Analysis of Rhizome Development in Oryza longistaminata, a Wild Rice Species

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Vegetative reproduction is a form of asexual propagation in plants. A wide range of plants develop rhizomes, modified stems that grow underground horizontally, as a means of vegetative reproduction. In rhizomatous species, despite their distinct developmental patterns, both rhizomes and aerial shoots derive from axillary buds. Therefore, it is of interest to understand the basis of rhizome initiation and development. Oryza longistaminata, a wild rice species, develops rhizomes. We analyzed bud initiation and growth of O. longistaminata rhizomes using various methods of morphological observation. We show that, unlike aerial shoot buds that contain a few leaves only, rhizome buds initiate several leaves and bend to grow at right angles to the original rhizome. Rhizomes are maintained in the juvenile phase irrespective of the developmental phase of the aerial shoot. Stem elongation and reproductive transition are tightly linked in the aerial shoots, but are uncoupled in the rhizome. Our findings indicate that developmental programs operate independently in the rhizomes and aerial shoots. Temporal modification of the developmental pathways that are common to rhizomes and aerial shoots may be the source of developmental plasticity. Furthermore, the creation of new developmental systems appears to be necessary for rhizome development.

Keywords: Axillary bud • Oryza longistaminata • Rhizome • Stem elongation • Vegetative reproduction.

Abbreviations: GA20ox, GA20-oxidase; LOG, LONELY GUY; MRI, magnetic resonance imaging; PAP2, PANICLE PHYTOMER2; QTL quantitative trait locus; SAM, shoot apical meristem.

Introduction

Rhizomes are modified stems that grow horizontally underground. Because of their high advantage for vigorous asexual propagation, rhizomes are observed in various perennial plant species (Wang et al. 2010, Navarro et al. 2011, Lee et al. 2013, Zhang et al. 2014, Yang et al. 2015). One interesting aspect of rhizome development is that new buds on rhizomes can grow underground as a new rhizome or emerge above-ground and grow as an aerial shoot. This growth habitat of rhizomatous species enables them to expand their territory rapidly and widely. Moreover, rhizomes can survive underground when growth conditions are undesirable and shoots can rapidly emerge when the conditions become suitable for growth. They are also important from the view point of crop production. Rhizomes are harvested as crops in potato, ginger, lotus and many others (Navarro et al. 2011, Yang et al. 2015). On the other hand, rhizomatous species are often noxious weeds invading wide regions due to their vigorous growth (Hu et al. 2003, Yun et al. 2014).

Morphogenesis is a lifelong process in plants. New stem cells generated in the axils of leaves form an axillary bud and grow as secondary shoots. Reiterating generation of axillary buds and their growth establish the elaborate structure of plants. The identity and growth pattern of each axillary bud are determined according to internal and external cues. In rhizomatous species, both rhizome buds and aerial shoot buds derive from the axillary buds. In contrast to progress in our understanding of molecular mechanisms underlying specification of axillary bud identity and determination of growth, little is known about rhizome bud initiation and development. Furthermore, how two distinct systems, rhizome buds and aerial axillary buds, are regulated in a single plant is not known.

A number of quantitative trait locus (QTL) regions responsible for or related to rhizome development have been reported in several rhizomatous species (Jang et al. 2006, Washburn et al. 2013, Yun et al. 2014). Moreover, large-scale analyses, such as transcriptome analysis and proteome analysis, have been performed in rhizomatous species (Hu et al. 2011, Barbier et al. 2015, Teichmann and Muhr 2015). Although these studies contributed significantly to improve our understanding regarding rhizome development at the molecular level, isolation of genes critical for rhizome development has not been reported. The complexity of the developmental patterns in rhizomatous species can be an impediment in the analysis of rhizome development. In addition, low seed fertility in rhizomatous species, which evolved for asexual reproduction, hampers the application of genetic approaches. Furthermore, a lack of model
Rhizomatous species is a serious problem for molecular genetic analysis of rhizome development. *Oryza longistaminata*, a wild species of rice originated in middle Africa, develops rhizomes and proliferates vigorously. *Oryza longistaminata*, containing the AA genome, which is the same as that of *Oryza sativa*, can be crossed with *O. sativa* and set fertile seeds, and thus has been considered one of the ideal materials to study mechanisms controlling rhizome development in grass species (He et al. 2014). In this study, we performed a detailed observation of rhizome morphology, growth and development. Our findings indicate that common modules of development are used in both the rhizome and aerial shoot, but modification of the temporal regulation of the modules may be the source of the developmental plasticity observed in the rhizome. In addition, we demonstrate that innovation of new developmental systems was adopted to establish the process of rhizome development.

Results

Rhizomes elongate during the vegetative phase

*Oryza longistaminata* develops rhizomes underground and exhibits a typical rice growth pattern at ground level (Fig. 1a). In this study, the above-ground shoot and stem of *O. longistaminata* are named the aerial shoot and aerial stem, respectively, and the underground stem is named the rhizome. Both the aerial stem and the rhizome formed an axillary bud at each node (Fig. 1b). An axillary bud on the aerial stem of *O. longistaminata* grows as an aerial shoot called a tiller, which constantly initiates leaves during the vegetative stage. The aerial stem did not show substantial elongation during the vegetative phase, and elongation occurred after the transition to the reproductive phase when a panicle is formed, as is the case in *O. sativa* (Fig. 1c, d). Axillary buds on the rhizome grew to form a new rhizome. In contrast to the aerial stem, the rhizome elongated when the aerial shoots were at the vegetative stage (Fig. 1b). This indicates that aerial stem elongation and rhizome elongation are controlled independently, i.e. two systems of stem elongation operate in a single plant of *O. longistaminata*—a developmental phase-dependent and a developmental phase-independent system. Gibberellic acid is a crucial determinant of stem elongation (Kusaba et al. 1998, Carrera et al. 2000, Magome et al. 2013). In particular, accumulation of active gibberellins, such as GA<sub>4</sub> and GA<sub>19</sub>, produced by GA20-oxidase (GA20ox), is required for rapid stem elongation before heading in rice (Magome et al. 2013). We analyzed expression of three GA20ox genes in *O. longistaminata*. As previously described, expression of GA20ox1 and GA20ox2 was low in an undeveloped stem during the vegetative phase and increased after the transition to the reproductive phase in *O. sativa* (Fig. 1e, f). In the aerial stem of *O. longistaminata*, expression of GA20ox1 and GA20ox2 was also low at the vegetative phase and increased at the reproductive phase (Fig. 1g, h). Expression of GA20ox1 was low in the rhizome, while GA20ox2 was strongly expressed in rhizomes, regardless of the developmental stage (Fig. 1i). We did not detect significant expression of the GA20ox3 gene in *O. sativa* and *O. longistaminata* (data not shown).

Rhizome buds rapidly change direction after their initiation

The morphology of axillary buds on rhizomes is similar to those on young aerial stems (Fig. 2a, b). They are small, flat and attached to the aerial stem or to the rhizome. The unique morphology of the rhizome buds becomes evident soon after the start of bud growth (Fig. 2c). Prior to elongation, axillary buds on the rhizome became rounder than those on the aerial stem (Fig. 2c; Supplementary Fig. S1c–e). Axillary buds on the rhizome then increased in size and became bulkier at the onset of outgrowth (Fig. 2d). In addition, the angle of the rhizome buds changed, accompanying an emergence of the tip of the bud from its center (Fig. 2d). The buds then grew at right angles to the original rhizome and elongated (Fig. 2e). A more detailed examination of the early stages of rhizome bud outgrowth revealed that the buds rapidly increased the number of leaves accompanying the change of angle (Figs. 2f–k, 3).

In order to confirm that these processes, which were observed in different buds, occur sequentially in a single bud, we observed the development of a single bud by making use of the magnetic resonance imaging (MRI) technique (Moriwaki et al. 2014). In this experiment, a rhizome piece containing a bud was cut and incubated in a glass tube in a magnetic field and was continuously observed for >65 h. This showed that the bud begins bending immediately after the start of bud growth. A rapid increase in the number of leaves and enlargement of the size of the whole bud took place concomitantly with the change of the bud angle (Fig. 4a–g; Supplementary Fig. S1; Supplementary Videos S1, S2). Interestingly, when a rhizome was excised and grown under light conditions, the rhizome turned green and the buds on the rhizome grew as new aerial shoots (Fig. 5a, b). In contrast, some buds on the aerial shoots adjacent to the border between the aerial shoot and the rhizome grew as new rhizomes when they were kept under dark conditions (Fig. 5c, d).

Rhizomes are maintained at the juvenile phase

The morphology of leaves subtending rhizome buds is different from that of leaves in the aerial shoots. Leaves of grass species consist of two parts, the blade, formed at a distal position, and the sheath, formed at a proximal position (Fig. 6a). The ratio of the leaf blade to the leaf sheath changes according to the developmental stage, and thus is used as an indicator of the growth phase during the vegetative phase (Fig. 6b). The first leaf of rice (*O. sativa*) consists of only the leaf sheath. The leaf blade is observed from the second leaf (Fig. 6b). From the third leaf, the leaf blade occupies nearly half the entire leaf. Leaves on the rhizome, called scale leaves, are small and vestigial (Fig. 6c). The scale leaves mostly consist of the leaf sheath, resembling the first leaf of *O. sativa* (Fig. 7a–f; Supplementary Fig. 52). These observations indicate that the rhizome is maintained in the juvenile phase. When buds on the rhizome emerge above the ground and start to grow as aerial shoots, the ratio of the blade to the sheath in newly initiated leaves increases gradually, indicating
a development phase transition (Fig. 6d, e). In axillary buds of *O. sativa*, the leaf blade differentiates in the initiating leaf primordia at stages P3–P4 (Fig. 6f). In contrast, several leaf primordia differentiated in an axillary bud on the rhizome, and no sign of blade differentiation was detected (Fig. 6g).

**The developmental phase is controlled independently below ground and above ground**

The transition to the reproductive phase in *O. longistaminata* is induced by exposure to short days. A panicle is formed at the top of each aerial shoot, as occurs in tillers of *O. sativa* (Fig. 8a, b). Remarkably, while developmental phase transition occurred in the aerial shoots, the developmental phase in the rhizomes was maintained at the vegetative stage (Fig. 8c–e). When leaves were removed from an aerial shoot before heading, an initiating inflorescence was observed at the top of the non-elongated stem (Fig. 8d). In contrast, a shoot apical meristem (SAM) at the top of the rhizome was maintained at the vegetative stage (Fig. 8e). To confirm further that the phase of the rhizome is vegetative, we used *PANICLE PHYTOMER2* (*PAP2*)

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**Fig. 1** Morphology and development of *O. longistaminata*. (a) Shoots and rhizomes of *O. longistaminata*. Rhizomes grow underground and shoots grow above the ground. The broken line indicates the ground surface. Soil was removed. Scale bar = 10 cm. (b) Axillary buds on rhizomes and aerial shoots. An axillary bud (arrow) is formed on a node of the rhizome which has several nodes. The first few buds on the aerial shoot are similar to rhizome buds (right panel). Internodes between nodes with axillary buds have elongated. Scale bars = 10 cm (left panel), 5 cm (right panel). (c and d) Shoot apical meristems (SAMs) of *O. sativa* (c) and *O. longistaminata* (d) shoots. The SAM is located at the base of the shoot (square in (b), with regard to *O. longistaminata*). After removal of leaves, the SAM at the vegetative phase (VM) is observed. Scale bars = 1 cm (left panel in c), 1 mm (right panel in c, d). (e, f) Expression levels of *GA20ox1* and *GA20ox2*, gibberellin synthesis genes, in *O. sativa* (e, f) and *O. longistaminata* (g, h). Values are represented relative to the expression level in the stem (*O. sativa*) and aerial stem (*O. longistaminata*) at the vegetative stage. Error bars show the SD of three replications. St, stem; Rh, rhizome; As, aerial stem; veg, vegetative stage; rep, reproductive stage.
molecular marker for the reproductive phase. PAP2, encoding OsMADS34, is one of the earliest genes induced in the SAM after phase transition (Kobayashi et al. 2010, Kobayashi et al. 2012). PAP2 was expressed in the shoot apex of the aerial shoot after the transition, while PAP2 was not detected in the shoot apex of the rhizome (Fig. 8f). We next examined expression of Hd3a, the rice ortholog of Arabidopis FT encoding florigen (Tamaki et al. 2007, Taoka et al. 2011). Hd3a expression was undetectable in the leaf blade and the leaf sheath of the aerial shoot, as well as in the scale leaf of the rhizome at the vegetative stage (Fig. 8g). After the transition to the reproductive stage, Hd3a expression was detected in the leaf blade but not in the leaf sheath in aerial shoots, as has been reported previously (Tamaki et al. 2007). In the scale leaf, Hd3a expression was also undetectable.

Discussion

Developmental timing is crucial for all organisms in establishing their body architecture (Geuten et al. 2013). Modulation of developmental timing often works as a strong driving force to generate morphological plasticity and evolution. Our analyses indicate that a temporal shift in developmental processes is critical in creating the complex developmental pattern of O. longistaminata. We propose that a heterochronic modification of developmental processes is the key in establishing the unique system of rhizome development.

Our results show clearly that developmental timing is regulated independently between above-ground and below-ground parts in O. longistaminata (Fig. 8h). In rice, the juvenile phase, which is characterized by a high ratio of leaf sheath to leaf blade, is relatively short and only the first few leaves exhibit this juvenile characteristic (Itoh et al. 2005). In contrast, leaves on rhizomes consist solely of the sheath, resembling the first leaf in aerial shoots in O. longistaminata and O. sativa. This means...
that the rhizomes are maintained in the juvenile phase, and phase progression is strongly suppressed. In spite of this, rhizomes elongate. In normal rice growth, stem elongation occurs only after the transition to the reproductive phase (Itoh et al. 2005). In tomato, axillary buds grew to tuber-like organs when LONELY GUY (LOG) a cytokinin biosynthesis gene, was ectopically expressed. Formation of the ectopic tubers was enhanced by expression of miR156, a positive regulator of the juvenile phase (Eviatar-Ribak et al. 2013). This implies that the effect of ectopic LOG expression was modified by the growth phase of the buds. This also indicates that the development and growth pattern of the axillary buds are affected by developmental phases of the buds.

The SAM of rhizomes is constantly maintained at the vegetative phase irrespective of the developmental phase in the aerial parts. This implies the existence of a system that blocks the SAM from entering the reproductive phase. In rice, the reproductive phase change is conferred by florigen, which is transported from the leaves to the SAM. Florigen is encoded by two genes in rice, Hd3a and RFT (Tamaki et al. 2007, Komiya et al. 2008). Hd3a and RFT expression in leaves is induced under short-day conditions. Intriguingly, Hd3a and RFT expression occurs only in the leaf blade and is excluded from the leaf sheath even under short-day conditions (Tamaki et al. 2007, Komiya et al. 2008). Therefore, the absence of Hd3a and RFT induction in the scale leaves of the rhizomes that contain only the leaf sheath may prevent the rhizome SAM from entering the reproductive stage. However, we cannot rule out the possibility that florigen proteins are transported from aerial leaves to the rhizome. In the stolon, the potato rhizome, the apical region expands into tubers in an environmentally independent manner (Nicolas et al. 2015). Potato tuber formation is controlled by florigen-related proteins transported from the aerial parts of the plant. Despite the induction of tuber formation, the SAM at the top of the stolon is not affected and remains in a vegetative stage. This suggests that there is a regulatory network controlling the spatial fine tuning of temporal development in the potato stolon. Understanding the molecular basis for the fine tuning of spatial regulation of developmental timing progression in O. longistaminata is an important challenge for the future.

Many plant species contain stems that grow horizontally. Horizontal stems grow above ground, at the soil surface, just below ground or underground. Strictly speaking, rhizomes are stems that grow underground and the others are called stolons or runners. One similarity between these rhizomatous plants is that two distinct systems of stem growth, namely vertical and horizontal growth, operate in a single plant. Horizontal growth of stems, in addition to normal vertical growth, is advantageous for rapid territory expansion and thus is a sensible adaptive strategy. Here, we showed that rhizome buds of O. longistaminata change their angle to grow horizontally. It will be of interest to know if this trait is conserved in other species with horizontal stems. Furthermore, unraveling the molecular and physiological mechanisms of this process is essential to improve our understanding of rhizomes. The rapid progress of genomic-based technologies has enabled the molecular and genetic analysis of plant species with unique and interesting growth habits, such as rhizome development (Cheng et al. 2013, Koo et al. 2013, Yun et al. 2014, Zhang et al. 2014, Barbier et al. 2015, Salvato et al. 2015, Teichmann and Muhr 2015, Yang et al. 2015). Indeed, model rhizomatous species, such as Brachypodium, Sorghum and Oryza as described here, are being established. These studies will provide the basic information needed to further our understanding of the molecular basis of rhizome development.
Materials and Methods

Plant materials

Rice strains *O. longistaminata* (IRGC10404) and *O. sativa* cv. Nipponbare were utilized. Plants were grown in soil under natural light conditions in the greenhouse. For the observation of axillary buds, leaves were removed manually and photos were taken under a microscope (Leica MCT20HD) and analyzed using the ImageJ free software (NIH).

Scanning electron microscopy

Axillary buds of the stem and rhizome were fixed in 2.5% glutaraldehyde overnight at 4 °C and dehydrated in a series of ethanol solutions, then the final ethanol solution was substituted with 3-methylbutyl acetate. The samples were then dried, coated with platinum and observed under a scanning electron microscope (Hitachi S-4000) at an accelerating voltage of 5 kV.

Histological analysis

Shoot apices of the stem and rhizome were fixed with FAA (5% formalin, 5% acetic acid, 45% ethanol and 45% water) overnight at 4 °C, and dehydrated in a series of ethanol solutions, then embedded in Paraplast plus (McCormick). Transverse sections of 10 μm thickness were made and stained with hematoxylin. After rinsing in water, stained sections were dehydrated in a graded ethanol series, replaced with hist-clear and mounted with Multi Mount (Matsunami Glass Ind. Ltd.).

Magnetic resonance imaging

Rhizome axillary buds of *O. longistaminata* were observed using a 4.7 T 89–1 mm vertical-bore superconducting magnet system. For imaging, a part of the stem including a bud was cut (3 cm long), terminated with moistened cotton wool, and placed in a sample tube (10 mm in diameter). A home-built solenoid radiofrequency coil (12 mm in diameter) was used. Planar gradient coils with gaps of 26–31 mm were constructed and the current efficiency for *x*, *y* and *z*-gradients was 46.2, 37.8 and 69.0 mT m⁻¹ A⁻¹, respectively.

Real-time RT–PCR

Total RNA was extracted using a Plant RNA Isolation mini kit (Agilent Technologies). After DNase I treatment, first-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). The primer sets used to amplify the transcripts were as follows: forward and reverse primers for *Hd3a*,
For PAP2, 5′-AGCTGACCTCCAGTGACATGC-3′ and 5′-CCTGCAATGATAGCATGC-3′; for GA20ox1, 5′-AGCAGCTCCACTGGCTACAAATGA-3′ and 5′-AGGTCGCAGAGTTGATCAAG-3′; for GA20ox2, 5′-CCTTCTCCGACTGGCTTAAT-3′ and 5′-GATGGATGGATGGATAATAGG-3′; for GA20ox3, 5′-AGGATGTCACCAGGAGTACTGCGA-3′ and 5′-GTAGTGGTTCAGCCGCATC-3′; and for Ubiquitin, 5′-AGAAGGAGTCCACGCTCCACC-3′ and 5′-GCCGGGCCATATAACCTTGACTT-3′. PCRs were performed with SYBR green I using the LightCycler®480 System II (Roche Applied Science).

Supplementary data

Supplementary data are available at PCP online.
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Disclosures
The authors have no conflicts of interest to declare.

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