh shoot base tissue was ground in liquid nitrogen for each sample. Sucrose extraction and colorimetric detection were performed following the detailed protocol provided in the sucrose assay kit from Solarbio (item no. BC2465, http://solarbio.net/goods-9298.html). The main processes are displayed below:

1. Grind the shoot base tissue in liquid nitrogen, then add 1 mL of extraction buffer.

2. Heat at 80°C for 10 minutes, vibrating 2-3 times during this period. Cool to room temperature afterward.

3. Centrifuge at 5000g for 10 minutes, then transfer the supernatant to a new centrifuge tube.

4. Add 2 mg of reagent IV and heat at 80°C for 30 minutes to decolorize the sample.

5. Add 50 μL solution I, 700 μL solution II, and 200 μL solution III to the sample to be detected, standard sample, and empty tube, respectively. Mix thoroughly and heat at 95°C for 30 minutes to develop color.

6. Transfer the reaction solution to a 1 mL glass cuvette and measure the absorbance at 480 nm. Recorded as $A_{sample,}$ $A_{standard}$ and A_{blank}. Calculate $\Delta A_{sample} = A_{sample} - A_{blank,}$ $\Delta A_{standard} = A_{standard} - A_{blank.}$ Note: The blank tube only needs to be measured 1-2 times.

7. Establishment of the Standard Curve: use 3.0, 2.0, 1.0, 0.5, 0.25, and 0.125 mg/mL as the x-axis (independent variable) and the corresponding $\Delta A_{standard}$ values as the y-axis (dependent variable) to plot the standard curve. The standard equation $y=kx+b$ is obtained, and the measured Δ Asample value is substituted into the equation to calculate x (mg/mL).

8. Caculate sucrose contents (mg/g) following the formula: content (mg/g) = x/w, where W is the sample weight in grams.

2.10. Dynamic observation of AM development and its subsequent outgrowth

To evaluate AM development in wheat, seedlings were examined at the developmental stages of 1) only coleoptile emerged, 2) two leaves, and 3) four leaves. At each stage, shoot base samples were collected randomly from several seedlings. After carefully removing the leaves, the shoot bases were directly visualized using a stereomicroscope. The number of visible axillary meristems was counted at each time point. We also examined the plants after the heading stage for axillary bud outgrowth to observe if they had ceased axillary buds, like the process of AM number counting.

2.11. Wheat RNA extraction

Wheat total RNA extraction is performed using the Trizol method (Chomczynski and Sacchi, 1987) modified as follows: Take approximately 0.1 g of wheat material, grind it in a mortar with liquid nitrogen, then transfer it to a 1.5 mL RNase-free centrifuge tube. Add 1 mL Trizol (Invitrogen, CA, USA) and mix well by shaking, then add 200 μL chloroform and mix well by shaking, centrifuge at 12000 rpm for 15 minutes at 4°C. Transfer the supernatant to a new 1.5 mL RNase-free centrifuge tube, add an equal volume of isopropanol, then centrifuge at 12000 rpm for 10 minutes at 4°C. Remove the supernatant, add 1 mL 70% ethanol to wash the pellet, then centrifuge at 7000 rpm for 5 minutes at 4°C. Remove the supernatant, air-dry the pellet, then dissolve it in 50 μL nuclease-free ddH2O. Determine the RNA concentration by measuring OD values using a UV spectrophotometer and assess RNA quality by 1.2% formaldehyde denatured agarose gel electrophoresis.

2.12. Quantitative RT-PCR

After total RNA extraction, remove DNA from the total RNA and reverse transcribe RNA to cDNA following the instructions of HiScript IV 1st Strand cDNA Synthesis Kit (+gDNA wiper, R412, Vazyme) as follows:

1. The genome DNA removal

Add the following components to remove genomic DNA:

Components	Volume
RNase-free ddH2O	To 10 μL
5× gDNA wiper Mix	2 μL
Total RNA	500 ng

Gently mix the components by pipetting, then incubate at 42℃ for 2 minutes.

2. First-strand cDNA synthesis

Prepare the cDNA synthesis reaction mixture as follows:

Components	Volume
The solution from the step 1	10 μL
4 × HiScript IV RT SuperMix	4 μL
Oligo (dT)20VN	1 μL
Random Primers	2 μL
RNase-free ddH2O	2 μL

Mix gently by pipetting. Incubate the reaction at 37℃ for 15 minutes, followed by heating at 85℃ to stop the reaction.

The fluorescent quantitative PCR reagent used is SsoFast^{TM} EvaGreen Supermix from BIO-RAD. The total reaction volume is 10 μL, including 5 μL Supermix, 1 μL cDNA from step 1, and 4 μL primers, with a final primer concentration of 0.4 μM. Set up at least 3 technical replicates for each reaction, using wheat *Actin* gene as an internal reference. The fluorescent quantitative PCR instrument used is BIO-RAD's CFX96. The PCR program is: 98°C for 30 seconds, 40 cycles (98°C for 5 seconds, 60°C for 5 seconds, read plate for 5 seconds), melt curve 65-95°C, 0.5°C/5 seconds, read plate for 5 seconds. Analyze experimental data using Bio-Rad CFX Manager software.

Primers used for qRT-PCR are listed in Table 5. The wheat *Actin* (*TraesCS1A02G020500*) (Table 5) gene served as an internal control. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each experiment was performed with at least three technical replicates.

Table 5. Primers used for qPCR

Primer name	**Primer sequence (5′–3′)**
LT1-F	ACATAATGGAAACTATTTGACGCAC

LT1-R	CCACGCGACTTAGGTTCTCA
ACTIN-F	GCCATGTACGTCGCAATTCA
ACTIN-R	AGTCGAGAACGATACCAGTAGTACGA
Ta3BFBPase-F	ACTCACTCCCATCACCTCAC
Ta3BFBPase-R	AAAGTGAGGCTGGGAGCGGAG
Ta3AFBPase-F	GAGAGGGTCGGCGTCGGAGA
Ta3AFBPase-R	GCGACGAACTTGCAGCCGAGG
Ta3DFBPase-F	GGGTCGTCCCCGGCCGGCCG
Ta3DFBPase-R	GCGACGAACTTGCAGCCGAGG

2.13. Co-expression analysis

For gene expression analysis, we harvested at least 50 shoot bases (around 3 mm in length) from C6878 and *lt1* wheat individuals, as the shoot base is the site where the tiller bud forms and subsequently grows out to form a tiller. Samples were collected at three developmental stages: 2-leaf, 3-leaf, and 4-leaf, to capture temporal gene expression changes. Due to the small size of individual shoot bases, we pooled them for each sample at each time point to obtain sufficient RNA for sequencing. For each time point of C6878 and *lt1*, we prepared three technical replicates to ensure statistical robustness. We then performed RNA-seq on these pooled samples, generating comprehensive gene expression profiles across the developmental stages. RNA sequencing was performed by ANOROAD company, generating approximately 800 million reads, namely up to 12 GB of clean data per sample. The subsequent data analysis pipeline (run in the Linux Platform) includes the following main steps:

1. Quality control and preprocessing of raw sequencing data were carried out as follows:

fastp -i ./sample-F.fq.gz -o sample_F.fq.gz -I ./sample-R.gz -O sample_R.fq.gz.

2. Align clean reads to the reference genome using the software STAR (Dobin et al., 2013). The code is indicated below:

2.1 Create the index of the wheat genome

STAR --runThreadN 100 # the threads used, which depends on the server's ability

--runMode genomeGenerate # read the genome and gene annotation information

--genomeDir ./index # the index of wheat generated by STAR

--genomeFastaFiles genome sequence

--sjdbGTFfile $gtf # the $gtf file containing all the gene information, including positions of UTR, CDS, and more.

--sjdbOverhang 149 # the length of reads

2.2 Perform alignment

STAR --runMode alignReads # Set STAR to alignment mode to align RNA-seq reads to the reference genome

--runThreadN number $T # Use multiple threads to accelerate processing, where $T specifies the number of threads

--genomeDir index # Specify the directory where the genome index files are stored

--outFileNamePrefix $d # Set the output file path and prefix, with $d being the output directory and prefix

--outSAMtype SAM # Specify the output file format as SAM; other options include BAM (compressed format)

--readFilesCommand zcat # Use 'zcat' to read gzip-compressed FASTQ files; skip this if files are uncompressed

--twopassMode Basic # Enable to detect novel splice junctions for more accurate alignment

--readFilesIn $r1 $r2 # Input RNA-seq read files; $r1 and $r2 represent paired-end FASTQ files

Then, the bam file was generated after discarding alignments with a MAPQ score less than 20. Below is the code: samtools view -bS -q 20 sample.sam | samtools sort --o sample.sorted.bam. samtools index -c sample.sorted.bam.

2.3 Generate read count files indicating the number of reads in genes by the htseq-count soft (Anders et al., 2015) as follows:

htseq-count -f bam input.sam gene.gtf -r pos -t gene -i ID -s no --max-reads-in-buffer 90000000 > input.count.txt; # gene.gtf is the file containing all genes information

Merge all the *count.txt to one fine (all.counts.xls) by Excel, including all the read counts of each sample.

2.4 Identification of differentially expressed genes across samples by using DEGseq2 (Love et al., 2014). The R codes are described as follows:

library(DESeq2)

countMatrix=read.delim(' all.counts.xls ', header=T, row.names=1,sep="\t")

group=read.delim('$group',header=T,sep="\t") #$group is the file indicating which sample is control and which is mutant or the sample be treated.

dds <- DESeqDataSetFromMatrix(countMatrix, colData=group, ~ Group)

dds <- dds[rowSums(counts(dds)) > 0,] #discard the genes undetectable

dds <- DESeq(dds)

res <- results(dds)

out=as.data.frame(cbind(res$log2FoldChange,res$pvalue,res$padj))

rownames(out)=row.names(res)

colnames(out)=c('log2_FoldChange','Pvalue','FDR')

diff <- dplyr::filter(out, abs(log2_FoldChange)>=log2(2) & FDR<=0.05) #FDR and FoldChange filtering

write.table(out,"DESeq2_AllResult.txt", sep = "\t", quote=F,row.names = T,col.names=T) # Output all the gene expressions

write.table(diff,"DESeq2_DEG.txt", sep = "\t", row.names = TRUE,quote=F) #only include the genes expressed significantly.

2.5 Perform GO analysis with our tailor-made application (Appendix 8). Here is the code: rna_seq_ref_only_gene_version2.pl --species wheat --pvalueCutoff 0.05 --file DESeq2_DEGlist.txt (generated by DESeq2) --db_build yes.

2.6 To calculate Transcripts Per Million (TPM) values from the raw count data, we employed a custom Perl script named 'counts2tpm.pl'. The script was executed as follows: counts2tpm.pl all.counts.xls $gff >tpm.txt #$gff-gene annotation file.

It is worth noting that I had integrated all the software required for steps from 2.1-2.6 into one application written by Perl. Here is the running code:

RnaSeqVersion2.pl \

--suffix1 R1.fq.gz --suffix2 R2.fq.gz \# all the samples required to be aligned.

--species wheat \ #indicate the species

--group group.txt \ #indicate which is the wild type plant and which is the mutant or be treated.

--FDR 0.05 \ # the modified p-value must be less than 0.05, but you can set others

--FC 2 #the expression changes compared to the wild-type plants, other values also can be used.

Note: this software can produce all the files mentioned in steps 2.1-2.6.

2.7 Clustering analysis was performed using the Mfuzz R package (Kumar and Futschik, 2007). The main code is described below:

Library (Mfuzz)

n_clust=8 #should indicate the number of gene expression trends

eset <- new("ExpressionSet", exprs = as.matrix(expr)) # expr contain all the TPM mean values at the three different stages.

```
eset <- filter.std (eset,min.std=0.0,visu = F) # discard the genes do not express significantly.
eset <- standardise(eset) # Make the Mean of Each Gene 0 and the Standard Deviation 1
cl <- mfuzz(eset, c = n_clust, m = mestimate(eset) )   # "m" as the Fuzzification Parameter
write.table(cl$membership, file='mfuzz.membership.txt',col.names = NA,quote=F,sep="\t")
write.table(cl$cluster,file='mfuzz.cluster.txt',col.names = F,quote=F,sep="\t")
# Output the image portraying
pdf('mfuzz.sep_final.pdf',h=4,w=8)
mfuzz.plot2(eset, cl, mfrow = c(2,4),
   xlab='Stage', #the name of X axis
   ylab='Expression changes', # the name of Y axis
   centre=T,       #draw the mean line
   time.labels=colnames(expr),
   colo='fancy'
)
dev.off()
```

2.8 Perform GO analysis of the genes belonging to the intersection between Cluster 2 and the 2-leaf stage using my custom application, rna_seq_ref_only_gene_version2.pl, mentioned above in step 2.5.

2.14. Protoplasmic preparation and transformation

The preparation of wheat protoplasts and PEG-mediated transformation were partially modified based on previously published methods (Bart et al., 2006). The reagents used are listed in Appendix 3. The specific steps are as follows:

(1) Using a sharp blade, cut the above-ground parts of wheat seedlings grown in a greenhouse into approximately 0.5-1 mm strips and place them in a 0.6 M mannitol culture medium for 15 minutes. After cutting all the material, transfer it to 20 mL of enzyme solution, wrap it in aluminum foil, and place it on a shaker at 23°C, 50 rpm for about 4 hours in the dark. In the last 2 minutes, increase the speed to 100 rpm.

(2) After enzymatic digestion, filter the cells through a 100-mesh sieve, then centrifuge at 150 g for 10 minutes to collect the protoplasts.

(3) Wash the cells twice with 10 mL of W5 solution, centrifuge at 150 g for 5 minutes to collect the protoplasts.

(4) Resuspend the protoplasts in an appropriate amount of MMG solution (200 μL per plasmid).

(5) Take the protoplasts resuspended in MMG from the previous step and add high-quality, endotoxin-free plasmid DNA (10 μg). Mix gently by tapping. Then, add an

equal volume of 40% (w/v) PEG4000 solution, mix gently by tapping, and let it lie down in the dark at 23°C for 15 minutes.

(6) Add 1.5 mL of W5 solution, gently mix the cells, centrifuge at 150 g for 3 minutes to collect the cells, and repeat this step once.

(7) Add 1.5 mL of W5 solution to resuspend the cells and culture in the dark in a 23°C incubator for 12-16 hours.

(8) Centrifuge at 150 g for 5 minutes to remove the upper W5 solution, gently tap the remaining liquid, and use for microscopic observation.

2.15. Subcellular localization assay

To investigate the subcellular localization of the LT1 protein, we performed an *in vitro* localization experiment using wheat protoplasts. We fused the C-terminal of LT1 from the Chinese Spring wheat landrace to GFP containing plasmid pBI121 (Appendix 7) and transformed the fusion construct LT1-GFP into wheat protoplasts via polyethylene glycol-mediated transfection as described in step 2.14. After incubating transformed protoplasts at 23°C for 12-16 hours, we visualized GFP fluorescence by confocal laser scanning microscopy (LSM 880, Carl Zeiss, Germany) to determine the intracellular localization of the LT1-GFP protein.

2.16. Phylogenetic analysis

All the protein sequences were obtained from Ensembl Plants (http://plants.ensembl.org/Triticum_aestivum/Tools/Blast) using the assigned protein as a query. Homologs that had more than 60% identity with the query protein were selected for analysis. After aligning the amino acid sequences using ClustalW with default parameters, the phylogenetic tree was constructed using MEGA 7.0 (Kumar et al., 2016) based on the neighbor-joining method, and the bootstrap values were estimated with 1,000 replicates.

2.17. Luciferase imaging assays

Luciferase (LUC) imaging assays were carried out as described previously (Zhang et al., 2022b) as follows:

The full-length LT1 fused with the untruncated LUC was cloned into the vector pCAMBIA1300-nLUC (Appendix 7). The positive control only containing LUC protein was cloned into pCAMBIA1300-nLUC. The *A. tumefaciens* strain GV3101 carrying different constructs was infiltrated into *N. benthamiana* leaves cultured either at 25℃ or 20℃ as follows:

1. Culture 1-3 positive *Agrobacterium* bacterial colonies in 5 mL LB medium. Add the corresponding antibiotics and rifampicin (final content 50 mg/L). Incubate at 28°C, 220 rpm, for about 16 hours.

2. Inoculate 3 mL of the *Agrobacterium* culture from step 1 into 20-50 mL of LB medium (containing plasmid resistance antibiotics, rifampicin, and AS). Grow until OD_{600} reaches approximately 1-1.5. For the empty vector control only containing the LUC gene and P19 plasmid (which promotes *Agrobacterium* cell transformation,

catalog: 68214, Addgene), more culture may be prepared as needed based on experimental requirements.

3. Mixing ratio of bacterial solutions: gene-LUC:P19 = 1:1. Inject 6 leaves when OD_{600} = 1.

4. Centrifuge the mixed bacterial solution at 3000 rpm/minute for 10 minutes to collect the bacterial cells.

5. Discard the supernatant and resuspend the pellet in 2.7 mL resuspension solution (Appendix 5). Let it stand at room temperature for 3 hours.

6. Inject an appropriate amount of resuspended solution into the back of tobacco leaves that have been growing for 30-40 days. Note: Dry the leaves for 7 days before injection to facilitate the process. After injection, water the plants and place them in a humid environment for 2 days, then treat them with low light for one day.

7. Pick the leaves, apply the LUC substrate D-luciferin (3‰ w/v.) in the back of the leaves, and take photos for observation after 6 minutes by using the CCD imaging apparatus (Tanon 5200 luminous imaging system, China) to capture the LUC signal. At least six independent leaves are used for each experiment, and at least three biological replicates generate similar results.

2.18. Yeast-two hybrid screening

A DUALmembrane Y2H screening was carried out to identify the proteins that interact with LT1, using the cDNA library from the shoot bases at the seedling stage where tillers are formed and grow out or dormant. LT1 was cloned into a pBT3-SUC vector to generate the bait plasmid LT1-pBT3-SUC (Appendix 7). The cDNA library was cloned into the pPR3-N vector to generate the prey plasmids. All of the cDNA library plasmids were transformed into the yeast strain NMY51, together with the LT1-pBT3-SUC plasmid, using a yeast transformation system. Then, the transformants were selected for growth on a synthetic defined (SD) medium lacking histidine (His), leucine (Leu), tryptophan (Trp), and adenine (Ade) (Clontech). Positive colonies were processed for sequencing to identify each bait-prey pair.

For every bait-prey pair identified by sequencing, pairwise Y2H testing was performed to ensure high reproducibility. The bait plasmid LT1-pBT3-SUC and prey plasmids were co-transformed into the yeast strain NMY51. The blue-positive colonies that were grown on Quadruple Dropout Medium (QDO) plates were supplemented with xgal, indicating interaction pairs. In pairwise Y2H testing, the pair of pTSU2-APP and pNubG-Fe65 was used as a positive control, and the combinations of the vector pPR3-N with LT1-pBT3-SUC and pTSU2-APP were included as negative controls. The reagents used in these experiments are listed in Appendix 6. The detailed steps are provided as follows:

1. Detection of autoactivation and toxicity of bait genes

Using the combination of the plasmid pTSU2-APP control vector and pPR3-N control vector as a negative control and the combination of the plasmid pTSU2-APP control vector and the pNubG-Fe65 vector as the positive interaction controls.

1. Streak NMY51 yeast cells on one YPDAplate and incubate at 30°C upside down for approximately 3 days until colonies reach 2-3 mm in size.

2. Pick several yeast colonies into one bottle containing 50 mL YPDA medium and incubate at 30°C with shaking at 250 rpm/min overnight until OD_{546} = 0.6-0.8.

3. Centrifuge at 2500g for 5 minutes to collect the cells.

4. Discard the supernatant and resuspend the pellet in 2.5 mL sterile water.

5. Prepare the PEG/LiOAc master mix as follows:

Components	Volume
50% PEG4000	2.4 mL
1 M/L LiOAc	360 μL
Single-stranded carrier DNA (10 μg/μL)	250 μL

6. Prepare reaction mixtures according to the table below:

Combinations	Plasmid 1 (each 1.5μg)	Plasmid 2 (each 1.5μg)
1	LT1-pBT3-SUC	pOst1-Nubl
2	LT1-pBT3-SUC	pPR3-N
3	pTSU2-APP	pNubl-Fe65
4	pTSU2-APP	pPR3-N

7. Add 300 μL PEG/LiOAc mix to each tube and gently vortex to mix.

8. Add 100 μL resuspended cells to each tube and vortex for 1 minute to ensure thorough mixing.

9. Incubate at 42°C for 45 minutes in a water bath.

10. Centrifuge at 700g for 5 minutes.

11. Discard the supernatant, resuspend the cells in 150 μL 0.9% NaCl, and plate as per the table below on 100 mm Petri dishes:

Combinations	SD/-Trp/-Leu	SD/-Trp/-Leu/-His	SD/-Trp/-Leu/-His/-Ade
1	50 μL	50 μL	50 μL
2	50 μL	50 μL	50 μL
3	50 μL	50 μL	50 μL
4	50 μL	50 μL	50 μL

12. Seal the plates with parafilm and incubate at 30°C for 4 days.

13. Calculate the colony growth rate on the selective medium:

%growth under selection = number of colonies on selective plate × 100/number of colonies on non-selective plate.

Notes:

1. After 4 days at 30°C, 100-1000 colonies should appear on all SD/-Trp/-Leu plates.

2. If the bait protein is expressed correctly, the growth rate in combination 1 on SD/-Trp/-Leu/-His and SD/-Trp/-Leu/-His/-Ade should reach 10%-100%.

3. In the absence of autoactivation, reaction 2 should show no significant growth on selective media.

4. If autoactivation occurs, transform the empty plasmid pPR3-N into yeast expressing the bait plasmid and streak on SD/-Trp/-Leu/-His/-Ade plates containing different 3-AT concentrations. The maximum working concentration of 3-AT can reach 100 mM/L.

2. Yeast two-hybrid library screening

1. Streak NMY51 yeast expressing validated bait protein on the SD/-Leu plate and incubate at 30°C upside down for approximately 3 days until colonies reach 2-3 mm in size.

2. Transfer fresh yeast colonies expressing bait protein into the 10 mL SD/-Leu liquid and shake at 30°C, 220 rpm/L for 8 hours.

3. Inoculate into 100 mL SD/-Leu liquid medium and shake at 30°C, 220 rpm/L overnight.

4. Collect 3 mL of overnight culture, centrifuge at 2500g for 5 minutes, and resuspend the pellet in 3 mL water. Use water as a blank control to measure OD_{546}.

5. Use an amount of yeast equivalent to 30 × OD_{546} and centrifuge at 700g for 5 minutes.

6. Resuspend the pellet in 10 mL pre-warmed 2× YPDA medium at 30°C and transfer to a 1L flask.

7. Wash the centrifuge tubes with 40 mL pre-warmed 2× YPDA, and transfer to the same flask.

8. Add 150 mL pre-warmed 2× YPDA and adjust the OD_{546} to 0.15.

9. Shake at 30°C, 220 rpm/min until OD_{546} = 0.6-0.7.

10. Prepare single-stranded carrier DNA, denature at 99°C for 5 minutes, then cool on ice for 2 minutes. Repeat once.

11. Prepare the LiOAc/TE and PEG/LiOAc master mixes as follows:

LiOAC/TE components	Volume	PEG/LiOAc components	Volume
1 M/L	1.1 mL	50% PEG	12.0 mL
10× TE (pH = 7.5)	1.1 mL	1 M/L LiOAc	1.5 mL
Water	7.8 mL	10× TE (pH = 7.5)	1.5 mL

12. Aliquot 200 mL culture into four 50 mL centrifuge tubes and centrifuge at 700g for 5 minutes.

13. Discard the supernatant, resuspend each pellet in 30 mL sterile water, and centrifuge at 700g for 5 minutes.

14. Discard the supernatant and resuspend each pellet in 1 mL LiOAc/TE master mix, then transfer to 1.5 mL tubes. Centrifuge at 700g for 5 minutes.

15. Add 600 μL LiOAc/TE master mix to each tube, vortex, and set aside.

16. Prepare 4 tubes of 15 mL centrifuge tubes and construct the reaction according to the following table:

Components	Volume
Single-strand carrier DNA	100 μL
Competent cells	600 μL
PEG/LiOAc master mix	2.5 mL

Library plasmids	7 μg

17. Vortex for 1 minute to mix thoroughly.
18. Incubate at 30°C for 45 minutes, mixing gently every 15 minutes.
19. Add 160 μL DMSO and mix gently.
20. Heat shock at 42°C for 20 minutes, mixing gently every 10 minutes.
21. Centrifuge at 700g for 5 minutes, collect the cells.
22. Resuspend in 3 mL 2× YPDA and shake at 30°C, 150 rpm/min for 90 minutes.
23. Centrifuge at 700g for 5 minutes, collect the cells.
24. Resuspend in 0.9% NaCl to a final volume of 4.8 mL.
25. Plate 200 μL per plate on X-gal plates (20 mg/L), spreading approximately 16 plates per tube.
26. Incubate at 30°C for 3-5 days. Positive clones will appear blue.
27. Pick the validated positive clones and validate with the following steps:

Extract plasmids from positive clones, co-transform with the bait plasmid into NMY51 yeast, and incubate at 30°C for 4 days. If positive clones appear, it confirms interactions between the prey and bait proteins.

2.19. Overexpression of *LT1*

Amplify the full-length CDS of *LT1* from whole seedling cDNA using KOD-FX-NEO (Takara, KFX-101) to generate blunt-ended products. Insert the *LT1* CDS into the pBM27 vector (Biomed, pBM27 Toposmart cloning kit) through a ligation reaction at 25°C for 15 minutes, using 50 ng DNA, 1 μL pBM27 vector, 1 μL 10× Toposmart buffer, and water to a total volume of 10 μL. Transfer the full *LT1* contained in the pBM27 vector to PC186 via LR reaction at 25°C for 1 hour, combining 3 μL pBM27 vector with *LT1*, 1 μL PC186 vector, TE buffer (pH 8.0) to 8 μL, and 2 μL LR Clonase II (Invitrogen, Cat. No.: 11791120). Finally, transfer the resulting *LT1*-containing plasmid to GV3101 *Agrobacterium* competent cells. For detailed information on the PC186 vector, refer to the appendix 7.

2.20. Determination of plant hormone content

Collect shoot base material 1 mg from wild-type and mutant seedlings at the 2-leaf, 3-leaf, and 4-leaf stages. Quickly freeze the samples in liquid nitrogen. After thorough grinding, the samples will be submitted to the hormone platform at the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, for the determination of multiple endogenous plant hormones using liquid chromatography-tandem mass spectrometry (LC-MS/MS) determination. This includes the measurement of auxin (Indole-3-Acetic Acid, IAA) and CK**s**. For each sample, five technical replicates will be used. The IAA content determination steps, referred from Di, 2021 (Di et al., 2021), are described as follows:

Plant material (200 mg, fresh weight) was ground to a fine powder in liquid nitrogen and extracted with 80% MeOH containing internal standards ($^2H_2$-IAA) at -20 ºC for 16 hours. After centrifugation at 15,000×g for 15 min, the supernatant was collected

and dried by evaporation. The residue was reconstituted using 5% NH_4OH and loaded onto an Oasis MAX (Waters) cartridge. The cartridge was sequentially washed with 5% NH_4OH, water, and MeOH, and then eluted with 5% FA in MeOH. The eluate was evaporated and re-dissolved in 80% MeOH for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. LC-MS/MS analysis was performed using a UPLC system (Waters) coupled to a 6500 QTrap system (AB SCIEX). LC separation used a BEH C18 column (1.7 mm, 2.1×100 mm; Waters) with mobile phase 0.1% formic acid (a) and ACN (b). The gradient was set with an initial 2% B and increased to 50% B after 6 min. IAA was detected in MRM mode with transitions set to 176/130, 291/130, 289/130, and 305/130, respectively.

The CK content determination steps, referred from Du, 2017 (Du et al., 2017), are described as follows:

Fresh plant tissues were frozen in liquid nitrogen and homogenized to fine powder. Around 400 mg of ground powder was extracted for 24 h in extraction solvent (methanol/water/formic: 15/4/1, v/v/v) with the internal standards of [2H5]tZ, [2H5]tZR, [2H5]DHZ, [2H6]iP, [2H6]iPR (400 pg, OlChemIm) added. The supernatant was centrifuged for 15 min at 15 000 g and dried with nitrogen stream, and then dissolved in 2 ml of formic acid (2 mol l^{-1}). Crude extracts were further purified by loading onto the Oasis MCX cartridge (500 mg 6^{-1} ml; Waters, Milford, MA, USA) preconditioned with 4 ml of methanol, water and formic acid (2 mol l^{-1}). The cartridge was sequentially washed with formic acid (1 mol l^{-1}), formic acid (0.5 mol l^{-1}), formic acid (0.5 mol l^{-1}) in 60% methanol, water, 5% methanol, ammonia solution (0.5 mol l^{-1}) in 5% methanol, ammonia (0.4 mol l^{-1}), formic acid (2 mol l^{-1}) and methanol. Cytokinins were eluted with 4 ml of 5% ammonia in methanol and dried with nitrogen gas. Dried elution was dissolved in 50% methanol for analysis on a LC–tandem MS system consisting of an Acquity UPLC (Waters) and Qtrap 5500 system (AB Sciex, Shinagawa-ku, Tokyo, Japan) equipped with Electron Spray Ionization source.

The separation was achieved on an Acquity UPLCTM BEHC18 column (100 mm × 2.1 mm, 1.7 μM; Waters) with the column temperature set at 30°C and the flow rate at 0.5 ml min^{-1}. The 8.5 min linear gradient runs from 98% to 75% A (solvent A, 0.05% acetic acid in water; solvent B, 0.05% acetic acid in acetonitrile) in 5.0 min, 75–20% A in the next 0.5 min and is returned to the initial condition for 3 min for equilibration. Cytokinins were detected in positive MRM mode, and the source parameters were set as follows: ion spray voltage, 5300 V; desolvation temperature, 600°C; nebulizing gas 1, 45; desolvation gas 2, 60; and curtain gas,30. The MRM transitions for cytokinins were as follows: 220.1 > 136.0 (tZ), 352.2 > 220.1 (tZR), 222.1 > 136.0 (DHZ), 204.1 > 136.0 (iP), and 336.1 > 204.1 (iPR).

2.21. Wheat vernalization treatment

The vernalization process for wheat is conducted as follows: first, the wheat seeds are soaked in water for 4 hours and then placed on 9 cm Petri dishes lined with several layers of filter paper, which are kept moist, and then the seeds are spread on top for germination until the seeds begin to sprout. Once the seeds have germinated and the

roots are visible, they are transferred to a growth chamber (NCERA-101, Greenfuture, China) for vernalization. The wheat plants are grown in the growth chamber under controlled conditions at 4°C (day/night), a 16-hour photoperiod, and approximately 300 μmol m^{-2} s^{-1} photosynthetically active radiation. The vernalization treatment is maintained for at least one month. After vernalization, the seedlings are transferred to either the greenhouse or the field for further growth.

3. Results

3.1. Phenotypes of the wheat tillering mutant *lt1*

The wheat variety C6878 has successfully passed both national and Shanxi provincial evaluations in China. In various regional trials, it demonstrated significant yield increases ranging from 15.66% to 29.58% compared to control varieties. C6878 is characterized by high and stable yields, as well as strong resistance to environmental stresses.

Building on these advantageous traits, our research aimed to further improve C6878's grain yield potential. We employed EMS mutagenesis to generate a population of mutants with diverse phenotypes. Our study specifically focused on precise control of tillering as a strategy to enhance grain yield in this variety. The *lt1* mutant exhibits reduced tillering, typically producing four tillers compared to eighteen of C6878 at the heading stage (Figure 15A-B). Additional pleiotropic defects in *lt1*, including decreased stature, short roots, chlorotic leaves, and wrinkled seeds, are concomitant (Figure 15G-I). To explain whether the reduced tillers are due to defects in bud initiation or bud elongation, we observed the dynamic development process of tiller buds. At first, we found that the number of AMs remained consistent during the coleoptile and 2-leaf stages but started to diverge by the 4-leaf stage, with four in the wild type and two in *lt1* (Figure 15C-D). This revealed that the reduced tillering of *lt1* is partially due to the defective AM initiation. Furthermore, we examined the number of ceased lateral buds at the heading stage. A higher ratio of ceased lateral buds (Ratio = Ceased buds/all buds) in *lt1* compared to C6878 indicated that the tiller buds were negatively regulated during tiller bud outgrowth stages in *lt1*(Figure 15E-F). Taken together, the tillering defect of *lt1* appears attributable to both its reduced AM formation and bud outgrowth.

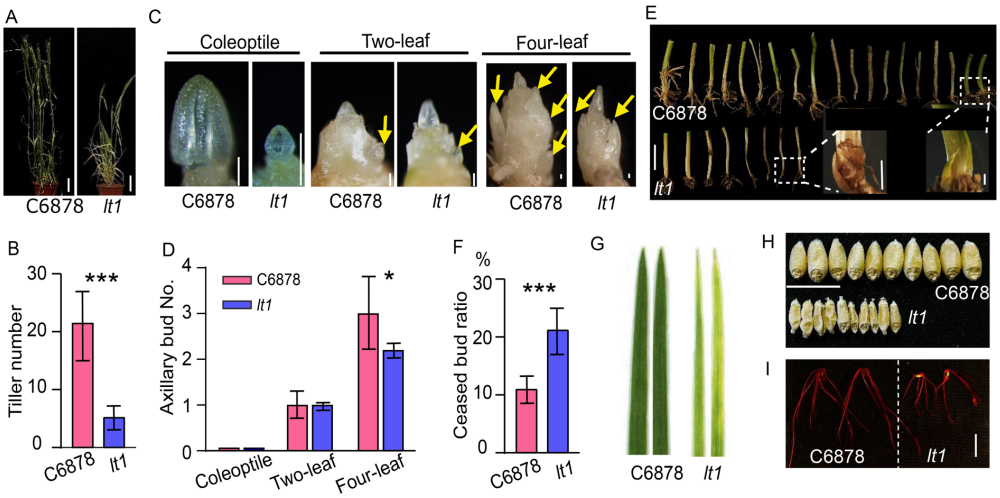

Figure 15. Phenotypes of *lt1*.(A) The tiller number of *lt1* is fewer than the wild-type plant C6878. Bar = 15 cm. (B) The statistical values representing the tiller number of *lt1* and C6878. Values are means ± SD (n = 10). *** $P < 0.001$, Student's t-test. (C) The dynamic observation of AM formation between *lt1* and C6878, namely, coleoptile, two- and four-leaf stages. The yellow arrow indicates the bud primordium. Bar = 200 μm. (D) Axillary bud number of *lt1* and C6878 at each stage. Values are means ± SD (n = 5). * $P < 0.05$, Student's t-test. (E) The tillers fully extended and ceased after the heading stage. Bar = 2 mm. The ceased buds are closed up in the white box. Bar = 1 cm. (F) The ceased bud ratios are represented by bar plots. Values are means ± SD (n = 4). ** $P < 0.01$, Student's t-test. (G)-(I) The chlorotic leaves, wrinkled seeds, and short root length in the *lt1* mutant, compared to the C6878. Bars = 1 cm in H and I, respectively.

3.2. Isolation of *LT1* by an upgraded bulked segregant method, uni-BSA

We backcrossed *lt1* to its parental line C6878 and performed self-crossing to generate the F_2 segregating population. All the F_1 individuals showed comparable tiller numbers as C6878, indicating that *lt1* has a recessive mutation. The low-tillering plants (one to four tillers) compared to the high-tillering plants (over 15 tillers) in the F_2 generation fit the Mendelian of 1:3 ratio (205 low-tillering plants:710 multi-tillering plants, $\chi^2 = 1.6367$, $p = 0.2008$). These results suggest that a recessive single gene controls the *lt1* mutant. Wheat's allohexaploid nature presents an interesting context for *LT1* functioning as a recessive gene controlling tillering. While such genes typically have two homologous copies across wheat's three genomes, our findings show clear recessive inheritance. This pattern, where a mutation in one copy leads to a phenotype despite potential functional compensation from homologs, has precedent in wheat tillering genes. For example, *TN1* shows classic 3:1 segregation between plants with fewer and more tillers (Dong et al., 2023), while *TIN* demonstrates a 1:3 ratio of low-tillering to high-tillering lines (Spielmeyer and Richards, 2004; Hyles et

al., 2017). These cases illustrate that despite wheat's genomic complexity, individual genes can exhibit clear Mendelian inheritance.

To expedite the cloning of *LT1*, we utilized the BSA-based method uni-BSA, using the sequence data from WES, making it cost-friendly and effective. Firstly, the WES data was used to minimize the genome size without the penalty of losing protein-encoding genes while guaranteeing enough SNPs to carry out linkage analysis. Secondly, to address the ambiguous mapping when alignment is performed due to the high duplication proportion of the wheat genome, which may result in aligning one read to multiple loci, we tailor-make a Perl script called Filter.ambi.pl integrated into the uni-BSA protocol (Figure 14). The principle of our filtering algorithm is based on the fact that one DNA fragment generates two sequences: a forward sequence and a reverse sequence. If either of these sequences aligns to one position, its mate should be nearby, regardless of whether it aligns to multiple sites (Figure 13). Discarding sequences with uniquely aligned mate reads, as done in strict filtering methods, is inefficient. Our algorithm, Filter.ambi.pl, addresses this by retaining such sequences, resulting in broader genome coverage (Figure 17A) in comparison to the strict filtering method. Accordingly, this filtering method retained 61% of total reads, compared to 48% when discarding all ambiguous reads (Figure 17B). The average percentage of each gene coverage was over 81%, with the majority of genes covered at 100% (Figure 17D). The average coding sequencing depth reached 70X (Figure 17C). Notably, this algorithm produces more accurate variants compared to the no-filtering method (Table 7). For example, a mutation site (C-T) identified by the no-filtering method is heterozygous. Collectively, the filtering algorithm of uni-BSA is powerful and efficient.

Uni-BSA application narrowed *LT1* to a 6 Mb region on the short arm of chromosome 2D (Figure 16B-C), compared to 8 Mb without ambiguous read filtering (Figure 16D). This interval contains 140 genes, of which 65 genes have variations, including SNPs and Indels. We excluded genes with heterozygous variants in the fewest-tillering pooled DNA samples, remaining 26 genes. Since EMS predominantly causes SNPs rather than Indels, we excluded 14 Indels, thereby eliminating 9 genes. We also excluded 12 genes containing 28 SNPs in *lt1* that matched the reference Chinese Spring, which exhibits normal tillering. Ultimately, five genes were identified as candidate genes (Table 6). Interestingly, one gene, TraesCS2D03G0082100, encoding a nucleotide-binding shared by APAF-1, R proteins, and CED-4 （NB-ARC） domain protein (Figure 18C), harbors an SNP mutation in the 793rd base (C-T), causing a premature of this gene in *lt1* (Figure 18C), while the other four genes had UTR mutations (Table 6). In addition, the individuals of the F_2 population with this homozygous mutation (Figure 18B) show *lt1* phenotypes. Further, TraesCS2D03G0082100 was not expressed in 2-leaf, 3-leaf, and 4-leaf stages of *lt1*, compared to the wildtype (Figure 18D). Of note, the gain-of-function of the *UNI* gene, which also shares the NB-ARC domain, of *Arabidopsis* can generate more axillary meristems (Figure 19), which gives a hint that this *TraesCS2D03G0082100* is the candidate gene. The amino acid similarity between *UNI* and *LT1* is only 13.11%. However, this low similarity does not preclude the possibility that *UNI* and *LT1* are

functionally related. Similar phenomena exist for other genes across species. For instance, the *LAZY1* gene, which regulates branching angles in rice (Li et al., 2007) and *Arabidopsis* (Yoshihara et al., 2013), shares only 14.22% identity between these species. Taken together, we initially considered *TraesCS2D03G0082100* the likely causal *LT1* gene, given its severe mutation, undetectable expression, and phenotypes of its homolog in *Arabidopsis*.

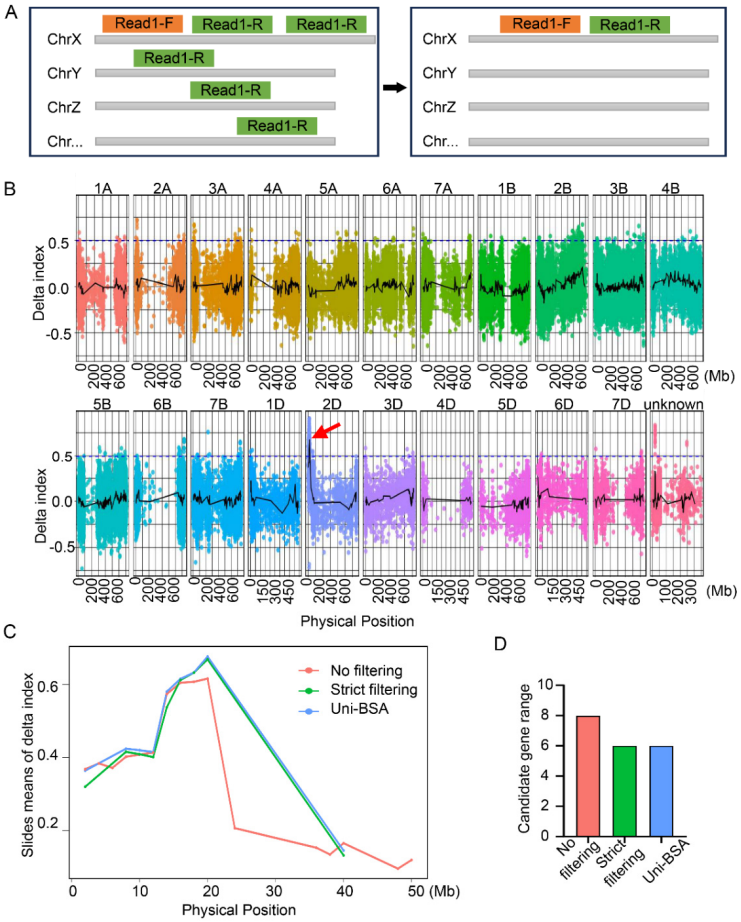

Figure 16. Features of uni-BSA. (A) The strategy of uni-BSA to exclude ambiguous reads. The Left is the original mapping result, and the right is the ideal effect after filtering. (B) Linkage analysis of *LT1* indicates that it is located on the short arm of chromosome 2D. The means of δ indexes in the 2M range are indicated by black lines. The points with distinct colors represent δ indexes of each chromosome. (C) The uni-BSA method is more sensitive in detecting and determining the linkage interval. Namely, its resulting mean δ indexes are bigger than other methods. (D) Candidate gene intervals of each method. Uni-BSA and strict filtering have smaller intervals than the No filtering method.

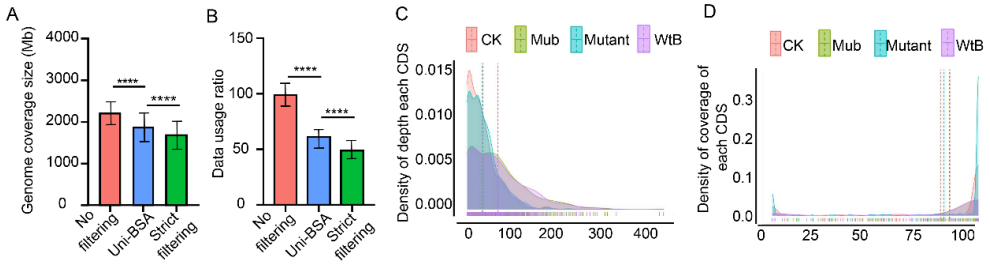

Figure 17. Comparisons of different filtering algorithms. (A) The genome coverage calculation comparison. The uni-BSA algorithm covers larger areas than that of the strict filtering method and smaller than that of No filtering. Values are means ± SD (n = 4). **** P < 0.001, Student's t-test. (B) Data usage ratio comparisons between different methods. Uni-BSA has the medium data usage ratio among the three methods. Values are means ± SD (n = 3). **** P < 0.001, Student's t-test. (C) Histograms indicate the density of mapping depth of each CDS from 4 samples: Control (CK), Mub (individuals from F_2 with mutant phenotypes), Mutant, and Wtb (individuals from F2 with wild-type phenotypes). The mean depth is more than 70X. (D) Histograms indicate the density of coverage of each CDS from 4 samples: CK, Mub, Mutant, and Wtb. The mean coverage is more than 81%.

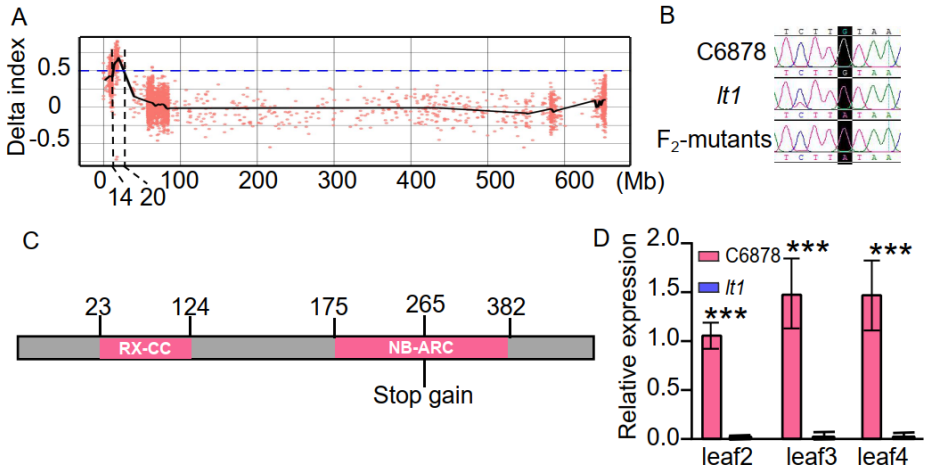

Figure 18. Cloning of *LT1*. (A) The linkage analysis indicates that *LT1* is framed in a 6 Mb interval on the short arm of the chromosome 2D between 14-16 Mb. (B) Mutation was validified by Sanger sequencing. The nucleotide change is shaded. (C) The schematic diagram of *LT1*. This protein contains a RX-CC (N-terminal coiled-coil) domain, and NB-ARC (nucleotide-binding domain) domain. The stop gain position is 265th amino acid. (D) Relative expression of *LT1* at three stages of *lt1* and the C6878. Values are means ± SD (n = 3). *** P < 0.001, Student's t-test.

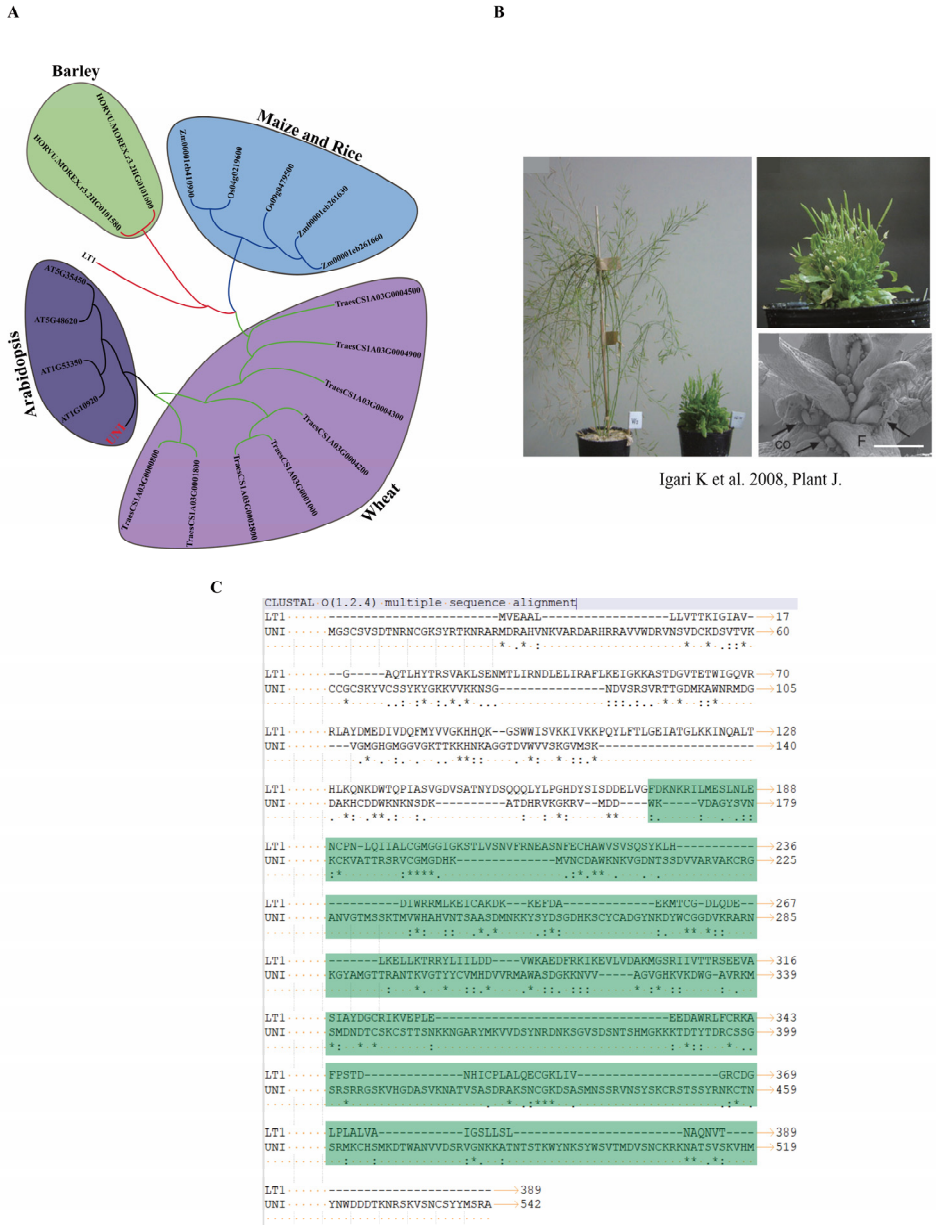

Figure 19. Phylogenetic tree analysis indicates LT1's involvement in tillering. (A) Phylogenetic tree analyzing homologs of LT1 of barley, maize, rice, and wheat. Of Note, the gain-of-function of *Arabidopsis* UNI shows more AMs depicted in B (Igari et al., 2008). (C) Multiple sequence alignment of LT1 and UNI shows that they all share the NB-ARC domain. The NB-ARC is shaded by green.

Table 6. Genes in the candidate interval

Gene number	Annotation	Mutation effect
TraesCS2D03G0080000	unknown function	UTR5
TraesCS2D03G0080400	Belonging to the GST superfamily	UTR3
TraesCS2D03G0080800	Ortholog to *Arabidopsis* Li-tolerant lipase 1	UTR5
TraesCS2D03G0082600	Disease resistance protein RPM1	UTR3
TraesCS2D03G0082100	Disease resistance protein RPM1	Stop gain

3.3. Verification of *LT1*

To validate TraesCS2D03G0082100 as the *LT1* gene regulating tillering in wheat, we used CRISPR/Cas9 to create knock-out mutants in the wheat cultivar Fielder. The three independent edited lines with different mutations within its coding sequences were obtained (Figure 20A). The *LT1-CR1* and *LT1-CR2* show the mutations at gRNA targeted sites, and *LT1-CR3* has 239 bp deletion (Figure 20A). Intriguingly, all three edited homozygous individuals produced fewer tillers than the wild type in both field and greenhouse experiments and reduced expression level of *LT1* (Figure 20B-E). Moreover, these three lines exhibit other defects of *lt1*, like yellow leaves (Figure 20B, D), thus confirming TraesCS2D03G0082100 as the *LT1* locus.

To elucidate the possible reasons for pleiotropic phenotypes of the *lt1* mutant, we assessed the expression levels of *LT1* in various tissues. qPCR analysis revealed ubiquitous expression of *LT1* (Figure 20G). Given its expression in leaves, it is not strange that *lt1* has yellow leaves once *LT1* is disrupted. *LT1* was detectable in tiller buds, implying its role in tillering (Figure 20G). The broad expression pattern of *LT1*

suggests its multiple roles in wheat development. Overall, these data indicate that *LT1* likely influences tillering and other developmental processes directly or indirectly.

To determine the sublocation of LT1, we carried out a transient expression experiment of LT1 in wheat protoplasts. In contrast with the control, which is ubiquitous in protoplast cells, the LT1-GFP fusion protein was predominantly localized in chloroplasts (Figure 20F). The chloroplast location of LT1 implies that LT1 may operate nutrition production, like sucrose, to control tillering.

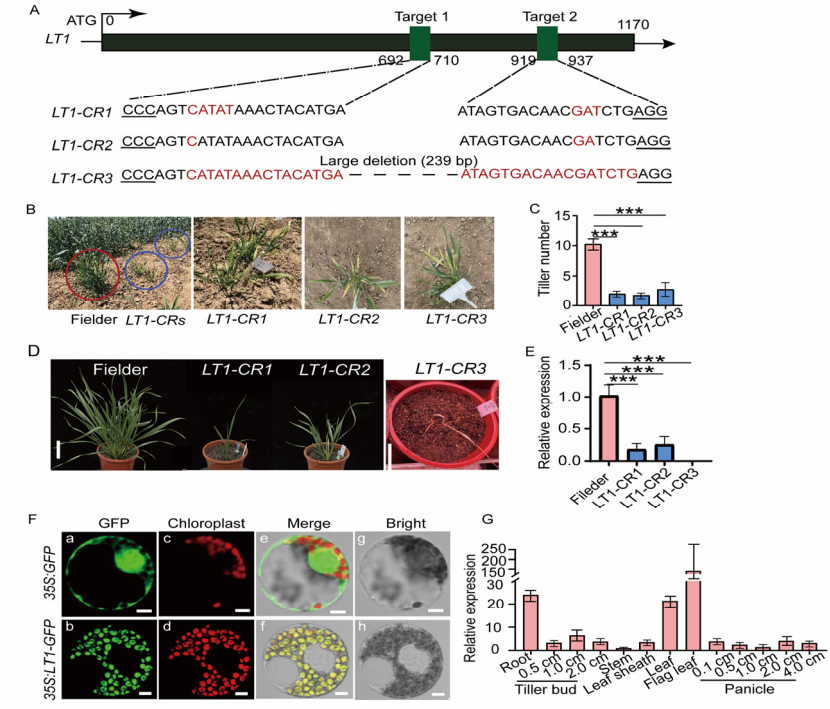

Figure 20. Verification of *LT1*. (A) Schematic diagrams indicating targets of CRISPR/Cas9 (*LT1-CRs*) and the mutations in each line. PAM sites are depicted with underlines. Deletions are indicated with words in red color, and *LT1-CR3* had a 293 deletion. (B) Phenotypes of *LT1-CRs.* All the *LT1-CRs* show *lt1* phenotypes, including yellow leaves and fewer tillers in the field conditions. The wild-type plants circled by red are Fielder. The *LT1-CRs* are boxed by blue circles and closed up in the right pictures. (C) Tiller number comparison between *LT1-CRs* and the wild type. Values are means ± SD (n = 3). *** P < 0.001, Student's t-test. (D) Phenotypes of LT1-CRs in the cabinet. Progenies of *LT-CR1* and *LT-CR2* grown in the growth cabinet at the seedling stage show fewer tillers. Progenies of *LT-CR3* are lethal in seedlings. Bar = 7.5 cm. (E) Relative expression levels of *LT1* in *LT1-CRs* and Fielder. The values are relative to *ACTIN*. Values are mean±SD (n= 4). *** P< 0.001, Student's t-test. (F) Sublocation of LT1 using wheat protoplasts. Scale bars correspond to 10 μm. (G) Relative expression levels of LT1 in various tissues. The values are relative to ACTIN. Values are mean± SD (n=3).

3.4. The regulatory pathways of *LT1* in tillering development

3.4.1. *LT1* control lateral bud formation by targeting *TaROX/TaLAX1* directly or indirectly

To further investigate modular relationships involving *LT1*, we conducted a co-expression analysis using TPM values from shoot base tissues at three developmental stages: the 2-leaf, 3-leaf, and 4-leaf stages. An initial survey of these RNA-seq datasets revealed that samples belonging to each group clustered well (Figure 21). By analyzing the TPM values of C6878, the transcripts were grouped into eight clusters, each representing distinct gene expression trends (Figure 22A). *LT1* expression, which belongs to cluster five, was highest at the 2-leaf stage and then decreased at the 3- and 4-leaf stages. We considered the 2-leaf stage to be crucial for AM initiation, as genes active at this stage showed a peak in expression, followed by a decrease in the subsequent stages. Therefore, we performed GO analysis on genes within the overlap between cluster five and the 2-leaf stage (Figure 22B), which showed significant expression changes between *lt1* and C6878 (absolute log2FoldChange >1, FDR<=0.05). This analysis revealed perturbations in various pathways in *lt1*. Notably, in this stage, various pathways (Figure 22C) related to AM formation shared the locus TraesCS3B02G383000, an ortholog of *Arabidopsis ROX* and *LAX1* in rice that regulate AM formation. These pathways include “morphogenesis of a branching structure”, “secondary shoot formation”, and “shoot axis formation”. Moreover, TraesCS3B02G383000, namely *Ta3BLAX1*, is undetectable in *lt1* (Figure 22D). This is consistent with our previous observation of significant differences in tiller numbers at the 4-leaf stage in *lt1* mutants (Figure 15C). Together, *LT1* might regulate AM initiation by affecting *TaROX/TaLAX1* directly or indirectly.

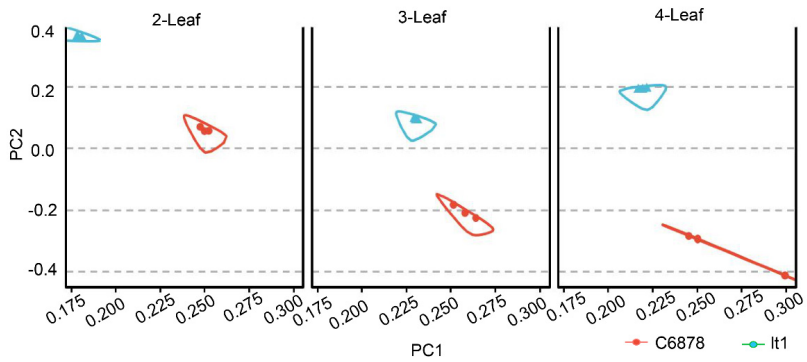

Figure 21. Principal Component Analysis (PCA) of individuals belonging to different growth stages. Different colors circle two obvious subpopulations of points. The values on the X and Y axes represent PC1 and PC2, respectively.

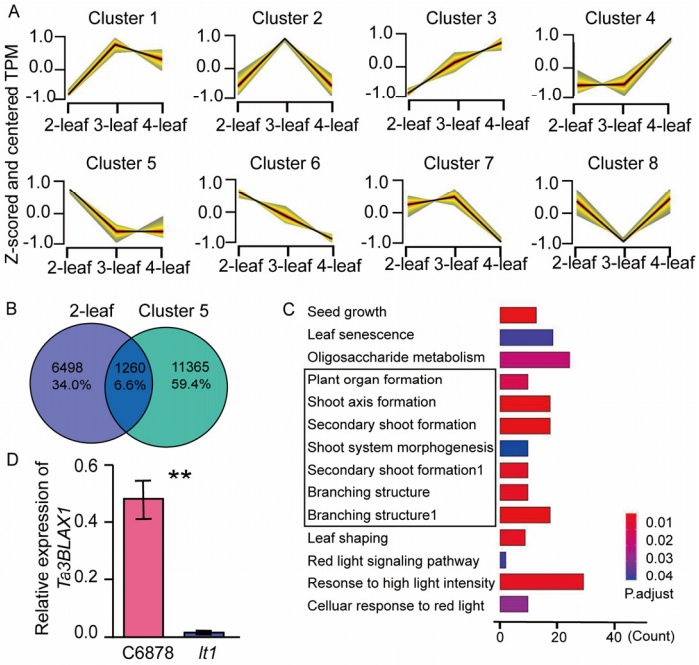

Figure 22. Co-expression analysis of DEGs in C6878 and *lt1*. (A) Eight clusters of all the gene expression are grouped distinctly. Cluster 5 contains *LT1*. (B) Venn diagram showing the proportion of overlapped genes between the 2-leaf stage (significantly changed) and cluster 5. (C) Go analysis of the genes belonging to the intersection between the 2-leaf stage and cluster 5 containing *LT1*. Pathways sharing *TaLAX1* are framed by the black box. P. adjust values indicating significance are colored gradually from blue to red. (D) Expression levels of *Ta3BLAX1*. Values are mean ± SD (n = 4). ** $P < 0.01$, Student's t-test.

3.4.2 Auxin and CKs are involved in tiller development in *lt1*

We performed GO analysis on the genes shared between the three developmental stages and cluster 5, respectively. The results revealed perturbation in several phytohormone-related pathways, including auxin, salicylic acid, and jasmonic acid (Figure 23A). Among these, the indole-containing compound biosynthesis process, through which auxin is biosynthesized, was enriched at all three developmental stages. For example, *TrpA* family genes *Ta5BTrpA* and *Ta5DTrpA* exhibited significant upregulation in *lt1* (Figure 23B), suggesting higher auxin levels may inhibit tillering. Indeed, the active auxin content of indole-3-acetic acid (IAA) is increased in *lt1* (Figure 24A), though no significant differences were observed at the 2-leaf stage due to low expression of IAA biosynthesis genes (Figure 23B). Collectively, these data indicate that *LT1* can affect auxin in regulating wheat tillering.

Auxin and CK antagonistically regulate tillering (Yuan et al., 2023b). In addition to auxin perturbation, CK levels appeared to decrease due to the upregulation of *TaCKX5* (cytokinin dehydrogenase 5) genes (*Ta3ACKX5*, *Ta3BCKX5*, and *Ta3DCKX5*) mediating CK degradation (Figure 23B). These CKX5 genes were also enriched in pathways related to secondary shoot formation (Bartrina et al., 2011), implying CK metabolism importantly regulates *LT1*-controlled tillering. Indeed, levels of active CKs like trans-zeatin riboside (tZR), trans-zeatin (tZ), and isopentenyladenine (iP) declined at the 2-leaf stage critical for tiller bud initiation (Figure 21B-D) but increased at the 4-leaf stage (Figure 24B-E), suggesting feedback regulation. Levels of inactive/precursor CKs like isopentenyladenosine riboside (iPR), trans-zeatin-9-glucoside (tZ9G), isopentenyladenine-9-glucoside (iP9G), cis-zeatin riboside (cZR) and trans-zeatin-O-glucoside (tZOG) also decreased at the 2-leaf stage (Figure 24 F-J), consistent with their active counterparts. Collectively, *LT1* appears to regulate tillering through perturbations in auxin and cytokinin pathways.

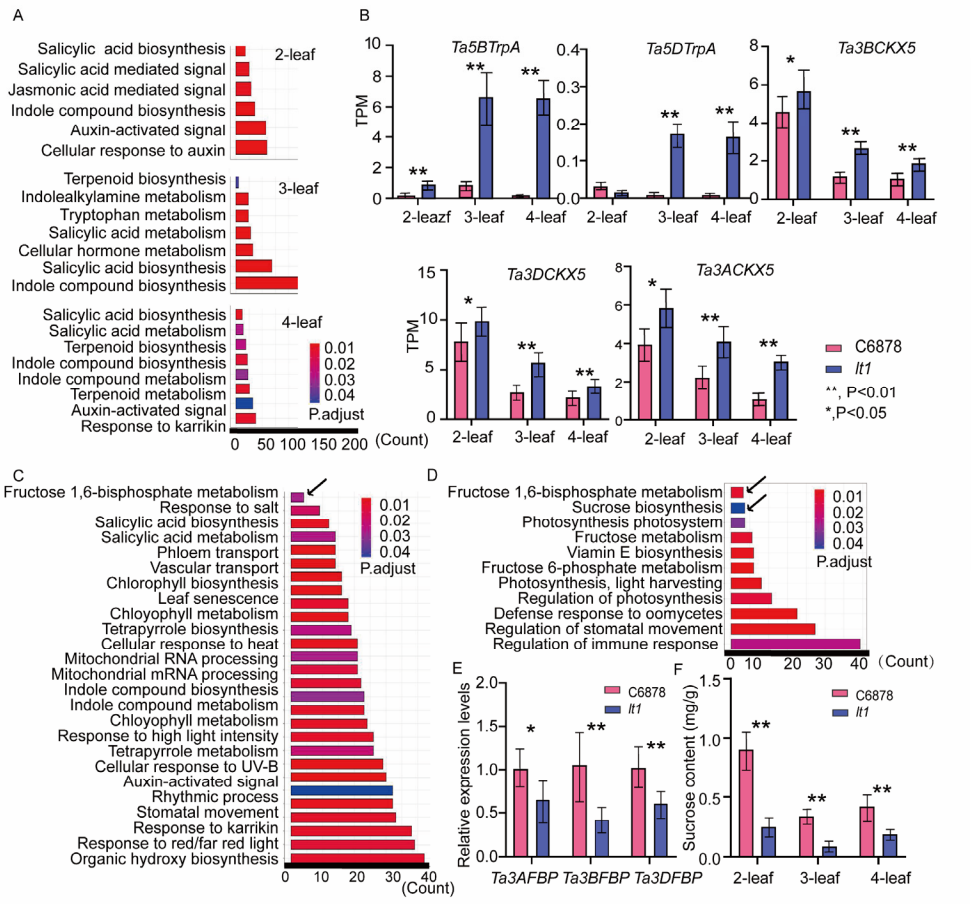

Figure 23. GO analysis of genes relative to auxin and sucrose and their endogenous levels. (A) Various phytohormones enriched by GO analysis. The P.adjust values are portrayed by blue to red gradient colors. The gene number represented by values on the X-axis is the count number belonging to the pathways enriched. (B) Auxin and cytokinin-related gene expression (TPM) values. Values are means ± SD (n = 3). *** P < 0.001, Student's t-test. (C) and (D) Go analysis of DEGs at the 4-leaf stage and DEGs from seedlings. The sucrose biosynthesis pathways perturbed are arrowed. The gene number represented by values of the X-axis is the count number belonging to the pathways enriched. P.ajust values indicating significance are colored gradually from blue to red. (E) Relative expression levels of FBPase. The Values are relative to ACTIN. (F) Sucrose content comparison. Sucrose levels of *lt1* in three developmental stages are all reduced significantly than that in C6878. Values are means ± SD (n = 3). *** P < 0.001, Student's t-test.

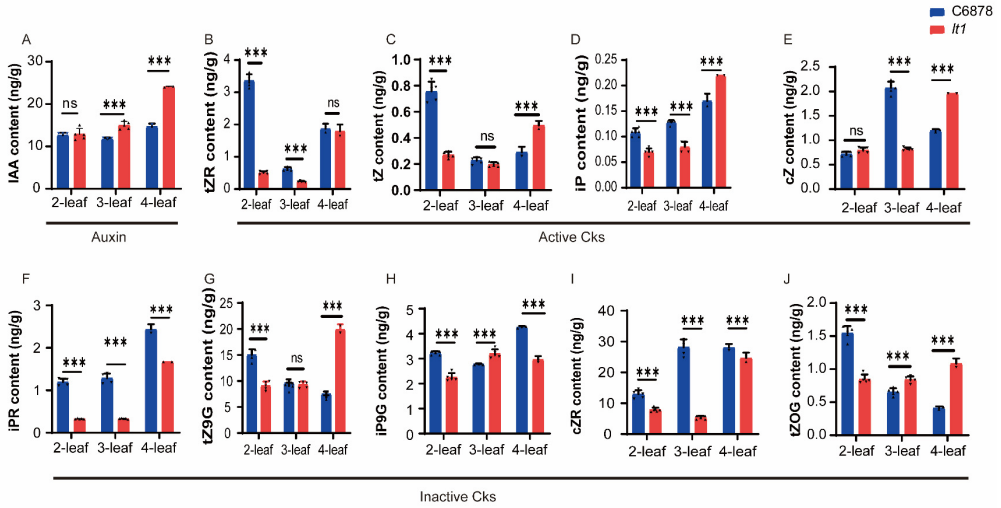

Figure 24. Phytohormone levels at three different stages. IAA levels are indicated in A. B-E depicts active forms of CKs, while F-J delineates inactive CK levels. Values are means ± SD (n = 5). *** $P < 0.001$, Student's t-test. Abbreviations: tZR: trans-zeatin riboside, tZ: trans-zeatin, iP: isopentenyladenine, cZ: cis-zeatin; iPR: isopentenyladenosine riboside, tZ9G: trans-zeatin-9-glucoside; iP9G: isopentenyladenine-9-glucoside; cZR: cis-zeatin riboside, tZOG:trans-zeatin-O-glucoside.

3.5. *LT1* may function through the sucrose biosynthesis pathway

As with all organisms, plants require energy for growth. They achieve this by intercepting light and fixing it into usable chemical forms via photosynthesis. The resulting carbohydrate (sugar) energy is then utilized as substrates for growth or stored as reserves (Eveland and Jackson, 2012), thus influencing various aspects of plant development, such as tillering (Rabot et al., 2012). Our co-expression analysis revealed perturbations in the *fructose 1,6-bisphosphate* (*FBP*) pathway at the 4-leaf stage, which is involved in sucrose biosynthesis (Figure 23C). Coincidentally, RNA-seq analysis using whole seedlings with two leaves also showed perturbations of the FBP pathway genes (Figure 23D). Within this pathway, three closely related *TaFBPase* genes involved in sucrose biosynthesis were down-regulated in *lt1* mutants (Figure 23E), implying lower sucrose levels. To determine if the sucrose levels have changed in the *lt1* mutant, we collected the shoot base at the 2-, 3-, and 4-leaf stages and measured the sucrose level. Indeed, it decreased significantly in *lt1* mutants compared to wildtype (Figure 23F). Together, these datasets suggest *LT1* may exert its influence on tillering and other phenotypes by targeting FBPases, thereby impacting sucrose levels.

3.6 Interaction proteins with LT1

To further the study of LT1, the screening of interacting proteins is a straightforward approach. We performed this by utilizing the yeast two-hybrid membrane system.

This experiment revealed that 34 interacting spots were identified (Figure 25A). Moreover, the DNA sequences and analysis of their functions indicated that various pathways were enriched, such as heat shock proteins, polyubiquitin, and more (Figure 25B).

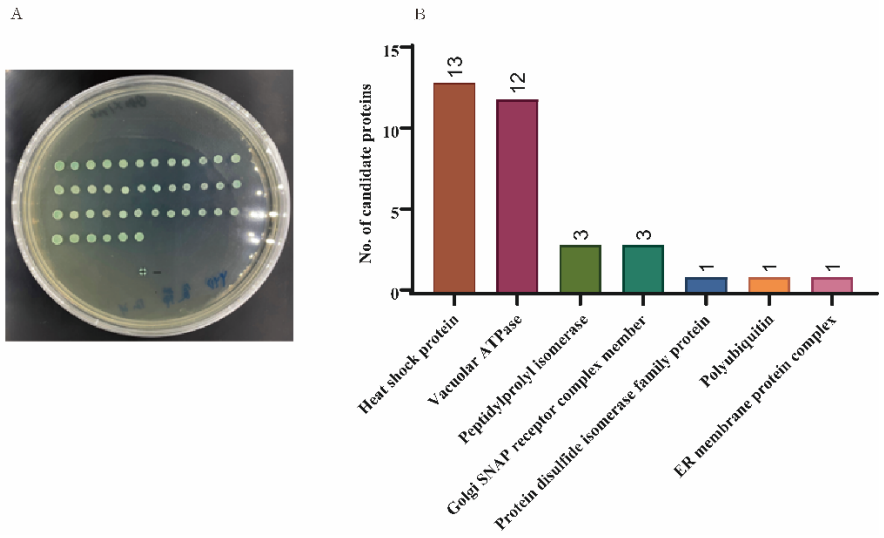

Figure 25. Analysis of candidate proteins. There are 34 candidate proteins interacting with LT1, as shown in A. Various GO pathways are enriched, like the heat shock proteins shown in B.

It is worth noting that heat shock protein 90s (HSP90s), a group of classic proteins sensitive to heat treatment, was first observed in the fruit fly and then identified and characterized in various species, such as bacteria, human cells, plants (Tichá et al., 2020). HSP90s can influence multiple plant developments, like hypocotyl length (Zeng et al., 2023), albinism (Jin et al., 2023), flowering time (Isaioglou et al., 2024), salt resistance (Zhang et al., 2022a), and axillary meristem formation which is essential for tillering (Isaioglou et al., 2024). The interaction between LT1 and HSP90s, namely, HSP90-5A, HSP90-5B, HSP90-5D, and HSP90-7D, was validated by the pairwise verification (Figure 26A).

Phylogenetic tree analysis of candidate HSP90s in wheat and *Arabidopsis* indicates that HSP90s are closely related to AtHSP90s of *Arabidopsis* (Figure 27). Intriguingly, *AtHSP90.1* and *AtHSP90.3* led to stunted growth, including the retarded emerging branches (Samakovli et al., 2014). This line of evidence gave us confidence, in combination with *lt1*'s stunted growth rate, to consider HSP90 is involved in tillering controlling with LT1. In addition, LT1 can be recognized by polyubiquitin proteins

(Figure 25B, 26B). The expression level of polyubiquitin genes is down-regulated (Figure 28) in *lt1*, suggesting that a positive regulation pathway exists.

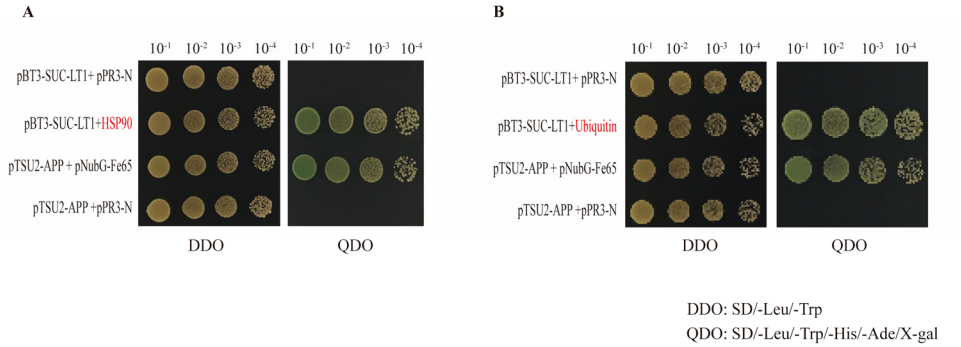

Figure 26. Pairwise verification of interaction between LT1 and HSP90s. The left panel shows the interaction between LT1 and HSP90. In this verification, the pBT3-SUC containing a part of sucrase was used to guarantee protein location on the membrane. The combination of pTSU2-APP and pNuBG-Fe65 works as the positive control, and the ones of pTSU2-APP and pPR3-N, pBT3-SUC-LT1 and pBR3-N work as the negative control. The candidate proteins are portrayed as red.

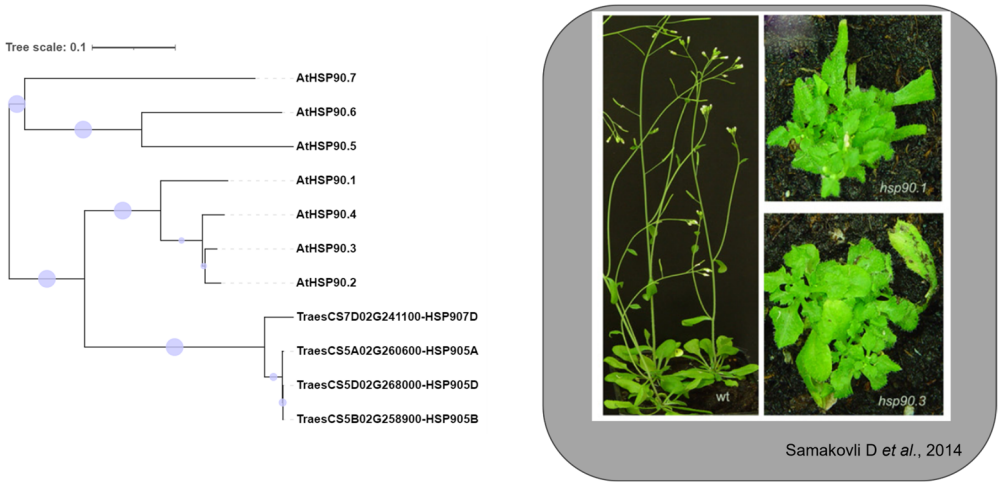

Figure 27. Phylogenetic tree analysis of HSP90 homologs in *Arabidopsis* and wheat. The phylogenetic tree of HSP90 was established by the neighbor-joining method. The scale bar shows the length of a branch that represents one nucleotide change in the genome. The disruption of HSP90.1 and HSP90.3 can lead to dwarfism, as shown in the right panel.

We should carefully consider whether high temperature affects LT1, given the temperature-dependent nature of HSP90s, which can interact with LT1. To investigate this, we tested LT1 expression levels in tobacco leaves by fusing it with the entire LUC reporter. Initially, LT1 was undetectable, but upon the addition of Bortezomib, a proteasome inhibitor, LT1 expression was observed (Figure 29A-B). This suggests that at higher temperatures (25°C), LT1 is degraded with the assistance of HSP90s. In contrast, at lower temperatures (20°C), LT1 exhibited high expression levels even without Bortezomib treatment (Figure 29C-D). Furthermore, the *lt1* mutant displayed significantly more tillers in Taian, which has a higher temperature than in Beijing at the tillering stage we measured in March (Figure 30). Interestingly, LT1 seems to be able to affect vernalization as *lt1* exhibited defective vernalization phenotypes, such as more tillers in different low-temperature duration treatments (Figure 31). Together, these results indicate that LT1 is degraded at higher temperatures with the help of HSP90s, thus promoting tillering.

An emerging working of LT1 is formed based on the lines of evidence stated herein. In this scenario, LT1 interacts with HSP90, thus inhibiting transcriptional factors that control tillering in low temperatures. The HSP90 in high temperatures can accelerate the degradation of polyubiquitinated LT1, therefore releasing the inhibitory role in tillering. As the LT1 gene is a recessive gene, it seems contradictory that LT1 plays an inhibitory due to fewer tillers of the *lt1* mutant. From our perspective, the truncated LT1 can also interact with HSP90s but cannot be degraded; thus, the inhibitory role persists.

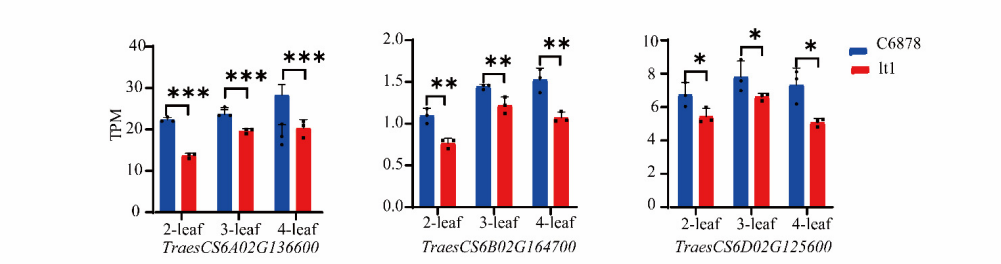

Figure 28. Relative expression of ubiquitin genes. Relative expression of ubiquitin genes is represented by TPM values. Values are mean ± SD (n = 3). Student's t-test. ***, p <= 0.0001.

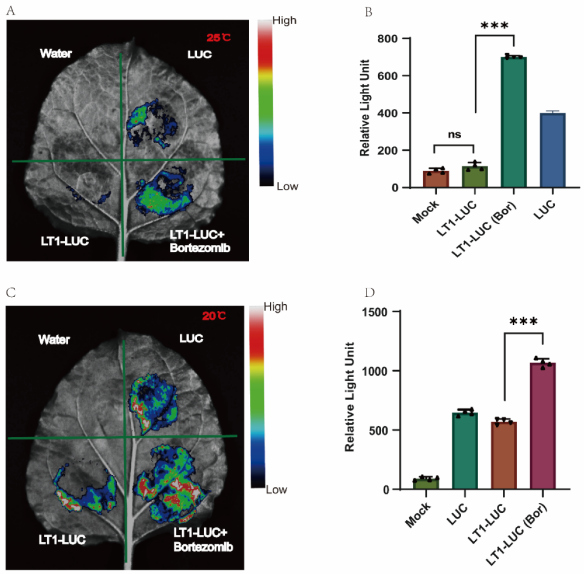

Figure 29. LT1 stability is influenced by temperatures. LT1's expression levels are depicted by light intensities. A and C show light intensities of LUC, LT1-LUC (with or without the addition of the Bortezomib) at either 25℃ or 20℃. B and D show quantified light intensity values. Values are means ± SD (n = 3). *** P < 0.001, Student's t-test.

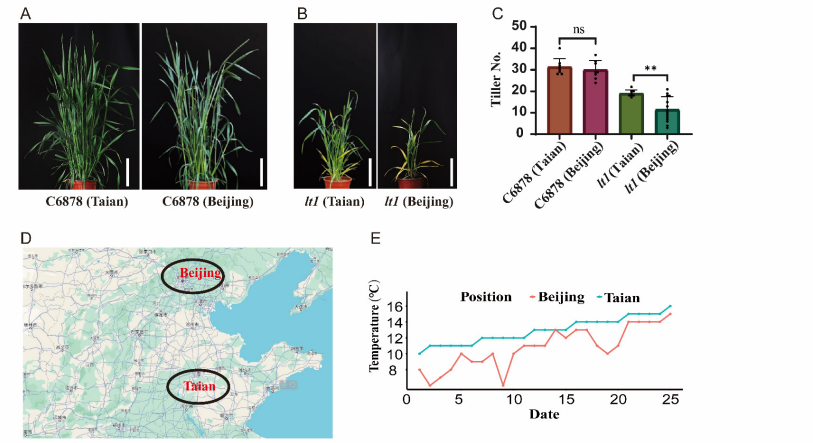

Figure 30. *lt1* shows distinct tiller numbers at different temperatures. *lt1* shows significant differences in tiller numbers when grown in distinct temperature conditions like in Beijing and Taian (D and E). The control of *lt1* was not affected by temperatures (A) but *lt1* (B). C shows the statistical values of tillers. Values are mean ± SD (n = 10). Student's t-test. **, p <= 0.001.

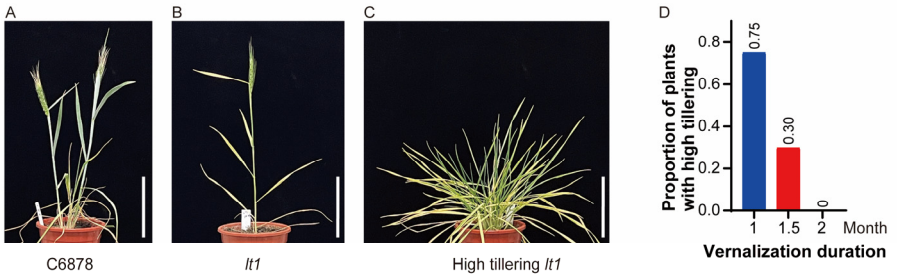

Figure 31. *LT1* can influence vernalization. A shows C6878, which serves as the control for *lt1*, exhibiting normal tillering capacity. While B shows *lt1* having fewer tillers under normal conditions, C demonstrates that *lt1* paradoxically produces more tillers. D presents quantitative data through bar plots showing the proportion of plants exhibiting high tillering phenotypes under different vernalization durations (Methods 2.21), such as 1, 1.5, and 2 months.

3.7 *LT1*-OE lines show fewer tillers but earlier flowing time

Further dissecting the role of *LT1* in wheat tillering, a line with constitutive overexpression of *LT1* displayed fewer tillers but flowered at least two weeks earlier than the wild-type (Figure 32). Theoretically, *lt1*-oe lines should exhibit the opposite phenotype of the original *lt1* mutant, with more tillers. However, considering the lines of evidence that HSP90 can help LT1 to be degraded, we propose that a high abundance of LT1 inhibits tillering, similar to the undegraded mutant LT1 protein. Interestingly, *lt1*-oe panicles exhibited less variability in panicle length than the wild-

type Fielder. Furthermore, *lt1*-oes shows a high grain yield, although it has fewer tillers (Figure 32I). Collectively, *LT1* could be a valuable genetic resource, as it does not penalize grain yield and confers early flowering time.

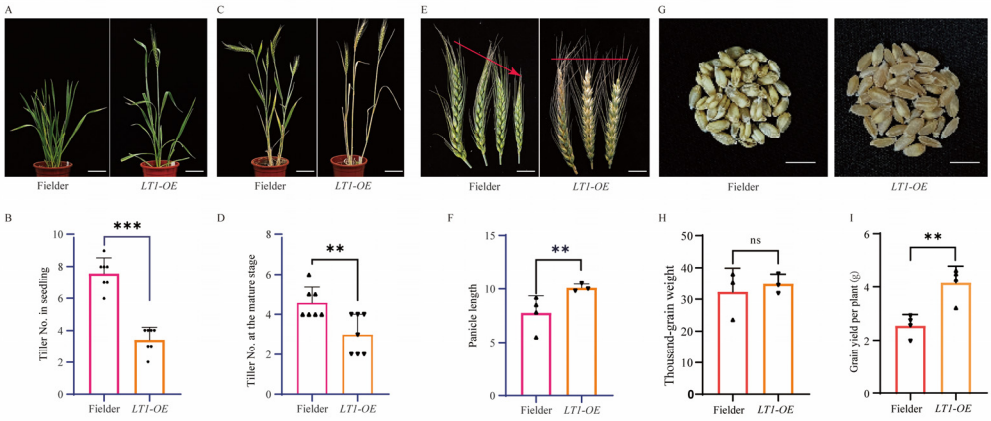

Figure 32. Phenotypes of *lt1*-oes. The *lt1*-oe line shows fewer tillers but earlier flowing time at the seedling stage in A and B. C and D display the tiller number of *lt1*-oe and its wild type at the maturing stage. E and F show the panicle length between *lt1*-oe and its wild type. G-H show aspects of grain yield. Values are mean ± SD (n =3). Student's t-test. **, p <= 0.001.

4. Discussion and future prospects

In higher plants, the degree and pattern of tillering/branching are major determinants of plant architecture and grain yield, especially in crops. Significant advances have been made in identifying genes controlling branching in model plants like *Arabidopsis* and rice, but fewer genes controlling tillering have been identified in wheat. This study used a new approach called uni-BSA to clone *LT1*, a chloroplast protein with an NB-containing domain. Functional analysis revealed that *LT1* modulates auxin, CK, and sucrose levels to control tillering in wheat (Figure 33).

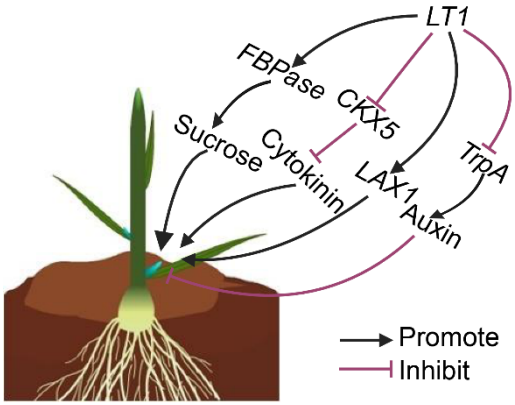

Figure 33. A proposed model of *LT1* regulating tillering. In this model, *LT1* promotes *TaLAX1* to regulate AM formation. *LT1* can affect *TaFBPase* expression levels, thus mediating internal sucrose content to facilitate axillary bud outgrowth. *LT1* also impacts phytohormone-related genes to control tillering, as it inhibits *TrpA* to reduce auxin levels and inhibits *TaCKX5s* to increase cytokinin levels.

4.1. Uni-BSA method is robust for wheat gene cloning

BSA is a cost-effective and robust approach for identifying causal genes from segregating populations. BSA-based methods, such as BSR-seq (del Viso et al., 2012), Mutmap (Abe et al., 2012), and Graded-seq (Wang et al., 2019b), enable the rapid development of genetic markers and gene cloning. However, few genes in wheat have been mapped using BSA-based methods. This is mainly due to the high cost of whole genome resequencing for BSA, which becomes prohibitive given the large genome size of wheat and the high proportion of repetitive regions that lead to ambiguous read mapping. To address these challenges, firstly, we implemented WES to identify variations while ensuring sufficient markers for the linkage analysis and, thus, reducing the genome from 17 Gb to 260 Mb. A similar approach, like BSE-seq (Dong et al., 2020) using WES data with the varBscore algorithm, has successfully cloned several genes. However, it did not address the issue of high repetitive sequence content inherent in the wheat genome. We developed an effective uni-BSA algorithm to filter out ambiguous reads while retaining as many reads as possible, improving mapping accuracy and narrowing down smaller candidate gene intervals (Figure 16D). Namely, uni-BSA can produce more sensitive δ index values than those with no-filtering or strict-filtering methods, making it easier to define linkage areas (Figure 16C). While the linkage interval defined by the strict-filtering method is the same as uni-BSA, the uni-BSA covers more genomic areas by using its algorithm (Figure 17A). Additionally, this algorithm produces more accurate variants compared to the no-filtering method (Table 7). For example, the mutation site (C-T) of *LT1* identified by the no-filtering method is heterozygous but homozygous by uni-BSA. Collectively, our uni-BSA method is a powerful and preferable approach for gene cloning in wheat.

Table 7. List of variants in the linkage interval

Methods	Chromosome	Position	F_2-Pool (mutant)	Description
No-filtration	2D	16721988	Heterozygous	Mutation site (C/T)
Uni-BSA	2D	16721988	Homozygous	Mutation site (T/T)
Absosulte-filtration	2D	16721988	Homozygous	Mutation site (T/T)

4.2. *LT1* shares an NB domain with plant resistance proteins

The NB domain is a common feature of many plant resistance proteins, also known as NB-LRR proteins, named after their central NB domain and C-terminal leucine-rich repeat (LRR) domain (Takken and Tameling, 2009). Because R proteins can trigger host cell death, their activity requires tight regulation. Studies of R protein interactions and mutagenesis revealed that both the NB and LRR domains play a role in the auto-inhibition of these proteins (Rairdan and Moffett, 2006; Rairdan and Moffett, 2007). Additionally, the LRR domain likely functions in recognizing avirulence effectors produced by pathogens (Takken and Tameling, 2009). Despite their role in disease resistance, dysregulation of R proteins also impacts developmental processes, resulting in phenotypes like stunted dwarfism (Yang and Hua, 2004; Michael Weaver et al., 2006), increased branching (Igari et al., 2008), early leaf senescence (Xie et al., 2023), altered plant height (Borrill et al., 2022), and abnormal panicle development (Pan et al., 2022).

Unlike other R proteins, the *LT1* gene identified in our study encodes only an NB domain, lacking the LRR domain. Our analysis showed 2035 NB domain-containing genes in the wheat genome, with 964 lacking LRR domains. The evolutionary mechanisms leading to the high number of NB-only proteins require further investigation. We hypothesize that disruption of *LT1* removes its auto-inhibition, thereby activating resistance responses and impacting developmental pathways like tillering. Alternatively, *LT1* may presumably play a direct role in the regulation of tillering, independent of disease resistance.

The chloroplast location of *LT1* provides a link between its effect on disease resistance and plant development. Chloroplasts are energy production sites, so localization to this organelle implies that *LT1* may impact developmental processes by influencing energy production. This is consistent with the pleiotropic phenotypes observed in *lt1*, such as reduced tillering, plant height, and short roots. Further investigation of how a chloroplast-localized protein like *LT1* influences energy production and downstream developmental pathways will shed important light on its roles in plant growth and disease resistance.

4.3 *LT1* is involved in temperature-controlling tillering in wheat

Winter wheat requires a long exposure to low temperatures (vernalization) to become competent for flowering (Dubcovsky et al., 2006); without this vernalization process, wheat produces minimal flowers and increases unproductive tillers. Similar phenotypes, like insufficient vernalization phenotypes, existed in *lt1* when it was vernalized for one month (Figure 31). This phenotype with more unproductive tillers of *lt1* indicates that *LT1* could interfere with vernalization, thus regulating tillering. Another hint we can get is that *LT1* can physically interact with HSP90s (Figure 26A), which is essential for plant development, such as flowering time (Isaioglou et al., 2024), albino (Jin et al., 2023), hypocotyl length (Zeng et al., 2023), salt resistance (Zhang et al., 2022a), and axillary meristem formation which is essential for tillering (Isaioglou et al., 2024). It is notable that HSP90s are sensitive to temperature. Namely, they express highly at elevated temperatures and lowly at reduced temperatures. In contrast, *LT1* expression levels are high in low temperatures and lower in increased temperatures (Figure 29). Combining this evidence with the observations that *LT1* can be recognized by polyubiquitin (Figure 26B) and that the *lt1* mutant produces more tillers at higher temperatures (Figure 30), we hypothesize that *LT1* promotes tillering with the assistance of HSP90s to degrade itself at elevated temperatures. Based on this hypothesis, it seems contradictory that the loss-of-function of *LT1* can inhibit tillering. We guessed that abnormal *LT1* could also interact with the HSP90 protein but could not be degraded easily; thus, the inhibitory role of *LT1* persists. This inhibitory role of *LT1* in regulating tillering can be sided by the *LT1* overexpression lines, which exhibited fewer tillers (Figure 32C-D). Taken together, *LT1* affects tillering in wheat dynamically depending on different temperatures (Figure 34). In this scenario, *LT1* can inhibit tillering in the low-temperature condition. In high-temperature conditions, with the help of HSP90 proteins, *LT1* can be degraded, thus releasing the repression of *LT1* to promote tillering in wheat. From our perspective, the deep dissection of how *LT1* is involved in temperature will benefit grain yield.

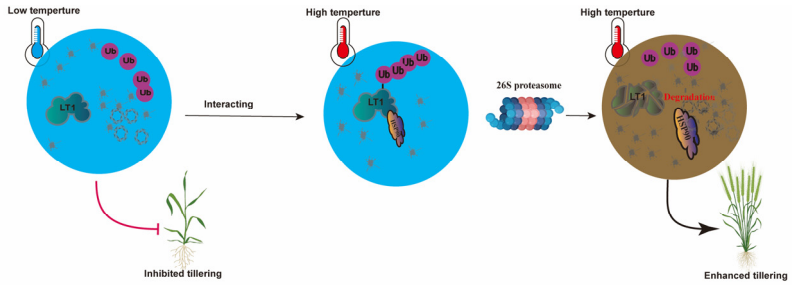

Figure 34. Work model of LT1 in different temperature conditions. In this model, LT1 interacts with the HSP90 protein at a relatively high temperature. In this scenario, LT1 is degraded through 26S proteasome after its ubiquitination, thus promoting tillering.

4.4 LT1 can mediate sugar content and phytohormones in controlling tillering in wheat

Sugars provide energy and carbon skeletons for metabolic processes in plant cells. They influence bud outgrowth, as axillary buds often remain dormant due to the growing shoot apex's repression. While auxin is considered the primary regulator of apical dominance, its transport is slower than the activation of lateral bud growth after decapitation. Before auxin levels decrease, the redistribution of sugars from source leaves results in their accumulation in lateral buds, inhibiting *BRC1* expression and promoting bud growth (Mason et al., 2014). Furthermore, auxin supplementation fails to fully restore apical dominance in some plants (CLINE, 1996; Morris et al., 2005), suggesting that auxin is not the first component inhibiting axillary bud outgrowth. Plants limit sugar supply to axillary buds to maintain apical dominance (Mason et al., 2014), as observed in the wheat *tin* mutant with reduced tillering and sucrose content in axillary buds (Kebrom et al., 2012), and the rice *moc2* mutant with disrupted sucrose biosynthesis and reduced tiller outgrowth (Kebrom et al., 2012). Sugar is required for bud outgrowth in rose (*Rosa hybrida*) nodes cultured in vitro (Rabot et al., 2012; Barbier et al., 2015a), and sucrose modulates the dynamics of bud outgrowth in a concentration-dependent manner (Barbier et al., 2015a), highlighting the trophic role of sugars in bud release.

Besides providing nutrients, sugars affect phytohormone homeostasis in *R. hybrida* nodes (Barbier et al., 2015a). Sucrose stimulates CK biosynthesis by upregulating related genes, modulates auxin metabolism, and promotes auxin export from buds to stems, favoring bud outgrowth according to the auxin canalization model. It also reduces the expression of *MAX2* and *RhBRC1* in a dose-dependent manner (Barbier et al., 2015a). Notably, the non-metabolizable sucrose analog palatinose can trigger bud outgrowth (Rabot et al., 2012), collectively demonstrating the crucial role of sugar signaling in regulating bud release.

In our study, the *lt1* mutant with low sucrose content coincided with a low bud outgrowth ratio (Figure 15E-F). The auxin level was increased, indicating an inhibitory role in regulating tillering in wheat. Furthermore, the CK concentration was significantly decreased at the 2-leaf stage, suggesting inhibition of axillary meristem initiation (Figure 24). Elucidating *LT1*'s roles in tillering, from energy metabolism to hormone signaling, will enable more targeted breeding efforts to optimize tiller number and wheat yields.

4.5. *LT1* is essential in controlling wheat developments, especially in tillering, and an excellent genetic resource for molecular breeding

Crop tillering is a trait closely linked to yield. *LT1* is a novel gene that regulates wheat tiller numbers by controlling both bud initiation and outgrowth. CRISPR/Cas9-generated transformants phenocopied *lt1* mutants, exhibiting reduced tiller number, shorter stature, yellow leaves, and additional traits. Moreover, some progeny derived from certain heterozygous transformants, especially those with large truncations of *LT1*, like *LT-CR3*, displayed lethal seedling phenotypes (Figure 20D), characterized by yellowing and withered leaves. This seedling lethality in some genotypes likely

explains the inability to find lines exhibiting *lt1* phenotypes in segregating populations in field conditions, as these lines died at early developmental stages.

Interestingly, the *LT1* overexpression line displayed fewer tillers, earlier flowering, and increased grain yield (Figure 32) grown in the greenhouse, indicating *LT1* is an excellent genetic resource for molecular breeding. Further exploration of *LT1* and its interacting partners will enhance our understanding of the intricate regulatory systems governing the complex networks that control tillering and grain yield in cereal crops.

Chapter IV

General Discussion, General Conclusion, and Perspective

1. General discussion

1.1. Introduction

Branching, also known as tillering in cereal crops, is a critical process in plant development characterized by the formation of lateral shoots from axillary buds. This phenomenon is crucial in major cereal crops such as wheat, rice, and barley. Tillering significantly influences plant architecture and directly impacts grain yield (Jiao et al., 2010), making it a vital trait for agricultural productivity. As global food demand rises (Dong et al., 2020), research into tillering mechanisms and their application in breeding programs can significantly increase crop yields and warrant global food security.

Tillering involves two key stages: AM initiation and the subsequent tiller bud outgrowth, both regulated by complex genetic interactions and environmental factors (Yuan et al., 2023b). AM initiation originates from SAM activity and involves key genes like *LAS*, *REV*, and *CUC* (Aida et al., 1999; Otsuga et al., 2001; Greb et al., 2003a; Hibara et al., 2006), as well as hormone interactions (Napoli and Ruehle, 1996; Beveridge et al., 1997; Beveridge, 2000; Chatfield et al., 2000; Foo et al., 2001; Morris et al., 2001; Turnbull et al., 2002; Sorefan, 2003; Beveridge, 2006; Reddy et al., 2013; Wang et al., 2014; Zhang et al., 2020b). Tiller bud outgrowth is controlled by genes such as *TB1* and influenced by various signals, including phytohormones, nutrients, and light (Aguilar-Martínez et al., 2007; Wang et al., 2019c). Understanding these mechanisms is crucial for crop improvement, potentially optimizing tiller number, enhancing stress resilience, and improving resource use efficiency.

The utilization of beneficial genes controlling tillering for molecular breeding has elevated grain yield, such as *IPA1* in rice (Wang et al., 2018a). Therefore, discovering and isolating more genes regulating tillering is imperative in plants. However, positive gene cloning methods like MBC, while effective, are tedious and challenging, particularly in complex genomes such as wheat. Recent advancements in high-throughput resequencing technologies by the next-generation resequencing platform have facilitated the identification of genes related to important traits in crops. High-throughput sequencing-based techniques, such as MutMap (Abe et al., 2012) and its derivatives (Fekih et al., 2013; Takagi et al., 2013b), have significantly accelerated gene isolation processes. However, wheat's large genome size (17Gb) and high repetitive sequence content (>80%) pose extra challenges for accurate gene identification by using next-generation sequencing data. Methods like BSE-seq (Dong et al., 2020) and BSR-Seq (Liu et al., 2012) have tried to use high-throughput data containing only protein-coding regions to reduce the order of magnitude of the wheat genome and successfully cloned genes, such as the leaf-color *YGL1* gene in wheat and the *GL3* gene of maize, respectively. While these two methods have reduced the size to a large extent, the large repetitive ratio of wheat’s genome remains to be resolved. That is why these methods are not widely used in wheat. So far, current BSA-based methods primarily address noise from sequencing platforms but lack specific algorithms to tackle wheat's inherent repetitive sequence noise. To deal with this problem, we have deliberately developed the uni-BSA method. This algorithm can

effectively filter out reads aligned to multiple genomic positions, theryby elevating variant calling accuracy. Therefore, in our project, we successfully mapped the wheat *LT1* gene. *LT1*, encoding a nucleotide-binding domain protein, was validated through CRISPR/Cas9 knockout lines and may regulate tillering via auxin, cytokinin, and sucrose pathways. It is worth noting that *LT1* might interfere with vernalization and is involved in temperature pathways, indicating its special role in controlling tillering in wheat.

Collectively, this study identifies a novel wheat tillering regulator and demonstrates the effectiveness of uni-BSA for gene cloning in wheat. The characterizing of *LT1* advanced the understanding of tillering in wheat. The development of uni-BSA offers a valuable program suite for molecular breeding in wheat, addressing the urgent need for efficient and precise gene cloning methods in this important crop.

1.2. The *LT1* is the first cloned gene controlling both AM initiation and bud outgrowth in wheat

The molecular mechanisms of AM formation remain elusive, partly due to a lack of mutants affecting AM development in plants like rice, *Arabidopsis*, and wheat. As discussed in Chapter II, recent methodologies have shed light on identifying more genes related to AM initiation. For example, using genetic backgrounds with reduced apical dominance can help identify AM initiation regulators, while CRISPR/Cas9 enables targeted genetic manipulation across various plant species. Emerging omics technologies, particularly single-cell approaches, are proving valuable. These techniques, especially when combined with clever genetics, provide promising avenues to elucidate the molecular control of AM development. They can help identify new genes and regulatory networks involved in AM initiation, potentially accelerating research in this critical area of plant development and agriculture. For our research, we successfully isolated the *LT1* gene regulating AM initiation, which is the first reported gene via the forward genetics in wheat.

In addition to AM initiation, bud outgrowth is also a determinant for tillering. Decades of research have sought to unravel the mechanisms of shoot branching. Current understanding suggests that endogenous factors and environmental signals interlock to regulate this process. Key genes influencing shoot branching include regulatory genes like *TB1*, which integrates various signaling networks, as well as environmental and physiological cues such as phytohormones, nitrogen availability, light conditions, and sugar concentrations (Yuan et al., 2023b). While substantial progress has been made, the underlying mechanisms remain elusive in wheat. Gene cloning faces significant challenges due to its complex genome. Only two tillering-related genes, *TN1* (Dong et al., 2023) and *TIN* (Spielmeyer and Richards, 2004; Hyles et al., 2017), have been isolated using MBC. Most research relies on reverse genetics, targeting genes like *TaD27* (Zhao et al., 2020b), *TaD14* (Liu et al., 2021a), and *TaMOC1* (Zhang et al., 2015). In our study, *LT1*, identified by uni-BSA (Yuan et al., 2024b), also influences bud outgrowth in various ways, such as controlling sugar levels and phytohormones and more. We believe that the study of *LT1*, which impacts both AM initiation and bud outgrowth, will contribute to understanding tillering and grain yield in wheat.

1.3. The characterization of *LT1* contributes to our understanding of tillering mechanisms in wheat

As discussed in Chapter II, tillering is a process controlled by internal and external cues. Significant advances have been made in identifying genes controlling branching/tillering in model plants like *Arabidopsis* and rice, but fewer genes controlling tillering have been identified in wheat. This study used a new approach called uni-BSA to clone *LT1*, a chloroplast protein with an NB-containing domain. Functional analysis revealed that *LT1* modulates auxin, CK, and sucrose levels to control tillering in wheat. These results are similar to the reports in the model plants like *Arabidopsis* and rice.

Intriguingly, the *lt1* mutant exhibits phenotypes similar to insufficient vernalization compared to its wild-type plant when vernalized for one month, suggesting *LT1*'s interference with vernalization. This was the first reported NB-containing gene related to vernalization. *LT1* interacts with HSP90s, which are temperature-sensitive and involved in various developmental processes (Tichá et al., 2020). Moreover, *LT1* expression is inversely related to temperature and can be recognized by polyubiquitins. Together, we hypothesize that *LT1* promotes tillering with HSP90 assistance. In this context, *LT1* is degraded at higher temperatures and kept stable at low temperatures. Furthermore, overexpression of *LT1* results in fewer tillers, indicating its inhibitory role.

Despite common regulations such as impacting phytohormones, the novelty of *LT1* is that it could dynamically affect wheat tillering depending on temperature. Namely, it inhibits tillering at low temperatures and promotes it at high temperatures. Prospectively, further investigation of *LT1*'s temperature-sensitive mechanisms could contribute to wheat yield improvement strategies.

1.4. Uni-BSA has the potential to accelerate the steps of gene cloning in wheat and other genomes with high repetitive ratios

The necessity to accelerate gene mapping for molecular breeding in wheat stems from its critical role in global food security and the unique challenges posed by its complex genome (IWGSC, 2014). As a staple crop providing sustenance for one-third of the world's population and contributing significantly to daily caloric intake (Zörb et al., 2018), improving wheat yield is crucial to meet the growing food demands. This ambitious goal requires a deeper understanding of the genetic basis of yield-related traits, which could be achieved through more efficient gene mapping techniques. However, gene cloning in wheat has lagged behind other crops due to its enormous genome size and high ratio of repetitive sequences, which exceed 85% of the total genome (IWGSC, 2014). This genomic complexity has made traditional positive gene mapping methods like MBC time-consuming and challenging, hindering the identification of genes regulating important traits such as tillering, plant height, and disease resistance.

Recent technological advancements, particularly NGS and new algorithms (Liu et al., 2012; Dong et al., 2020), offer promising opportunities to overcome the challenges posed by wheat's complex genome. However, these methods need to be adapted and

optimized specifically for wheat. Our uni-BSA method offers several significant advances for gene mapping in wheat, addressing key challenges posed by its complex genome (Yuan et al., 2024b). Uni-BSA tackles the major hurdle of wheat's high proportion of repetitive sequences by employing a unique mapping algorithm. This approach significantly reduces the impact of repetitive sequences, which have historically complicated gene cloning efforts in wheat. The effectiveness of uni-BSA was demonstrated through its successful isolation of the *LT1* gene controlling tillering in wheat. This achievement highlights the method's ability to navigate the wheat genome's complexities and pinpoint genes of interest more precisely. Uni-BSA primarily focuses on filtering out ambiguous reads that align to multiple genomic regions while maximizing the use of uniquely mapped reads. This strategy ensures higher accuracy in variant calling, which is crucial for reliable gene identification.

Moreover, uni-BSA's approach can be extended to other species with high ratios of repetitive sequences, broadening its applicability beyond wheat. By providing a more accurate and efficient means of gene mapping in complex genomes, as indicated by our thesis, uni-BSA represents a significant advancement in forward genetics techniques. This method would contribute to accelerating the pace of gene discovery in wheat.

1.5. *LT1* is involved in tillering by regulating the contents of sugars and phytohormones

Sugars, particularly sucrose, play a dual role in plants as both a carbon source and essential signaling compounds (Rolland et al., 2006; Smeekens et al., 2010; Wind et al., 2010). Recent research has highlighted their importance in regulating axillary bud outgrowth (Mason et al., 2014; Barbier et al., 2015b). While apical dominance has traditionally been attributed to auxin, evidence suggests that sugar redistribution to axillary buds precedes auxin depletion following decapitation, indicating a primary role for sugars in bud release (Mason et al., 2014; Barbier et al., 2015b). Studies in various plant species, including wheat (Kebrom et al., 2012) and rice, have shown that reduced tillering is associated with decreased sucrose content in axillary buds (Koumoto et al., 2013). In vitro experiments with rose plants demonstrate that sugar is necessary for triggering bud outgrowth and can modulate its dynamics in a concentration-dependent manner (Rabot et al., 2012; Barbier et al., 2015a). Beyond their trophic role, sugars also influence phytohormone homeostasis. In rose, sucrose stimulates cytokinin biosynthesis, modulates auxin metabolism and transport, and affects the expression of genes involved in strigolactone signaling and bud outgrowth regulation (Barbier et al., 2015b).

From a broader perspective, axillary buds are sink organs competing for sugars. The interplay between the apical and lateral buds in sugar demand is crucial for systemic regulation of shoot branching, involving nutritional support and signaling mechanisms. This sugar-centric model provides a new framework for understanding the complex regulation of plant architecture.

Our study on the *lt1* mutant in wheat provides further evidence for the intricate relationship between sugar content and tillering. This mutant, characterized by low

sucrose content, exhibits a reduced bud outgrowth ratio. Interestingly, it also shows increased auxin levels, suggesting an inhibitory role of auxin in wheat tillering. Moreover, the significant decrease in CK concentration at the 2-leaf stage indicates potential inhibition of axillary meristem initiation. These findings collectively highlight the complex interplay between sugar metabolism, hormone signaling, and tillering in wheat. Understanding *LT1*'s role in these processes, from energy metabolism to hormone signaling, opens new avenues for targeted breeding efforts aimed at optimizing tiller number and, consequently, wheat yields.

1.6. *LT1* is involved in tillering by responding to temperature

Explicating the relationship between ambient conditions and tillering is crucial for several reasons. Firstly, it enhances our fundamental understanding of plant biology and adaptive strategies. Secondly, this knowledge is vital for developing crop varieties that can maintain optimal tiller numbers and yield under varying environmental conditions, especially in the face of climate change. Lastly, understanding these mechanisms can lead to more targeted and efficient breeding strategies.

Environmental factors, particularly temperature, are crucial in regulating bud outgrowth and tillering in plants (Prasanth et al., 2017). Both high and low temperatures can negatively impact tiller numbers, as observed in various species. In *B. distachyon*, tiller numbers decrease linearly with increasing temperatures (Harsant et al., 2013), while in rice, heat-stress-related genes like *miR159* influence tillering under high temperatures (Wang et al., 2012). Cold stress also affects tillering, with several genes identified as key players in this process. For instance, *OsMADS57* in rice maintains tiller growth under chilling stress by activating defense genes and modulating tillering inhibitors (Chen et al., 2018). Other genes, such as *OsCYP19-4*, *OsRAN1*, and *miR393*, have improved cold tolerance and influence tillering (Chen et al., 2018).

The discovery of *LT1*'s role in wheat further underscores the intricate connection between temperature and tillering. *LT1*'s temperature-sensitive expression and its interaction with HSP90s demonstrate how plants fine-tune their developmental processes in response to ambient conditions. The *lt1* mutant's phenotypes under different temperatures and vernalization conditions reveal its specific role in temperature-mediated regulation of wheat tillering.

By elucidating the molecular pathways through which genes like *LT1* mediate environmental responses, researchers can develop wheat varieties with improved adaptability to temperature fluctuations. This knowledge not only contributes to basic plant science but also has significant implications for molecular breeding, as it can help create more resilient and productive wheat crops capable of thriving in diverse and changing climates.

1.7 *LT1* plays a versatile role in plant development and has potential applications in crop improvement

The versatility of genes like *LT1* in controlling various aspects of plant development is indeed crucial. *LT1*'s role in wheat exemplifies how a single gene can influence

multiple developmental processes, making it a valuable target for crop improvement. *LT1* regulates not only tiller numbers through bud initiation and outgrowth but also affects plant stature, leaf color, and potentially even survival at the seedling stage. The fact that CRISPR/Cas9-generated transformants phenocopied *lt1* mutants in multiple traits demonstrates this gene's broad influence on plant development. The observation of the transformant's lethality at the seedling stage in some genotypes with large *LT1* truncations further underscores its essential role in early plant development. Intriguingly, *LT1* overexpression led to fewer tillers but increased grain yield, suggesting a complex relationship between tillering and productivity. This multifaceted impact of *LT1* on plant architecture, development, and yield makes it an excellent candidate for molecular breeding efforts.

The versatility of *LT1* is critical in plant development for several reasons. Firstly, it allows plants to coordinate multiple aspects of growth and development through a single regulatory point. Secondly, it provides opportunities for fine-tuning various traits simultaneously through breeding or genetic engineering. Lastly, understanding these versatile genes can offer insights into the complex networks governing plant development, potentially revealing new strategies for crop improvement.

In conclusion, the discovery and characterization of versatile genes like *LT1* not only advance our understanding of plant biology but also open new avenues for crop improvement. By manipulating such genes, breeders and researchers can potentially develop wheat varieties with optimized architecture and enhanced yield.

2. General conclusion

Despite the challenges posed by wheat's large genome size and high repetitive sequence content, our tailor-made uni-BSA represents a significant advance in wheat gene cloning. This innovation enables more precise identification, overcoming longstanding obstacles in this field. By utilizing uni-BSA, researchers can progressively surmount the inherent challenges of wheat gene cloning. This progress would ultimately facilitate the development of improved wheat varieties with desirable agronomic traits.

Tillering, the process of producing side shoots, significantly influences the spike number per plant and, consequently, grain yield. Identifying additional tiller-controlling genes in wheat is crucial for advancing our understanding of plant architecture's genetic regulation and optimizing yields. However, the genetic and molecular mechanisms underlying wheat tillering remain incompletely understood, necessitating the identification of more genes involved. In this context, the identification and functional characterization of the *LT1* gene, as presented in this thesis, represent a significant advance in this field.

LT1, identified as a novel regulator of wheat tillering using our innovative uni-BSA method, demonstrates its complex nature of governing critical agronomic traits. It influences not only tillering but also plant stature, leaf color, and potentially seedling survival. *LT1*'s involvement in sugar metabolism, phytohormone regulation, and response to environmental factors like temperature and vernalization underscores the intricate networks governing plant growth and development.

The versatility of *LT1* in regulating multiple aspects of plant development illustrates the potential for targeted genetic manipulation to optimize crop architecture and yield. This discovery not only advances our fundamental understanding of plant biology but also opens new possibilities for developing wheat varieties with improved adaptability to diverse environmental conditions.

Altogether, our innovative uni-BSA method demonstrates significant potential in advancing wheat gene cloning, overcoming genomic challenges, and enabling precise identification of important genetic loci. The discovery of *LT1*, a novel tiller-controlling gene regulating both AM initiation and bud outgrowth, exemplifies its significance. Moreover, *LT1*'s influence extends beyond tillering to multiple agronomic traits, including plant stature, leaf color, and grain yield, indicating its potential to act as a versatile gene for crop improvement. Its involvement in complex biological processes such as sucrose metabolism, phytohormone regulation, and temperature response (Figures 33 and 34) underscores its significance in controlling plant growth and development. This multifaceted gene highlights the potential for targeted genetic manipulation to optimize wheat architecture and yield, opening new avenues for developing more adaptable wheat varieties.

3. Perspectives

The discovery of *LT1* and the development of the uni-BSA method mark significant advancements in wheat genetics and breeding. While our study has revealed *LT1*'s multifaceted role in wheat development, there is still much to learn about its precise mechanisms of action. Further research should focus on elucidating the complete signaling pathways and molecular interactions involving *LT1* and refining uni-BSA.

3.1 The uni-BSA is deliverable for gene mapping and further refinement of uni-BSA is required

The development and validation of the uni-BSA method represents this thesis's most significant technical achievement, marking a substantial advancement in gene mapping for complex genomes like wheat. This innovative approach enhances precision and accuracy by effectively mitigating alignment noise caused by high repetitive sequence content, enabling more accurate identification of candidate genes. Uni-BSA, which integrated several soft applications and its featured algorithm, streamlines gene mapping by focusing on uniquely aligned reads. This method's robustness is demonstrated by successfully identifying *LT1*, showcasing its potential for widespread adoption in cloning genes governing important traits in wheat and other plants with complex genomes. The codes of uni-BSA for gene cloning can be downloaded at https://1drv.ms/f/s!ApLKMOp4Q8-cgdYk7YtwhXNgzvH6kA?e=RFq0zr.

While uni-BSA has proven effective, future refinements could focus on enhancing computational speed and increasing accuracy. This could be achieved by incorporating additional parameters, such as the length of fragmented DNA sequences in sequencing libraries. These improvements would further strengthen uni-BSA as a powerful tool for genetic research.

3.2 How to further understand *LT1* involved in tillering

While our study revealed *LT1*'s multifaceted role in regulating plant development, including tillering, future research should focus on elucidating its complete signaling pathways and molecular interactions. Particular emphasis should be placed on *LT1*'s involvement in sugar metabolism, phytohormone regulation, and temperature response.

To build a comprehensive picture of *LT1*'s function, future studies could employ techniques such as protein-protein interaction assays, transcriptomics, and metabolomics. Given *LT1*'s importance in wheat, investigating potential homologs in other cereal crops could reveal conserved tillering regulation mechanisms across species. This approach may also identify new targets for crop improvement, such as beneficial haplotypes.

3.3 The potential application of *LT1* in molecular breeding

The translation of our *LT1* research findings into practical breeding applications represents a critical next step, encompassing several key strategies. Developing molecular markers for *LT1* and integrating them into marker-assisted selection programs will enable more efficient breeding for desirable traits. Concurrently, exploring gene editing techniques such as CRISPR/Cas9 to modulate *LT1* expression or function could lead to optimized wheat architecture and enhanced yield potential. Given *LT1*'s involvement in temperature response and interference with vernalization, studying its performance under diverse environmental conditions could facilitate the development of climate-resilient wheat varieties. This approach includes investigating how different *LT1* variants influence wheat's adaptability to temperature fluctuations, potentially contributing to varieties with improved environmental resilience. Crucially, mining and characterizing *LT1* haplotypes that promote optimal tillering and grain yield is necessary to maximize the gene's potential in breeding programs. By pursuing these research avenues, we aim to leverage our understanding of *LT1* to create wheat varieties with improved architecture, yield stability, and adaptability to diverse and changing climatic conditions. While the discovery of *LT1* and the development of uni-BSA represent significant progress, they also serve as a starting point for a new phase of research in wheat genetics and breeding. The coming years promise exciting developments as these findings are translated into practical applications, potentially accelerating wheat cultivation.

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Publications during Ph.D

During my Ph.D., several articles have been published, including reviews and research articles, as first-author and co-author. Below is the list:

1. **Yuan Y**, Du Y, Delaplace P (2024) Unraveling the molecular mechanisms governing axillary meristem initiation in plants. Planta 259 (5):101. http://doi.org/10.1007/s00425-024-04370-w.
2. **Yuan Y**, Khourchi S, Li S, Du Y, Delaplace P (2023) Unlocking the multifaceted mechanisms of bud outgrowth: advances in understanding shoot branching. Plants-Basel 12 (20):3628-3652. http://doi.org/10.3390/plants12203628.
3. **Yuan Y,** LB, Qi Juan, Liu Xin, Wang Yuanzhi, Delaplace Pierre, Du Yanfang (2024) A novel regulator of wheat tillering *LT1* identified by using an upgraded BSA method, uni-BSA. Mol. Breed 44 (7):47. http: //doi.org/10.1007/s11032-024-01484-7.
4. Cai Y, Huang L, Song Y, **Yuan Y**, Xu S, Wang X, Liang Y, Zhou J, Liu G, Li JJPBJ (2023) LAZY3 interacts with LAZY2 to regulate tiller angle by modulating shoot gravity perception in rice. Plant Biotechnol. J. 21 (6):1217-1228. http://doi.org/10.1111/pbi.14031.
5. Gong X, Huang Y, Liang Y, **Yuan Y**, Liu Y, Han T, Li S, Gao H, Lv B, Huang XJMP (2022) OsHYPK-mediated protein N-terminal acetylation coordinates plant development and abiotic stress responses in rice. Mol. Plant. 15 (4):740-754. http://doi.org/doi: 10.1016/j.molp.2022.03.001.
6. Huang L, Wang W, Zhang N, Cai Y, Liang Y, Meng X, **Yuan Y**, Li J, Wu D, Wang YJNP (2021) LAZY2 controls rice tiller angle through regulating starch biosynthesis in gravity-sensing cells. New Phytol. 231 (3):1073-1087. http://doi.org/doi: 10.1111/nph.17426.
7. Li Z, Liang Y, **Yuan Y**, Wang L, Meng X, Xiong G, Zhou J, Cai Y, Han N, Hua LJMP (2019) *OsBRXL4* regulates shoot gravitropism and rice tiller angle through affecting *LAZY1* nuclear localization. Mol. Plant. 12 (8):1143-1156. http://doi.org/doi: 10.1016/j.molp.2019.05.

Appendix

1. CTAB solution

CTAB DNA extraction solution	
2% (w/v)	CTAB
100 mM	Tris-HCl (pH 8.0)
20 mM	EDTA
1.4 M	NaCl

2. LB culture medium

LB culture medium (1 L)	
10 g	Tryptone
5 g	Yeast extract
15 g or 0 g (liqiud)	Agar
10 g	NaCl

3. Wheat protoplasmic system prepared buffer

Enzymatic hydrolysate	
0.6 M	Mannitol
10 mM	MES (pH 5.7)
1.5% (w/v)	Cellulase R-10
0.75% (w/v)	Macerozyme R-10
10 mM	CaCl2
5 mM	β-Mercaptoethanol
50 mg	Ampicillin
0.1% (w/v)	BSA (optional)

W5 solution	
154 mM	NaCl
125 mM	CaCl2
5 mM	KCl
2 mM	MES (pH 5.7)

PEG4000 solution	
40% (w/v)	PEG4000
0.2 M	Mannitol
100 mM	$CaCl_2$

4. Solutions related to wheat genomic transformation

NB medium (1 L)	
2830 mg	KNO_3
463 mg	$(NH_4)_2SO_4$
400 mg	KH_2PO_4
185 mg	$MgSO_4•7H_2O$
166 mg	$CaCl_2•2H_2O$
27.8 mg	$FeSO_4•7H_2O$
37.5 mg	Na_2EDTA
10 mg	$MnSO_4•4H_2O$
3 mg	H_3BO_3

2 mg	$ZnSO_4•7H_2O$
0.25 mg	$Na_2MoO_4•2H_2O$
0.025 mg	$CuSO_4•5H_2O$
0.025 mg	$CoCl_2•6H_2O$
0.75 mg	KI
10 mg	Vitamin B1
1 mg	Vitamin B6
1 mg	Nicotinic acid
100 mg	Myo-inositol
300 mg	Casein hydrolysate
500 mg	Glutamine
2 mg	Glycine
1000 mg	Proline
2 mg	2,4-D
10 g/	Agar

Inoculation solution (1 L)	
1×	NB solution
2 g	Inositol
2 g	Glutamine
500 mg	Casein hydrolysate
10 mL	10% (w/v) Synperonic PE
100 μM	Acetosyringone

Differentiation medium (1 L)	
1×	NB solution
100 mg	Inositol
2 g	Casein hydrolysate
0.2 mg	NAA
0.2 mg	Kinetin
2 mg	6-BA
30 g	Sorbitol
30 g	Sucrose
3 g	Hygromycin
50 mg	Gelrite
10 g	Agar

Rooting medium (1L)	
1×	NB solution
1.0-5.0 mg	Methionine
0.5 mg	IBA
10 g	Agar

5. Solution related to tobacco leave inoculation

Resuspending solution (1 L)
0.1 M $MgCl_2$
0.1 M MES (2-(N-morpholino) ethanesulfonic acid) (PH = 5.6)
1 mM AS

6. Yeast two-hybrid membrane system reagents

6.1 main mediums bought from Clontech

SD/-Leu solid medium	SD/-Leu/Trp solid medium
YPDA solid medium	SD/-Trp/-Leu/-His medium
YDPA liquid medium	SD/-Trp/-Leu/-His/-Ade/X-gal solid medium
SD/-Leu liquid medium	

6.2 Yeast strain: NMY51 (bait)

6.3 Plasmid：

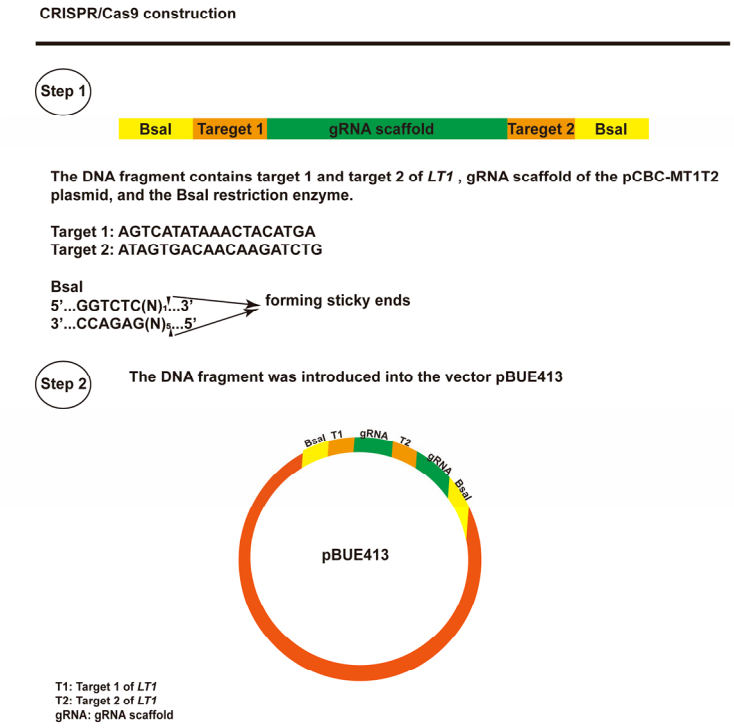
pTSU2-APP positive bait control	pOst1-Nubl wild-type prey
pNubG-Fe65 positive prey	pPR3-N empty plasmid

6.4 Main reagents:

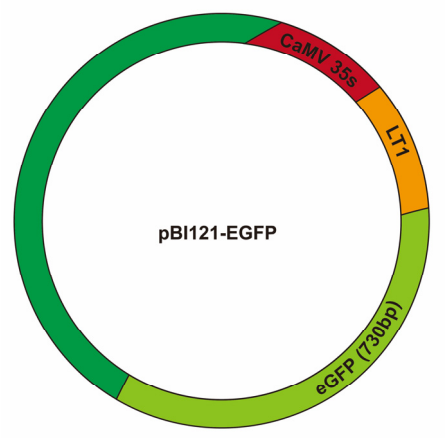
DMSO	10 x TE Buffer
1 M/L LiAc	50% (w/v) PEG4000
0.9% (w/v) NaCl	Single-stranded carrier DNA
X-Gal	3-AT (3-Amino-1,2,4-triazole)

7. Vector maps

7.1 The schematic maps for CRISPR/Cas9



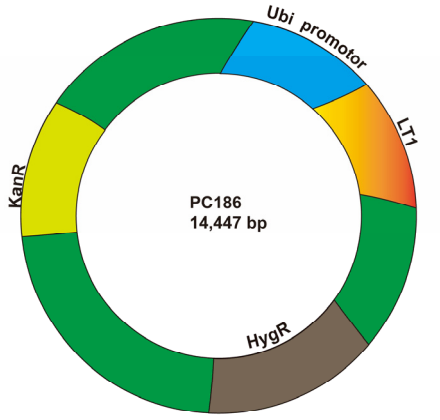
7.2 Subcellular localization vector PBI121 introduced with *LT1*



eGFP: enhanced GFP

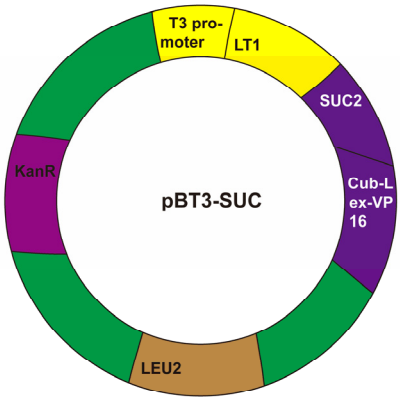
CaMV 35s: Cauliflower Mosaic Virus 35S Promoter

7.3 Vector over-expresses *LT1*



Ubi: Ubiquitin
HygR: Hygromycin B resistance
KanR: Kanamycin resistance

7.4 The pBT3-SUC used in yeast two-hybrid membrane system



SUC2: Sucrose invertase 2
LEU2: Isopropylmalate Dehydratase
KanR: Kanamycin Resistance
a fusion domain responsible for activating transcriptional responses upon interaction between the bait and prey proteins.

8. The bioinformatics applications and data sets

8.1 The uni-BSA-related applications and the datasets are deposited in Onedrive network disk due to their huge sizes: https://1drv.ms/f/s!ApLKMOp4Q8-cgdYkWA7Ai4IrsH8Zvw?e=K1YUPc

8.2 The complete RNA-seq analysis pipeline and related tools are available at: https://1drv.ms/f/s!ApLKMOp4Q8-cgdcnmpj0hkTS-Z3QJA?e=l6qrLY

8.3 All the predicted positions of HSP90 proteins are available at: https://1drv.ms/u/s!ApLKMOp4Q8-cgeYKNow5jc9ZzMCcTg?e=Necbag