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LAZY4 acts additively with the starch–statolith-dependent gravity-sensing pathway to regulate shoot gravitropism and tiller angle in rice

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ABSTRACT

Rice tiller angle is a key agronomic trait that has significant effects on the establishment of a high-yield rice population. However, the molecular mechanism underlying the control of rice tiller angle remains to be clarified. Here, we characterized the novel tiller-angle gene *LAZY4* (*LA4*) in rice through map-based cloning. *LA4* encodes a C3H2C3-type RING zinc-finger E3 ligase localized in the nucleus, and an *in vitro* ubiquitination assay revealed that the conserved RING finger domain is essential for its E3 ligase activity. We found that expression of *LA4* can be induced by gravistimulation and that loss of *LA4* function leads to defective shoot gravitropism caused by impaired asymmetric auxin redistribution upon gravistimulation. Genetic analysis demonstrated that LA4 acts in a distinct pathway from the starch biosynthesis regulators LA2 and LA3, which function in the starch–statolith-dependent pathway. Further genetic analysis showed that *LA4* regulates shoot gravitropism and tiller angle by acting upstream of *LA1* to mediate lateral auxin transport upon gravistimulation. Our studies reveal that *LA4* regulates shoot gravitropism and tiller angle upstream of *LA1* through a novel pathway independent of the *LA2–LA3*-mediated gravity-sensing mechanism, providing new insights into the rice tiller-angle regulatory network.

Key words: auxin, shoot gravitropism, tiller angle, LAZY4, rice

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INTRODUCTION

Rice (*Oryza sativa* L.) is a major cereal crop that feeds more than half of the world's population. New elite rice varieties with ideal rice architecture can produce much higher grain yields to meet the increasing demand for rice production. Rice tiller angle, an important agronomic trait defined as the angle between the vertical line and the side tillers with maximum inclination (Wang et al., 2022), controls plant density by affecting the efficiency of light capture and thus photosynthesis. In practice, as an important determinant of efficient plant architecture in rice, tiller angle has long attracted the attention of breeders for the design of ideal rice architecture (Wang and Li, 2008).

The prostrate-to-erect growth transition is a key domestication trait in rice (Gao et al., 2019; He et al., 2021; Xu and Sun, 2021; Wang et al., 2022). In recent years, some key domestication genes related to tiller angle, such as *PROSTRATE GROWTH1* (*PROG1*), *PROG7*, and *RICE PLANT ARCHITECTURE DOMESTICATION*, have been shown to regulate the growth-habit transition in rice (Jin et al., 2008; Tan et al., 2008; Hu et al., 2018; Wu et al., 2018). The tiller angle of Asian cultivated rice

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experienced further post-domestication selection, and the two major subspecies, *O. sativa* ssp. *japonica* and *indica*, show significantly different tiller angles (Wang et al., 2022). Some key regulators, such as Tiller Angle Control 1 (TAC1), TAC3, TAC4, TILLER INCLINED GROWTH 1, and DWARF2, have been shown to be responsible for the different tiller angles of *japonica* and *indica* rice (Yu et al., 2007; Jiang et al., 2012; Dong et al., 2016; Zhang et al., 2019; Li et al., 2021a).

Shoot gravitropism plays an essential role in determining tiller angle (Wang et al., 2022). When plants are inverted to a given angle, the shoots and roots orient their growth direction upward (negative gravitropism) and downward (positive gravitropism), respectively (Blancaflor and Masson, 2003; Morita and Tasaka, 2004; Morita, 2010; Toyota and Gilroy, 2013). After sensing the gravistimulation, plants can restore their appropriate growth angle through differential growth (Toyota and Gilroy, 2013). The gravitropic response is composed of the following sequential steps: gravity perception, signal transduction and transmission, and differential organ growth, resulting in curvature (Perbal and Driss-Ecole, 2003; Morita and Tasaka, 2004). Studies have provided molecular evidence in support of two major hypotheses about the mechanism of gravitropism: the starch–statolith hypothesis and the Cholodney–Went hypothesis (Sack, 1991; Kiss, 2000; Friml et al., 2002; Hashiguchi et al., 2013). The starch–statolith hypothesis suggests that sedimentable amyloplasts containing high-density starch granules play a key role in sensing the direction of gravity (Sack, 1991; Kiss, 2000). This hypothesis is strongly supported by studies of starchless mutants (Kiss et al., 1997; Fujihira et al., 2000; Morita and Tasaka, 2004). The rice mutant *agpl1*, which lacks a large subunit of ADP-glucose pyrophosphorylase (AGP), exhibits a defective gravitropic response and thus an increased tiller angle (Okamura et al., 2013, 2014). A study of the double mutant *agpl1 agpl3* implied that the expansion of rice tiller angle was positively correlated with a reduction in starch content of the culm (Okamura et al., 2014). Recent studies found that the loss-of-function mutant of *O. sativa plastidic phosphoglucomutase* (*OspPGM*), which encodes a starch biosynthetic enzyme, showed impaired starch biosynthesis and a larger tiller angle (Huang et al., 2021; Lee et al., 2016). The YbaB protein LAZY2 (LA2) can interact with OspPGM in chloroplasts to regulate shoot gravitropism and tiller angle in rice (Huang et al., 2021). Another study demonstrated that LA3 can interact with LA2 to form an LA3–LA2–OspPGM protein complex to modulate starch biosynthesis in gravity-sensing tissues (Cai et al., 2023). Starch granules were completely absent from amyloplasts in the gravity-sensing tissues of both *la2* and *la3* mutants, causing a larger tiller angle and defective shoot gravitropism (Huang et al., 2021; Cai et al., 2023). These studies demonstrated that LA2 and LA3 are both indispensable for the starch–statolith-dependent gravity-sensing mechanism in the control of shoot gravitropism and rice tiller angle. In rice, *Loose Plant Architecture1* regulated the sedimentation rate of amyloplasts to affect shoot gravitropism and thus inhibits the formation of a large tiller angle, implying that amyloplast sedimentation may also be involved in the regulation of shoot gravitropism and tiller angle. In *Arabidopsis*, the RING-type E3 ligase SHOOT GRAVITROPISM9 (SGR9) modulates amyloplast dynamics, and the *sgr9* mutant did not show sedimentation with increased saltatory movement, resulting in reduced gravitropism (Nakamura et al., 2011). However, few genes have been identified as acting in the starch–statolith-independent pathway (SSIDP) to date.

The Cholodney–Went hypothesis proposes that asymmetric auxin distribution causes the differential growth of tropism (Friml et al., 2002; Morita and Tasaka, 2004). Auxin transport plays an essential role in regulating tiller angle (Gao et al., 2019; Wang et al., 2022). *LA1*, the first identified tiller-angle gene, regulates shoot gravitropism and tiller angle by modulating asymmetric auxin distribution upon gravistimulation in rice shoots (Li et al., 2007; Yoshihara and Iino, 2007). Screening for suppressors of *la1* revealed the novel function of strigolactones, a group of carotenoid-derived branching-inhibiting hormones that can also attenuate shoot gravitropism to modulate rice tiller angle by inhibiting local auxin biosynthesis (Sang et al., 2014). Further studies revealed that the LA1-interacting protein Brevis Radix-Like 4 could regulate shoot gravitropism and tiller angle by affecting the nuclear localization of LA1 (Li et al., 2019). Dynamic transcriptome analysis identified a core tiller-angle regulatory pathway in which HEAT STRESS TRANSCRIPTION FACTOR 2D (HSFA2D) functions as an upstream positive regulator of *LA1* to initiate asymmetric redistribution of auxin and induces asymmetric expression of the redundant downstream transcription factor genes *WUSCHEL-RELATED HOMEOBOX6* (*WOX6*) and *WOX11* to regulate rice tiller angle (Zhang et al., 2018). A recent study found that OsHOX1 and OsHOX28 positively regulate rice tiller angle by acting upstream of *HSFA2D*, directly binding to its promoter to suppress its expression (Hu et al., 2020). In addition, OsmiR167a–OsARF12/17/25 may participate in the *HSFA2D*- and *LA1*-dependent asymmetric auxin distribution pathway to control rice tiller angle (Li et al., 2020). Recent studies have found that the domestication factor PROG1, as a repressor, can directly bind the promoter of *LA1* to inhibit its expression, and LA1 can also repress the expression of *PROG1* by binding to its promoter (Wang et al., 2023a, 2023b; Zhang et al., 2023); these studies established the genetic regulatory relationship between the key domestication gene *PROG1* and the classical tiller-angle gene *LA1*. Polar auxin transport and auxin accumulation were reduced at the base of shoots of the *fuct-1* mutant, causing a defective gravitropic response and a large tiller angle (Harmoko et al., 2016). The bZIP-family transcription factor OsbZIP49 can also regulate rice tiller angle by controlling local auxin homeostasis (Ding et al., 2021). Overexpression of the auxin transporter OsPIN2 or suppression of OsPIN1 alters polar auxin transport to increase rice tiller angle (Xu et al., 2005; Chen et al., 2012).

In this study, we identified a novel rice tiller-angle gene, *LA4,* that encodes a C3H2C3-type RING finger E3 ligase. We demonstrated that *LA4* functions upstream of *LA1* to modulate lateral auxin transport (LAT), shoot gravitropism, and thus tiller angle in rice. We also showed that LA4 acts in a different pathway from the starch biosynthesis factors LA2 and LA3 through a novel SSIDP. This study greatly broadens our current understanding

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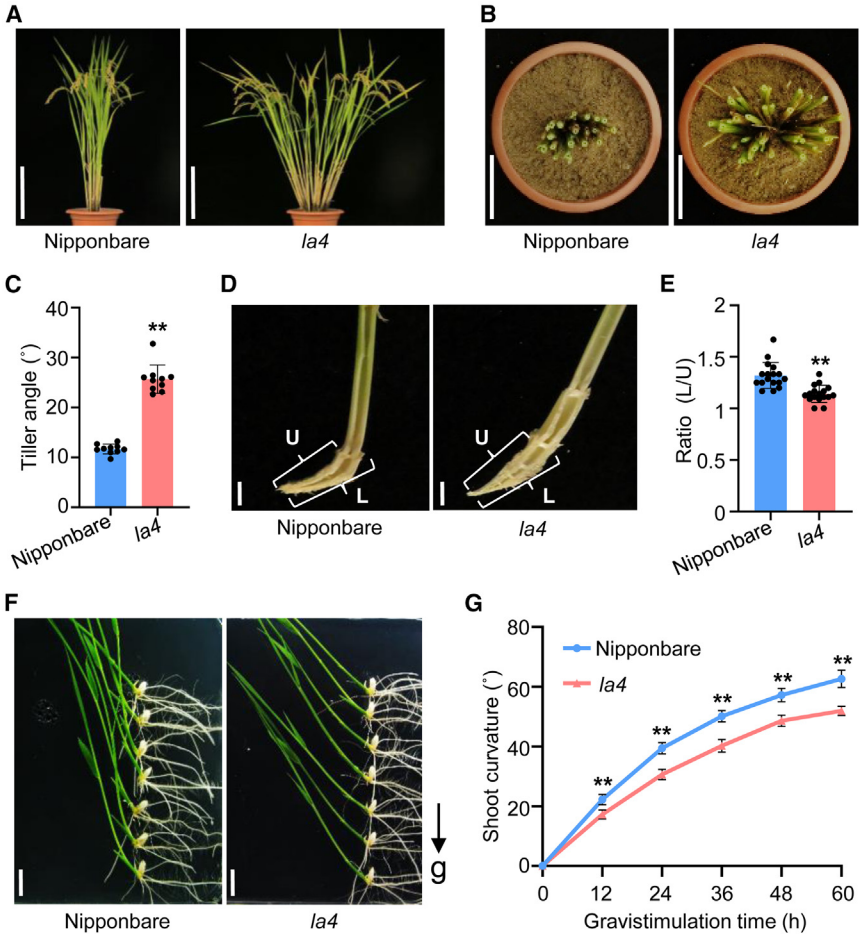

Figure 1. Phenotypic characterization of the rice mutant *la4*.

(A) Gross morphology of Nipponbare (NP) and the *lazy4 (la4)* mutant at the adult stage. Scale bars, 20 cm.

(B) Comparison of the culm bases of NP and the *la4* mutant shown in vertical view. Scale bars, 10 cm.

(C) Statistical analysis of tiller angle of NP and the *la4* mutant. Data are presented as mean ± SD (n = 10). Student's t-test, $^{**}P < 0.01$.

(D) Magnified tiller bases of NP and the *la4* mutant. L, lower side; U, upper side. Scale bars, 1 cm.

(E) Ratio of the length between the lower side (L) and upper side (U) in NP and the *la4* mutant. Data are presented as mean ± SD (n = 17). Student's t-test, $^{**}P < 0.01$.

(F) Shoot curvature of NP and *la4* seedlings after gravistimulation for 60 h. The arrow indicates the direction of gravity (g). Scale bars, 2 cm.

(G) Kinetic analysis of shoot curvature of NP and *la4* seedlings upon gravistimulation. Data are presented as mean ± SD (n = 15). Student's t-test, $^{**}P < 0.01$.

of the molecular mechanisms underlying shoot gravitropism and rice tiller angle and provides new insights into the genetic regulatory network of rice tiller angle.

RESULTS

Phenotypic characterization of the rice tiller-angle mutant *la4* in the Nipponbare background

To decipher the molecular mechanisms underlying rice tiller angle, we screened for new components that controlled this trait. A spontaneous rice mutant with loose plant architecture, designated *la4*, was isolated from the *japonica* cultivar Nipponbare (*Oryza sativa* L.) background. The *la4* mutant exhibited a larger tiller angle than the wild type (Figure 1A–1C). Phenotypic characterization and statistical analysis revealed that the large tiller angle of *la4* was caused by defective asymmetrical growth of the shoot bases compared with that of the wild type, and the length ratio between the lower side and upper side of the shoot bases was significantly lower in *la4* than in the wild type (Figure 1D and 1E). Rice tiller angle is closely associated with shoot gravitropic response (Wang et al., 2022). To check whether the enlarged tiller angle of *la4* was caused by defective shoot gravitropism, we examined the gravitropic responses of seedlings. Compared with wild-type seedlings, the *la4* seedlings displayed significantly less shoot curvature upon gravistimulation (Figure 1F and 1G). These results indicated that *LA4* positively regulates shoot gravitropism in rice and that the defective shoot gravitropism of *la4* is responsible for its large tiller angle.

Cloning and functional confirmation of the *LA4* gene

Genetic analysis revealed a 3:1 segregation ratio of tiller angle in the F_2 progeny of a cross between *la4* and its wild type, Nipponbare (Supplemental Table 4), suggesting that the large tiller angle of *la4* is controlled by a single recessive gene. To isolate the *LA4* gene, we took a map-based cloning approach using an F_2 population derived from a cross between *la4* (Nipponbare background) and ZF802 (a wild-type *indica* variety). *LA4* was initially mapped to the region between the two molecular markers M1 and M2 on chromosome 5 (Figure 2A). By screening for more recombinant individuals in the F_2 and $F_{2:3}$ segregating populations using newly developed markers, we narrowed down *LA4* to a 107-kb region between markers M8 and M9 (Figure 2A). Among the 20 candidate genes in this region, sequencing analysis revealed only a single base substitution (C→A) in the exon of *LOC_Os05g33830* in *la4*, which produced a premature stop codon (Figure 2A). To verify whether the base substitution in the exon of *LOC_Os05g33830* was responsible for the large tiller angle, we performed a complementation test and confirmed that the complemented line *pLA4C/la4* rescued the large tiller angle of *la4* (Figures 2B and 2C). To further confirm the function of *LA4*, we generated loss-of-function mutations in *LA4* in the *japonica* rice variety ZH11 through CRISPR–Cas9 (CR) gene editing. Using two single guide RNAs targeting different regions of the exon, we generated multiple independent transgenic lines for each target site (Supplemental Figure 1A). Compared with wild-type ZH11 plants, all the CR-engineered *la4* mutant lines (*CR-la4*) had significantly enlarged tiller angles (Supplemental Figure 1B and 1C). These results indicated that *LOC_Os05g33830* is the *LA4* gene.

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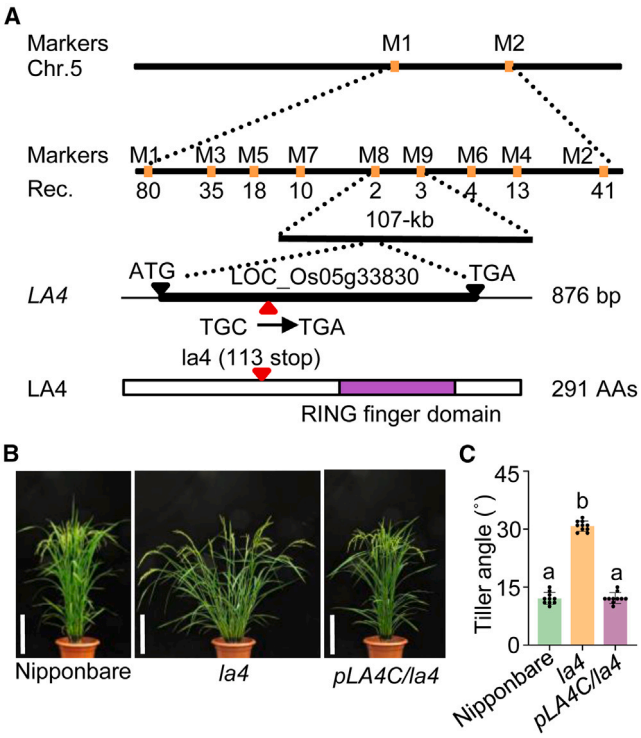

Figure 2. Map-based cloning and complementation test of *LA4*.

(A) Map-based cloning of *LA4*. *LA4* was narrowed down to a 107-kb candidate region between molecular markers M8 and M9. Numbers under the markers indicate recombinants. The red triangles indicate the 1-bp substitution from C (in NP) to A (in *la4*) in the exon of *LA4*, which results in premature protein termination.

(B) Gross morphology of NP, *la4*, and representative *pLA4/la4* complemented transgenic plants at the adult stage. Scale bars, 20 cm.

(C) Statistical analysis of the tiller angle of NP, *la4*, and *pLA4/la4* complemented transgenic plants. Means with different letters are significantly different ($P < 0.05$, ANOVA and Tukey's honestly significant difference).

Expression patterns of *LA4* and subcellular localization of LA4

To define the temporal and spatial expression patterns of *LA4*, we performed RT–qPCR analysis to check the expression of *LA4* in various tissues. *LA4* transcript levels were high in the roots and pulvinus; moderate in nodes, leaves, leaf sheaths, and shoots; and lower in young panicles (Supplemental Figure 2A). Previous studies have shown that the pulvinus of rice seedlings plays a crucial role in shoot gravitropism and tiller angle (Yoshihara and Iino, 2007; Wu et al., 2013); the higher expression levels of *LA4* in the pulvinus were thus consistent with its control of tiller angle. To test whether the transcription of *LA4* was sensitive to gravistimulation, we performed a time-course treatment in which we rotated the seedlings by 90°, and we found that *LA4* was significantly upregulated by gravistimulation (Supplemental Figure 2B). To determine the subcellular localization of LA4, GFP-tagged LA4 (LA4–GFP), was transiently expressed in rice protoplasts (Supplemental Figure 2C). In contrast to the GFP control, which was distributed in the cytoplasm and nucleus of rice protoplast cells, the LA4–GFP fusion protein was located exclusively in the nucleus (Supplemental Figure 2C), suggesting that *LA4* encodes a nuclear protein.

LA4 encodes a C3H2C3-type RING finger E3 ligase

Sequence analysis indicated that LA4 was a typical Cys-3/His-2/Cys-3 zinc-finger protein that contained a C3H2C3-type RING finger domain in the C-terminal region (Supplemental Figure 3). The RING finger domain is composed of two zinc-finger motifs and includes six highly conserved cysteines and two histidine residues (Supplemental Figure 3). Multiple sequence alignment showed that LA4 shares a conserved RING finger domain at the C terminus with *Arabidopsis* SGR9 (Supplemental Figure 3), a known E3 ubiquitin ligase (Nakamura et al., 2011). Because RING-type E3 ubiquitin ligases are characterized by a conserved RING finger domain, we carried out an *in vitro* ubiquitination assay. We constructed a recombinant protein, MBP–LA4 (RING), in which MBP was fused to the RING finger domain of LA4. High-molecular-weight bands of ubiquitinated LA4–MBP were detected by immunoblotting with both anti-MBP and anti-Myc antibodies after incubation of MBP–LA4 (RING) with an E1-activating enzyme, an E2 (UbcH5c)-conjugating enzyme, and Myc-tagged ubiquitin in an *in vitro* ubiquitination reaction system in the presence of ATP (Figures 3A and 3B). In negative control reactions, no ubiquitinated MBP–LA4 (RING) was detected when E1, E2, or MBP–LA4 (RING) was absent (Figures 3A and 3B). RING-type E3 ligase activity has been reported to depend on the RING finger domain (Katoh et al., 2005). We therefore performed site-directed mutagenesis to generate two E3 ligase-inactivated derivatives with Cys236 to Ala ($LA4^{C236A}$) and Trp248 to Ala ($LA4^{W248A}$) mutations in the conserved RING finger domain. *In vitro* ubiquitination analysis showed that both the C236A and the W248A mutation diminished the E3 ligase activity of LA4 (Figure 3A and 3B). We also examined MBP–LA4 (RING) E3 activity in the presence of different concentrations of MBP–LA4 (RING) protein when all other reagents were present in sufficient quantities, and the results showed that MBP–LA4 (RING) was ubiquitinated in a dose-dependent manner (Supplemental Figure 4A and 4B). By contrast, no E3 activity was detected for all concentrations of MBP–LA4 (RING) protein examined when E1 was absent (Supplemental Figure 4A and 4B). These results suggested that the conserved RING finger domain of LA4 is essential for its E3 ligase activity.

LA4 regulates shoot gravitropism and tiller angle through a novel pathway independent of the *LA2–LA3*-mediated gravity-sensing mechanism

Previous studies have found that starch granules are completely absent from the amyloplasts of gravity-sensing tissues in *la2* and *la3* mutants (Huang et al., 2021; Cai et al., 2023). To determine whether *LA4* and *LA2* act in the same pathway, we crossed *CR-la4-1* with *CR-la2-1* to generate the *CR-la2-1 CR-la4-1* double mutant. We first examined starch granules, the major inclusions of amyloplasts, in the gravity-sensing leaf sheath base of the double mutant *CR-la2-1 CR-la4-1* and the two single mutants. Large numbers of starch granules were stained purple and aggregated in cells of the leaf sheath base in ZH11 and *CR-la4-1* (Figure 4A and 4C). By contrast, starch granules were completely absent from amyloplasts in the gravity-sensing cells of *CR-la2-1* and *CR-la2-1 CR-la4-1* (Figure 4B and 4D). Phenotypic analysis revealed that the *CR-la2-1 CR-la4-1* double mutant had a significantly larger tiller angle than either *CR-la2-1* or *CR-la4-1* (Figure 4E and 4G). In

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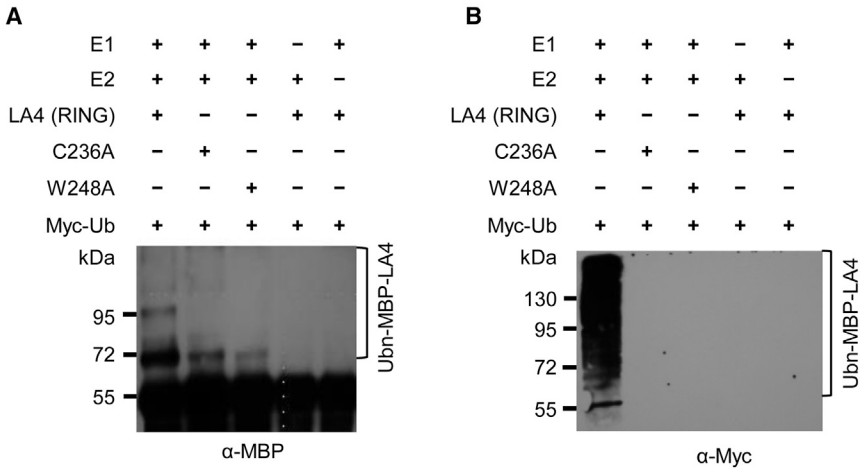

Figure 3. LA4 (RING) shows ubiquitin E3 ligase activity *in vitro*.

(A and B) E3 ligase activity of wild-type and mutated LA4 (RING). For the ubiquitination assay, we prepared MBP–LA4 (RING), in which MBP was fused to the RING finger domain of LA4, and the amino acid substitution W248A or C236A was introduced into the RING finger domain. An anti-MBP antibody was used to detect MBP–LA4 (RING) or mutated MBP–LA4 (RING) **(A)**, and an anti-Myc antibody was used to detect ubiquitinated MBP–LA4 (RING) or mutated MBP–LA4 (RING) with Myc-tagged ubiquitin **(B)**.

addition, defects in shoot gravitropism were much more severe in *CR-la2-1 CR-la4-1* seedlings than in those of the *CR-la2-1* and *CR-la4-1* single mutants (Figure 4F and 4H). These results revealed that *LA4* and *LA2* act through different pathways to regulate shoot gravitropism and tiller angle in rice. To confirm this result, we crossed *CR-la4-1* with *CR-la3-6* to generate the double mutant *CR-la3-6 CR-la4-1*. The double mutant *CR-la3-6 CR-la4-1* also showed significantly larger tiller angle than either single mutant (Supplemental Figure 5A and 5C). Compared with ZH11, the double mutant *CR-la3-6 CR-la4-1* and the two single mutants all displayed a reduced gravitropic response, and the defective gravitropism was more severe in the double mutant than in the two single mutants (Supplemental Figure 5B and 5D). These results confirmed that *LA4* regulates tiller angle in rice through a novel pathway, independent of the shoot gravitropism regulatory mechanism mediated by *LA2–LA3*.

LA4 and LA2 act additively to regulate asymmetric auxin distribution upon gravistimulation

Auxin asymmetric distribution plays a key role in regulating shoot gravitropism and tiller angle in rice (Li et al., 2007). To check whether *LA4* regulates auxin asymmetric distribution upon gravistimulation, we carried out ^{3}H-IAA transport assays in etiolated coleoptiles of the *CR-la4-1* mutant. The radioactivity ratio between the lower and upper halves of the coleoptiles was lower in *CR-la4-1* than in ZH11, revealing that LAT was attenuated in *la4* upon gravistimulation (Figure 5A). Treatment with the control compound ^{3}H-benzoic acid produced no significant differences between the *CR-la4-1* mutant and ZH11 in the radioactivity ratio of the lower to upper coleoptile halves (Figure 5A). Interestingly, although the defective LAT of *CR-la4-1* was similar to that of *CR-la2-1*, the *CR-la2-1 CR-la4-1* double mutant showed much more severely defective LAT than either single mutant (Figure 5A). To further confirm that *LA4* and *LA2* act additively to regulate asymmetric auxin distribution upon gravistimulation, we also checked the expression of the auxin-responsive genes *OsIAA20*, *WOX6*, and *WOX11* in seedlings upon gravistimulation. Compared with the single mutants, the *CR-la2-1 CR-la4-1* double mutants showed significantly lower expression of *OsIAA20*, *WOX6*, and *WOX11* in the lower sides of shoot bases upon gravistimulation (Figure 5B–5D). These results confirmed that asymmetric auxin distribution was much more severely impaired in the *CR-la2-1 CR-la4-1* double mutant than in either single mutant. *LA4* and *LA2* thus act additively to regulate asymmetric auxin distribution upon gravistimulation and function in different pathways to modulate shoot gravitropism and tiller angle in rice.

LA4 acts upstream of *LA1* to regulate tiller angle in rice

LA1 is a key component that regulates tiller angle by modulating asymmetric auxin distribution in rice (Li et al., 2007). To study the genetic relationship between *LA4* and *LA1* in the regulation of rice tiller angle, we crossed *CR-la4-1* with *la1* to generate the *la1 CR-la4-1* double mutant. Phenotypic characterization and statistical analysis revealed that the tiller angle of the double mutant *la1 CR-la4-1* was similar to that of the single mutant *la1*, and both were significantly larger than that of the single mutant *CR-la4-1* (Figure 6A and 6C). We next examined the gravity response of the seedlings. Compared with *CR-la4-1* seedlings, *la1* and *la1 CR-la4-1* mutant seedlings had significantly reduced gravitropic responses, whereas *la1 CR-la4-1* and *la1* seedlings showed similar levels of shoot gravitropism (Figure 6B and 6D). To confirm the genetic relationship between *LA4* and *LA1* in regulation of rice tiller angle, we also crossed *CR-la4-1* with *LA1*-overexpressing transgenic plants (*LA1 OE-1*) to generate *LA1 OE-1 CR-la4-1* plants. Phenotypic characterization and statistical analysis showed that overexpression of *LA1* restored the tiller-angle phenotype of *CR-la4-1* (Supplemental Figure 6 and Figure 6E). These results implied that *LA4* acts upstream of *LA1* to mediate LAT, shoot gravitropism, and thus tiller angle in rice.

Haplotype analysis of *LA4* in rice

To investigate allelic variations of *LA4* in rice accessions, we analyzed the haplotypes of *LA4* in *japonica*, *indica*, and wild rice populations (Supplemental Figure 7A–7C). The haplotypes of *LA4* could be grouped into 4 haplotype clusters (HCs), which were strongly associated with rice species. HC1 and HC4 were mainly composed of *Oryza rufipogon* individuals and *O. sativa* ssp. *indica* individuals, respectively. However, most *O. sativa* ssp. *japonica* individuals were grouped into HC2 and HC3 (Supplemental Figure 7B). Further haplotype network analysis revealed that the two *O. sativa* ssp. *japonica* HCs (HC2 and HC3) were separated from each other by various haplotypes in *O. rufipogon* (Supplemental Figure 7C), indicating possible functional differentiation in *japonica* rice groups.

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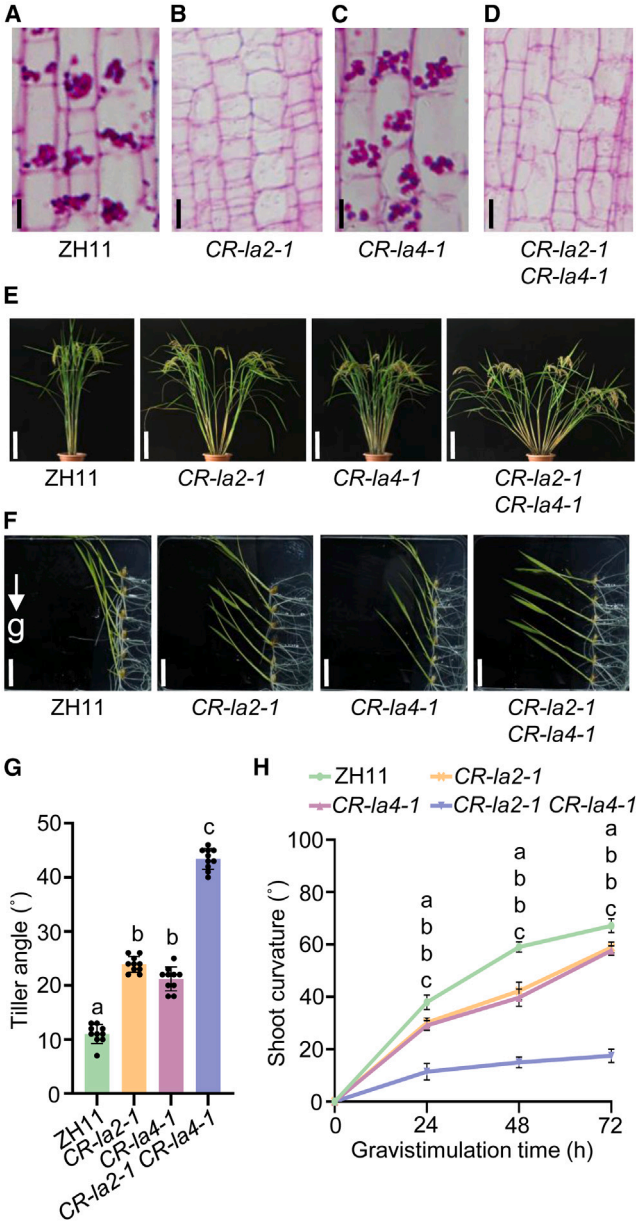

Figure 4. *LA4* acts additively with the starch–statolith-dependent g-sensing pathway mediated by *LA2* to regulate rice tiller angle.

(A–D) Starch granule staining of leaf sheaths from 7-day-old ZH11 **(A)**, *CR-la2-1* **(B)**, *CR-la4-1* **(C)**, and *CR-la2-1 CR-la4-1* **(D)** seedlings. Scale bars, 10 μm.

(E) Gross morphology of ZH11, *CR-la2-1*, *CR-la4-1*, and *CR-la2-1 CR-la4-1* plants at the adult stage. Scale bars, 20 cm.

(F) Shoot gravitropism of ZH11, *CR-la2-1*, *CR-la4-1*, and *CR-la2-1 CR-la4-1* plants after gravistimulation for 72 h. Scale bars, 2 cm. The arrow indicates the direction of g.

(G) Statistical analysis of tiller angle in the lines in **(E).** Data are presented as mean ± SD (n = 10). Means with different letters are significantly different (P < 0.05, ANOVA and Tukey's honestly significant difference).

(H) Kinetic analysis of shoot curvature of the lines in **(F)** upon gravistimulation. Data are presented as mean ± SD (n = 15). Means with different letters are significantly different (P < 0.05, ANOVA and Tukey's honestly significant difference).

DISCUSSION

Rice tiller angle is a dominant factor that determines the efficiency of light capture for photosynthesis, and an appropriate tiller angle is essential for ideal plant architecture in rice. Although significant progress has been achieved recently in the characterization of corresponding regulatory genes, the molecular mechanisms that underlie rice tiller angle remain to be clarified. In this study, we identified the novel tiller-angle gene *LA4* that acts upstream of *LA1* to modulate LAT and thus shoot gravitropism and rice tiller angle. *LA4* and *LA2* (or *LA3*) exhibit additive effects on the regulation of shoot gravitropism and tiller angle: *LA4* acts in an *LA2–LA3*-independent pathway for control of rice shoot gravitropism and tiller angle, whereas the LA3–LA2–OspPGM protein complex functions in the SSDP. Both the *LA2–LA3*-independent pathway and the SSDP modulate shoot gravitropism by acting upstream of *LA1* to mediate asymmetric auxin distribution and thus the asymmetric expression of *WOX6* and *WOX11*, thereby determining rice shoot gravitropism and tiller angle in rice (Figure 7).

LA4 acts in a novel pathway independent of the *LA2–LA3*-mediated gravity-sensing mechanism

The classical starch–statolith hypothesis proposes that gravity is sensed in specialized cells through the sedimentation of amyloplasts that contain starch granules (Sack, 1997). However, an increasing number of studies have suggested that the starch–statolith pathway may not be the only mechanism for gravity perception. Previous studies found that gravitropism of maize coleoptiles was not completely lost upon surgical removal of the vascular bundle sheath, the presumed gravity-sensing organ that contains the gravity-sensing amyloplasts (Edelmann, 2018). In *Arabidopsis*, the starch-deficient mutants *pgm* and *early starvation1* showed only partially defective shoot gravitropism, indicating that alternative factors in addition to starch-filled amyloplasts were involved in gravity perception (Periappuram et al., 2000; Yu et al., 2000; Song et al., 2021). Some studies have proposed that starch-deficient amyloplasts may also trigger gravity sensing by sedimentation in response to their own inherent mass and that the increased mass associated with starch accumulation would accelerate amyloplast sedimentation for a full gravitropic response (Morita, 2010; Nakamura et al., 2019). However, a recent study confirmed that empty endodermal plastids in the *early starvation1* mutant did not sediment in the direction of gravity, thus disproving the above hypothesis (Song et al., 2021). Although starch granules were completely absent in amyloplasts of gravity-sensing cells in the *la2 and la3 mutants*, they showed moderate defects in shoot gravitropism and a limited increase in rice tiller angle (Huang et al., 2021; Cai et al., 2023). On the basis of this new genetic evidence, we proposed the existence of an SSIDP in gravity perception in our previous studies (Huang et al., 2021; Wang et al., 2022; Cai et al., 2023). In the present study, we found that both the *CR-la2-1 CR-la4-1* and *CR-la3-6 CR-la4-1* double mutants showed much more severely impaired shoot gravitropism and a much larger tiller angle than the *CR-la4-1*, *CR-la2-1*, and *CR-la3-6* single mutants (Figure 4E–4H and Supplemental Figure 5), suggesting that *LA4* may act in a novel pathway independent of the *LA2–LA3*-mediated gravity-sensing

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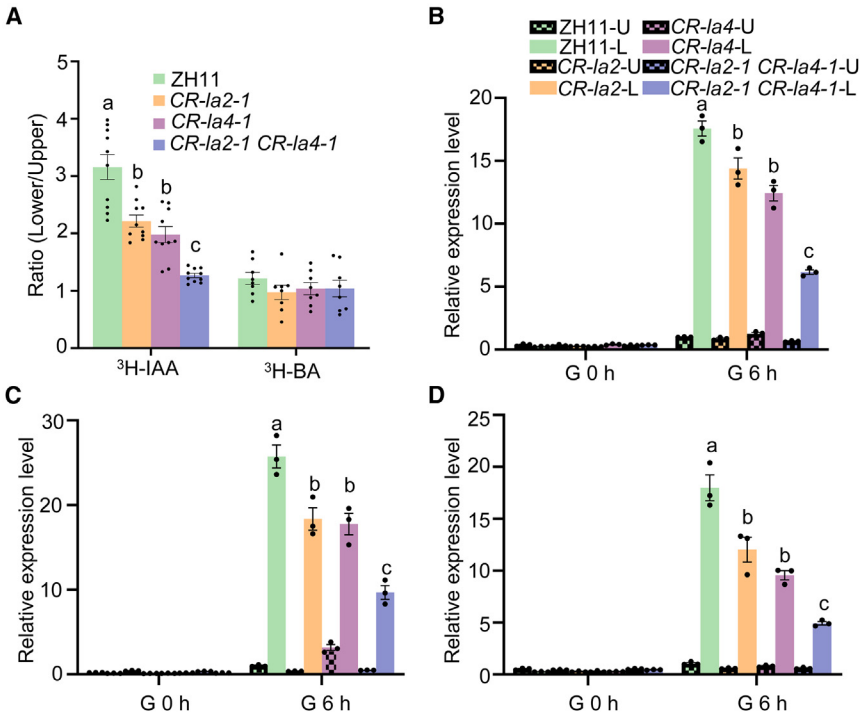

Figure 5. *LA4* and *LA2* act additively to regulate asymmetric auxin distribution upon gravistimulation.

(A) Comparison of lateral auxin transport (LAT) in ZH11, *CR-la2-1*, *CR-la4-1*, and *CR-la2-1 CR-la4-1* coleoptiles. The Counts per minute (Cpm) ratio indicates the radioactivity of the lower side to that of the upper side of coleoptiles upon gravistimulation for 2.5 h. Data are presented as mean ± SE (n = 8–10). Means with different letters are significantly different ($P < 0.05$, ANOVA and Tukey's honestly significant difference).

(B–D) Expression levels of *OsIAA20* **(B)**, *WOX6* **(C)**, and *WOX11* **(D)** at the upper sides and lower sides of shoot bases from ZH11, *CR-la2-1*, *CR-la4-1*, and *CR-la2-1 CR-la4-1* seedlings upon gravistimulation. Data are presented as mean ± SE (n = 3). Means with different letters are significantly different ($P < 0.05$, ANOVA and Tukey's honestly significant difference).

mechanism. In plants, the cytoskeleton is a key candidate for transmission of the force exerted by gravisensors to the mechanoreceptors, a process that is thought to play an important role in gravity sensing (Najrana and Sanchez-Esteban, 2016). A recent study found that gravity could trigger the asymmetric redistribution of actin filaments in the tip of the *Physcomitrella patens* protonema and that this redistribution of actin filaments was dependent on the microtubule-based cellular motor gravitropism group C to mediate plant gravitropism (Li et al., 2021b). These studies suggest important cues for further exploration of the SSIDP.

We propose that LA4 may act in the SSIDP, given that loss of LA2 and LA3 function resulted in the absence of starch granules in the gravity-sensing cells and that LA4 and LA2 (or LA3) had additive effects on shoot gravitropism and rice tiller angle. We confirmed that the LA4-involved SSIDP and the LA3–LA2–OspPGM-determined SSDP converge at LA1-mediated asymmetric auxin distribution in the control of rice shoot gravitropism and tiller angle, thus identifying not only a key regulator of the novel SSIDP but also the node at which these two gravity-perception pathways interact.

We also found that the tiller angle of *la4* in the Nipponbare background was larger than that of the *CR-la4* mutant in the ZH11 background (Figure 1, 2, 4, and 6). This variation is likely attributable to their different genetic backgrounds, given that tiller angle is controlled by multiple genes. Further identification of the responsible genes would shed light on the SSIDP and/or SSDP gravity perception pathways. In addition, haplotype analysis showed that *LA4* has four different haplotypes and that the HCs were strongly associated with individual rice species (Supplemental Figure 7). HC1 of *LA4* was composed mainly of wild rice. It would be interesting to analyze the function of *LA4* in the wild rice lines, and identification of beneficial *LA4* alleles selected during tiller angle domestication would further broaden our understanding of the molecular basis underlying rice tiller angle.

LA4 is the functional ortholog of *SGR9* but has distinct features

The *Arabidopsis* ortholog of *LA4* is *SGR9*, which can inhibit branch angle by positively regulating shoot gravitropism (Nakamura et al., 2011). Here, we found that *LA4* can inhibit rice tiller angle by enhancing LAT and shoot gravitropism (Figures 1 and 5), suggesting the functional similarity between *SGR9* and *LA4*. Our study revealed that *LA4* was significantly induced by gravistimulation to positively regulate LAT (Supplemental Figure 2B and Figure 5). However, it is not yet known whether expression of *SGR9* can respond to gravistimulation, and this is worthy of further exploration. The *la4* mutant (Nipponbare background) exhibits a slightly enlarged tiller angle, and its shoot gravitropism is also reduced to a lesser extent (Figures 1A–1C, 1F, and 1G). By contrast, the *sgr9* mutant shows a much larger branch angle, and its branches grow almost horizontally (Nakamura et al., 2011). Consistent with its branch angle, the shoot gravitropism of *sgr9* is greatly reduced compared with that of the wild type (Nakamura et al., 2011). Higher plants sense the direction of gravity using the sedimentation of amyloplasts in statocytes during gravitropism (Blancaflor et al., 1998; Fukaki et al., 1998; Morita and Tasaka, 2004). In the *sgr9* mutant, amyloplasts move dynamically around the cell before and after gravistimulation, and this movement appears to be random (Nakamura et al., 2011), implying that SGR9 is more likely to act in the SSDP. Our study revealed that LA4 may act in the SSIDP, given the additive effects of LA4 and LA2 (or LA3) on shoot gravitropism and rice tiller angle. Moreover, LA4 and SGR9 also showed different subcellular localizations (Supplemental Figure 2C), which may also contribute to their distinct roles in regulating shoot

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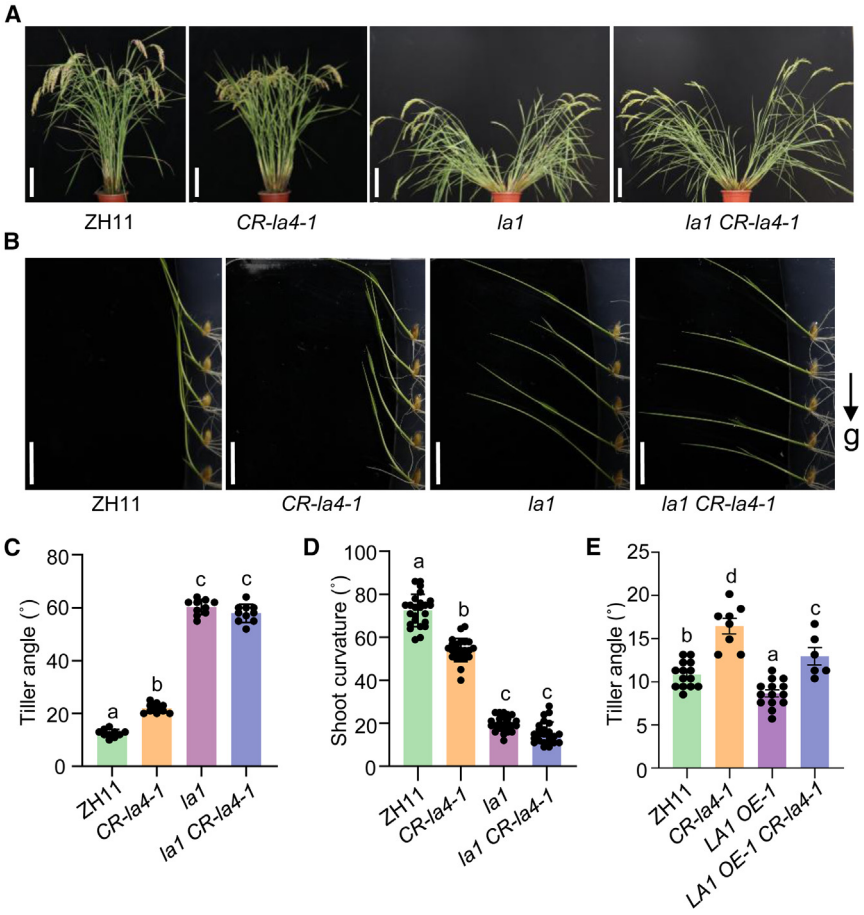

Figure 6. *LA4* acts upstream of *LA1* in regulating rice tiller angle.

(A) Gross morphology of ZH11, *CR-la4-1*, *la1*, and *la1 CR-la4-1* plants at the adult stage. Scale bars, 20 cm.

(B) Shoot gravitropism of ZH11, *CR-la4-1*, *la1*, and *la1 CR-la4-1* plants after gravistimulation for 48 h. Scale bars, 2 cm. The arrow indicates the direction of g.

(C) Statistical analysis of tiller angle of the lines in **(A)**. Data are presented as mean ± SD (n = 10). Different letters above the columns represent statistically significant differences at $P < 0.05$ (one-way ANOVA, Tukey's honestly significant difference).

(D) Statistical analysis of shoot curvature of the lines in **(B)**. Data are presented as mean ± SD (n = 24). Means with different letters are significantly different ($P < 0.05$, ANOVA and Tukey's honestly significant difference).

(E) Statistical analysis of tiller angle in ZH11, *CR-la4-1*, *LA1 OE-1*, and *LA1 OE-1 CR-la4-1* lines grown in Hainan. Data are presented as mean ± SD (n = 6–14). Different letters above the columns represent statistically significant differences at $P < 0.05$ (one-way ANOVA, Tukey's honestly significant difference).

gravitropism and tiller/branch angle. Taken together, our results suggest that *LA4* is a functional ortholog of *SGR9* but has distinct features in rice.

The genetic regulatory network mediated by rice *LA* genes

LA1 regulates shoot gravitropism and tiller angle by modulating LAT upon gravistimulation (Li et al., 2007). LA2 can interact with OspPGM to regulate starch biosynthesis in rice gravity-sensing tissues, and the interaction between LA2 and LA3 is associated with starch granules, implying that LA3 might function as a bridge to connect the OspPGM–LA2 complex to the starch granules during starch biosynthesis (Huang et al., 2021; Cai et al., 2023). LA2 and LA3 can sense gravity to act upstream of LA1 in mediating asymmetric auxin distribution upon gravistimulation (Cai et al., 2023). Interestingly, two research groups recently found that *Arabidopsis* LA/LZY can localize on the amyloplast and plasma membrane, and LA/LZY can translocate from statoliths to the plasma membrane in response to gravistimulation in roots (Chen et al., 2023; Nishimura et al., 2023). Therefore, the LA/LZY proteins are also associated with starch granules, and amyloplast sedimentation can repolarize LA/LZY proteins (Chen et al., 2023; Nishimura et al., 2023). In rice, it will be worth checking whether LA1 can localize to the amyloplast to interact with LA2 and LA3. Although we found that LA4 also acts upstream of *LA1* (Figure 6), like LA2 and LA3, to mediate LAT (Figure 5), LA4 functions in a different pathway from LA2 and LA3 in regulating asymmetric auxin distribution, shoot gravitropism, and tiller angle. There are thus two independent pathways responsible for gravity sensing that act upstream of *LA1* in rice (Figure 7). Our study establishes the framework of a rice tiller-angle regulatory pathway, providing new insights into the rice tiller-angle regulatory network.

METHODS

Plant materials and growth conditions

la4 was a spontaneous mutant in the Nipponbare background. The mutants of *CR-la4* were generated by CR technology in the ZH11 (*O. sativa* L. subsp. *japonica*) background. The *la1*, *CR-la2-1*, *CR-la3-6*, and *LA1 OE-1* mutants were reported in our previous studies (Huang et al., 2021; Cai et al., 2023; Zhang et al., 2023) and are all in the same ZH11 background. The *LA1 OE-1 CR-la4-1*, *CR-la2-1 CR-la4-1*, *CR-la3-6 CR-la4-1*, and *la1 CR-la4-1* double mutants were generated by crossing *CR-la4-1* with *LA1 OE-1*, *CR-la2-1*, *CR-la3-6*, and *la1*, respectively. Rice plants were grown in paddy fields in Tai'an or Hainan (China). For greenhouse experiments, rice plants were grown under a 16-h light/8-h dark photoperiod at 28°C in Tai'an.

Analysis of shoot gravitropism

The gravistimulation and measurement of gravitropic response were performed as described previously (Li et al., 2007), with some modifications. The seeds were germinated at 37°C for 2 days and then planted on 0.4% agar in square plates (13 × 13 cm). After growth at 28°C for 4 days, the plates were rotated by 90° for gravistimulation. The gravitropic response was determined by measuring the shoot curvature after

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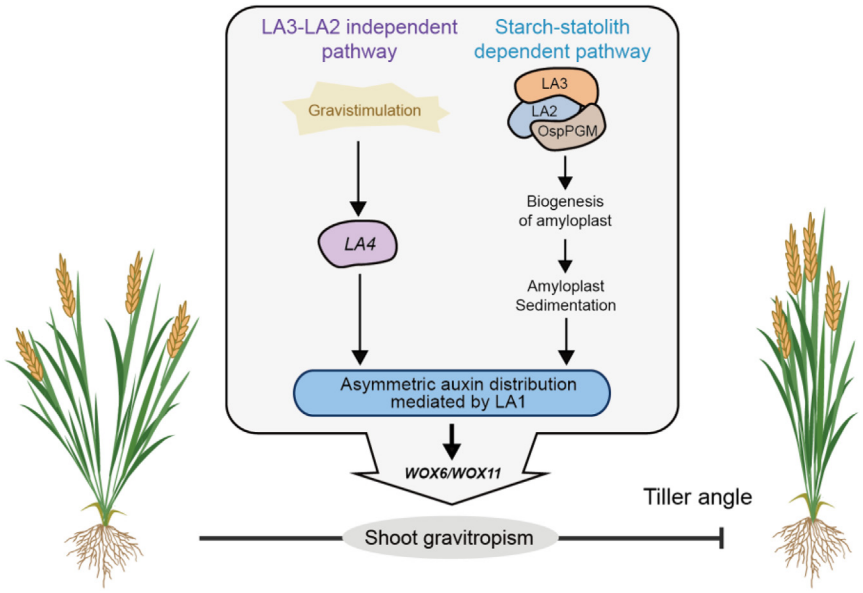

Figure 7. A proposed working model for the control of rice tiller angle by *LA4*.
In rice, the LA3–LA2–OspPGM complex modulates g perception by regulating the biogenesis of amyloplasts, thus controlling rice tiller angle via the classical starch–statolith-dependent shoot gravitropism regulatory pathway. The gravity-responsive gene *LA4* acts in a distinct pathway to positively regulate shoot gravitropism and thus inhibit rice tiller angle. These two pathways converge at LA1-mediated asymmetric auxin distribution, inducing the asymmetric expression of *WOX6* and *WOX11* upon gravistimulation to control rice shoot gravitropism and tiller angle in an additive manner.

reorientation at the indicated time points. To examine gene expression upon gravistimulation by RT–qPCR analyses, 7-day-old light-grown seedlings were gravistimulated, and 1.5 cm of the basal shoot was dissected into lower and upper sides along the direction of gravistimulation for RNA extraction.

Map-based cloning of *LA4*

To isolate *LA4*, we carried out map-based cloning. The *la4* mutant (Nipponbare background) was crossed with ZF802, an *indica* variety, to develop the mapping population. The insertion or deletion markers were developed based on nucleotide polymorphisms between the genome sequences of Nipponbare and 93-11, an *indica* variety. The primers used for mapping are listed in Supplemental Table 1.

Vector construction and transformation

To generate the complementation vector *pLA4C* for *LA4*, a 3.913-kb genomic DNA fragment containing the 2.033-kb upstream sequence, the entire 876-bp *LA4 gene, and a 1.003-kb downstream region* was amplified using primer pair LA4–comp-F/LA4–comp-R and cloned into pCAMBIA1300. To generate *CR-la4* mutants in the ZH11 background, two single guide RNAs targeting different exons were designed for each gene and cloned separately into the pYLCRISPR/Cas9Pubi-H vector as reported previously (Ma and Liu, 2016). The plasmids were then introduced into *Agrobacterium tumefaciens* EHA105, and *la4*, Nipponbare, or ZH11 was transformed as previously reported (Hiei et al., 1994). PCR product sequencing and hygromycin selection were used to identify Cas9-free plants with homozygous mutations. The primers used for gene cloning are listed in Supplemental Table 2.

RNA extraction and RT–qPCR

One-week-old seedlings were gravistimulated by 90° reorientation in the greenhouse. Shoot bases (1.5 cm in length) were split evenly with a scalpel into upper- and lower-side samples 6 h after the start of gravistimulation and were immediately frozen in liquid nitrogen. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from rice shoot bases. The total RNA was treated with DNase I (Ambion, Austin, TX, USA) and subjected to reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). RT–qPCR was performed using the CFX96 Real-Time System (Bio-Rad) with SsoFast EvaGreen Supermix Kit (Bio-Rad). Rice *UBIQUITIN* (*LOC_Os03g13170*) was used as the endogenous control gene. Primers used for RT–qPCR are listed in Supplemental Table 3.

Subcellular localization analysis of LA4

The full-length coding sequence of *LA4* without a stop codon was amplified from ZH11 cDNA and cloned into the pSCYCE–GFP vector to generate the 35Spro:LA4–GFP plasmid. The plasmid was transformed into rice protoplasts. After incubation at 28°C for 12 h, GFP fluorescence was observed under a confocal laser scanning microscope at an excitation wavelength of 488 nm (FluoView 1000; Olympus, Tokyo, Japan). Primers used for subcellular localization analysis are listed in Supplemental Table 2.

Protein expression and purification for *in vitro* assay

The coding sequence of the RING finger domain of LA4 was amplified using primer pair MBP–LA4-F/MBP–LA4-R, purified, and cloned into the pMal-C2E vector. LA4 ($RING^{C236A}$) and LA4 ($RING^{C248A}$) were generated by site-directed mutagenesis. For preparation of recombinant proteins, the plasmids were transformed into *Escherichia coli* strain BL21. Bacterial cells were cultured at 37°C to an optical density of ~0.8, and then isopropyl β-D-thiogalactopyranoside (0.5 mM) was added to the growth medium. After growth for an additional 12 h at 28°C, cells were harvested by centrifugation at 4000 *g* for 20 min, resuspended in 50 ml column buffer (20 mM Tris–HCl [pH 7.5], 200 mM NaCl, 1 mM EDTA, and 1 mM DTT), and lysed with a JN-3000 PLUS low-temperature ultra-high-pressure cell disrupter (JNBIO). The lysates were centrifuged at 4°C and 9000 *g* for 30 min, and the supernatant was collected. Recombinant proteins were purified with amylose resin (3 mg protein/1 ml amylose resin) according to the manufacturer's instructions.

In vitro ubiquitin assay and immunoblot analysis

The *in vitro* ubiquitination assay was performed according to a previously described protocol, with modifications (Xie et al.,

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2002). Each reaction contained 500 ng of purified MBP–LA4 (RING), MBP–LA4 (RING^{C236A}), or MBP–LA4 (RING^{W248A}), human E1 (250 ng), human E2 (UbcH5c) (250 ng), Myc-tagged ubiquitin (2 μg), and 1.5 μl 20× reaction buffer (1 M Tris [pH 7.5], 40 mM ATP [Sigma], 100 mM $MgCl_2$, and 400 mM DTT). The reaction mixture (30 μl) was incubated for 1.5 h at 30°C and 900 rpm in an Eppendorf Thermomixer. After 5× SDS sample buffer was added to the reaction and boiled at 100°C for 5 min, the samples were separated by SDS–PAGE. For detection of ubiquitinated proteins, the membranes were probed with anti-MBP and anti-Myc antibodies at a 1:5000 dilution for 1 h. After washing, the membranes were incubated with a secondary anti-rabbit immunoglobulin G or anti-mouse immunoglobulin G antibody at a 1:10 000 dilution, respectively.

Starch staining assay

The starch staining assay was performed as described previously (Wu et al., 2013). For starch staining, a periodic acid–Schiff kit (Sigma-Aldrich) was used according to the manufacturer's directions. Paraffin sections (10 μM) were deparaffinized and hydrated, immersed in periodic acid solution for 5 min, rinsed with distilled water, and immersed in Schiff's reagent for 15 min. After dehydration, clearing, and mounting, the sections were observed under a microscope.

LAT assay

The LAT assay was performed as described previously, with some modifications (Li et al., 2007). In brief, apical segments (1 cm) of 5-day-old coleoptiles grown in darkness were harvested and deprived of endogenous IAA. The apical ends of the coleoptiles were then submerged into agar blocks that contained 100 nM ^{3}H-IAA. ^{3}H-benzoic acid was used as a control. Segments from the apex were split evenly into upper and lower halves after transport in darkness at 28°C for 2.5 h. After 24 h incubation in 600 μl scintillation liquid, the radioactivity of each half (n = 10) was counted with a liquid scintillation counter (1450 MicroBeta TriLux, PerkinElmer).

Protein sequence analysis

All protein sequences were acquired by searching GenBank (http://www.ncbi.nlm.nih.gov/genbank/) using the LA4 sequence as a query. Protein sequences were aligned using DNAMAN.

Haplotype analysis of *LA4*

To investigate the haplotype of *LA4* among the rice accessions, a high-density SNP genotype set including both wild and cultivated rice germplasms (acquired from the National Genomics Data Center, China National Center for Bioinformation, accession number GVM000285) was used for the haplotype and selective signal analyses. Variants located 500-bp upstream and downstream of the *LA4* gene were extracted for haplotype analyses. For haplotype clustering, the homozygous reference, homozygous alternative, and heterozygous genotypes were recoded to "−1," "0," and "1," respectively. The pairwise Euclidean distances were then calculated on the basis of the silhouette analysis of k-means clustering using R/factoextra. The haplotype network analysis was performed on the basis of the whole-genotype matrix (retaining variants with heterozygous genotypes or missing genotypes) using a random minimum span tree embedded in R/pegas.

ACCESSION NUMBERS

Sequence data for this article can be found at the Rice MSU Genome Annotation Project under the following accession numbers: *LA1* (LOC_Os11g29840), *LA2* (LOC_Os02g08380), *LA3* (LOC_Os03g04100), *LA4* (LOC_Os05g33830), *OspPGM* (LOC_Os10g11140), *OsIAA20* (LOC_Os06g07040), *WOX6* (LOC_Os03g20910), *WOX11* (LOC_Os07g48560), and *Ubiquitin* (LOC_Os03g13170).

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

W.W., L.H., and Y.S. designed research, performed experiments, and analyzed data; W.W. and L.H. wrote the manuscript; S.G., J.Cao., H.Z., M.D., J.Chen., Z.W., J.Z., and X.M. performed some of the experiments; J.L. and D.Z. analyzed data and wrote the manuscript; and Y.W. supervised the project, designed research, analyzed data, and wrote the manuscript. W.W., L.H., and Y.S. contributed equally to this work.

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