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Short title: GA-control of rice leaf length via cell division

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Title: GA-mediated spatial control of cell division expounds the leaf size variation between cultivated and wild rice

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Summary: High Gibberellic Acid level promotes cell division and cell production rate in rice leaves to increase the leaf length.

Author contributions: V.J. and A.R, conceptualized the study. V.J., K.S., and A.R. designed experiments. V.J. and A.C. performed experiments. V.J., R.R., Y.I., and A.R. analyzed transcriptomic data. V.J. and K.S. analyzed leaf kinematics data. V.J. and A.R. compiled figures and wrote the manuscript. V.J., K.S. and A.R. edited the final manuscript with the contribution of all the authors. A.R. agrees to serve as the author responsible for contact and ensures communication.

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Abstract

Leaf size is a major determinant of crop performance by influencing leaf physiological processes, such as light capture, transpiration, and gas exchange. Therefore, understanding the genetic basis of leaf size regulation is imperative for crop improvement. Natural variation in leaf size for a crop plant is a valuable genetic resource for a detailed understanding of leaf size regulation. We investigated the mechanism controlling the rice leaf length using cultivated and wild rice accessions that showed remarkable differences for the leaf features. Comparative transcriptomic profiling of the contrasting accessions suggested the involvement of Gibberellic Acid (GA), Growth Regulating Factor (GRF) transcription factors, and cell cycle in the rice leaf size regulation. Leaf kinematics studies showed that the increased domain of cell division activity along with a faster cell production rate drove the longer leaves in the wild rice *Oryza australiensis* compared to the cultivated varieties. Higher GA levels in the leaves of *Oryza australiensis*, and GA-induced increase in the rice leaf length via an increase in cell division zone emphasized the key role of GA in rice leaf length regulation. Zone-specific expression and silencing of the GA biosynthesis and signaling genes confirmed that OsGRF7 and OsGRF8 function downstream to GA for controlling cell cycle to determine the rice leaf length. The GA-GRF-cell cycle module for rice leaf length regulation might have contributed to optimizing leaf features during the domestication and could also be a way for plants to achieve leaf plasticity in response to the environment.

Keywords: Cultivated and wild rice, Leaf length, Leaf Kinematics, Leaf growth zones, Cell division, Gibberellic acid (GA), Growth regulatory factors (GRFs), Cyclin.

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Introduction

The basic leaf development program includes leaf primordia initiation on the flanks of shoot apical meristem that follows growth and patterning by multiple transcription factor families (Townsley and Sinha, 2012; Bar and Ori, 2014; Du et al., 2018). While leaf initiation and patterning are regulated by a suite of transcription factors and complex hormonal interactions, the final leaf size – length and width – is determined by the coordination of cell division and expansion (Li et al., 2007; Gonzalez et al., 2012; Chitwood and Sinha 2016; Sarvepalli et al., 2019). Considering leaf as the major plant organ for light-harvesting and perception of environmental signals, the genetic understanding of leaf size regulation could be of paramount importance in crop improvement programs. Though leaf initiation and subsequent growth follow a similar basic program, a remarkable variation in leaf shape and size is observed within a plant species and across different species.

The regulation of leaf size is extensively studied for simple leaves of Arabidopsis and compound leaves of tomato, however detailed mechanistic studies are relatively scarce for monocot cereals except for maize (Efroni et al., 2010; Rodriguez et al., 2013; Vercruysse et al., 2020a). The spatial and temporal regulation of cell proliferation and cell elongation to determine the final leaf size varies between monocots and dicots. Cell proliferation and expansion are temporally separated in dicots as cell proliferation predominates in a young developing leaf, thereafter cells expand before reaching maturity (Beemster et al., 2005; Piazza et al., 2005; Andriankaja et al., 2012). In contrast, cell proliferation and expansion are spatially separated in monocots, such as maize, where division, elongation, and maturation zones are present together at steady-state leaf growth (Fina et al., 2017, Tardieu and Garnier 2000). Cells at the leaf base are actively dividing and smaller in size, whereas cells in the elongation zone primarily grow by expansion followed by cell maturation at the leaf tip (Granier and Tardieu, 2009). Monocot leaves with such spatial resolution of the growth zones allow the systematic study of the zones and their contribution in the leaf size control. Leaf kinematics, quantifying cell division and elongation, is a powerful tool to define a high-resolution map of leaf growth zones, and for studying the leaf growth differences across the genotypes (Sprangers et al., 2016).

Changes in the size of division and/or elongation zone alter the final leaf size in Arabidopsis and maize (Tardieu and Granier, 2000; Tardieu et al., 2000; Gonzalez et al., 2010; Kalve et al., 2014; Nelissen et al., 2018). Several phytohormones play important roles in the regulation of cell division, elongation, and transition between division and elongation (Avramova et al., 2015; Takatsuka and Umeda, 2014). The interplay of auxin and cytokinin to

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control cell division is long established (Inze and De Veylder, 2006; Su et al., 2011). GA is classically known for mediating cell-elongation response, however recent studies in Arabidopsis and maize have shown its key roles in determining cell division (Achard et al., 2009; Nelissen et al., 2012). GA, after being perceived by a receptor protein GIBBERELLIN INSENSITIVE DWARF1 (GID1), promotes degradation of DELLA repressor proteins, resulting in activation of GA-responsive transcription factors, such as GROWTH-REGULATING FACTORs (GRFs) (Willige et al., 2007; Davière and Achard, 2013; Omidbakhshfard et al., 2015). GRFs are reported to directly regulate cyclins to control cell division in different plant species (van der Knaap et al., 2000; Choi et al., 2004; Nelissen et al., 2015; Fina et al., 2017). Arabidopsis plants overexpressing, *AtGRF1*, *AtGRF2*, and *AtGRF3* develop longer leaves and larger organ size, whereas *grf* mutants produce smaller and narrower leaves (Kim et al., 2003; Beltramino et al., 2018). Moreover, *GRF* expression modulation in maize alters leaf length with associated changes in division zone size (Wu et al., 2014; Nelissen et al., 2015). Thus, GA along with downstream GRF transcription factors could be a key to the genotypic differences in the leaf size across multiple accessions of a crop plant.

The genetic basis of leaf size differences and underlying hormonal control is poorly investigated in rice. GA is known to regulate stem elongation in rice, and manipulation of GA-biosynthesis and signaling genes have optimized plant height during the green revolution (van der Knaap et al., 2000; Spielmeyer et al., 2002). However, a possible involvement of GA in the regulation of the rice leaf size is not systematically investigated. Cultivated rice varieties along with their wild relatives provide a tremendous genetic variability to study the genetic basis of leaf size differences. Several wild relatives of rice accumulate higher biomass associated with larger organ size including leaf size (Sanchez et al., 2013; Mathan et al., 2021). Rice domestication that primarily targeted the grain features and plant architectural traits has significantly reduced the genetic variability in the cultivated varieties (Huang et al., 2012; Chen et al., 2021). Therefore, a comparative study of leaf size and associated growth zone differences across cultivated and wild rice would provide a comprehensive understanding of genetic and hormonal regulation of rice leaf size.

We performed detailed phenotyping of leaf features of seven cultivated varieties and four wild rice accessions followed by leaf kinematics analyses of the four accessions that captured the entire variation for the leaf length. RNAseq differential gene expression analysis for the selected accessions suggested the major roles of phytohormones, transcription factors, and cell cycle genes in determining the rice leaf size. Leaf kinematics results showed a strong association of the final rice leaf length with the size of the division zone. We further attributed

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GA-content differences and downstream transcription factors to the variation in the division zone and rice leaf size between the cultivated and wild rice. Finally, silencing of GA biosynthesis and signaling genes established the key role of GA and downstream signaling components in controlling rice leaf size. The knowledge could be used to optimally control the rice leaf growth and size for improving crop performance.

Results

Leaf size variation across the selected cultivated and wild rice accessions

Quantification of the leaf length and the maximum leaf blade width of the fully-grown second to eighth leaves of the eleven rice accessions showed a strong variation in leaf length and width (Supplemental Table S1). The length of fully-grown eighth leaf varied from 44.8 cm in a cultivated rice *Oryza sativa ssp. indica* cv. IR64 to 87.8 cm in a wild rice *Oryza australiensis* (Table 1). The wild rice accessions generally developed longer leaves compared to the cultivated varieties. Significant variation in leaf width was also observed, with the wild rice *O. latifolia* showing maximum and cultivated variety *O. sativa ssp. indica* cv. Vandana showing minimum leaf width (Supplemental Table S2). We then selected four genetically diverse accessions, which captured the leaf length variations across the investigated accessions, for detailed phenotypic and genetic characterization. The four selected accessions were: IR64 (an *indica* variety of the cultivated species *O. sativa*) and Nipponbare (a *japonica* variety of the cultivated species *O. sativa*) with smaller leaves, the wild rice *O. australiensis* with the longest leaves, and *O. glaberrima* (the African cultivated rice) with intermediate leaf length between *O. sativa* and *O. australiensis* (Table 1; Figure 1A). The leaf phenotype of second to tenth leaves of the selected four accessions further confirmed the variation in leaf length pattern (Figure 1B, Supplemental Figure S1). The average leaf length of a matured leaf was remarkably higher for the wild rice *O. australiensis* and was moderately higher for the African cultivated rice *O. glaberrima* compared to Asian cultivated varieties IR64 and Nipponbare.

Quantification of the leaf four lengths of the selected four accessions consistently showed longer leaf in the wild rice *O. australiensis* compared to the cultivated accessions at different days of emergence (Figure 1, C and D). *O. glaberrima* showed intermediate leaf length between the wild rice *O. australiensis* and cultivated Asian rice varieties at different days of emergence. Consistent with the leaf length, the leaf elongation rate (LER) was higher for the wild rice than the cultivated accessions at different days of emergence (Figure 1E). Taken together, the wild rice *O. australiensis* showed remarkably longer leaves than the

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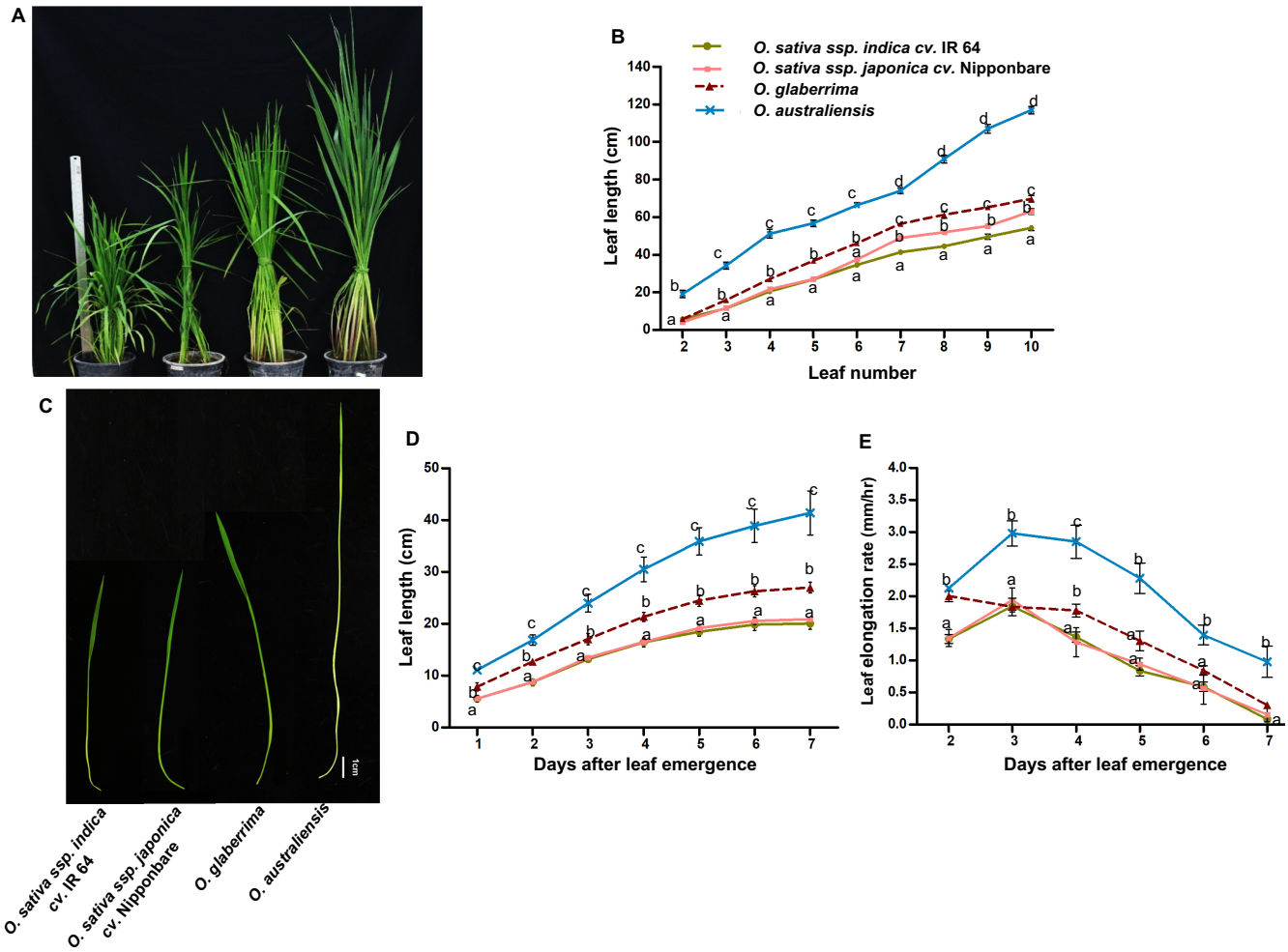


Figure 1. Wild rice *Oryza australiensis* grows longer leaf compared to the selected cultivated rice accessions. A, Representative photographs of the selected cultivated and wild rice accessions showing leaf size variations. B, Quantification of mature leaf length of leaf two to leaf ten. C, Representative leaf four images at the second day of emergence. D, Quantification of leaf four lengths at the different days of emergence. E, Leaf elongation rate of the selected rice accessions. Values in graphs represent mean±SD (n=15). Different letters indicate statistically significant differences according to one-way ANOVA followed by post-hoc Tukey HSD at $P < 0.05$.

cultivated varieties, and *O. glaberrima* also developed significantly longer leaves than IR64 and Nipponbare.

Transcriptome profiling to identify GA and cell cycle as the key regulators of rice leaf length

To identify genes and pathways controlling the leaf length differences across the selected accessions, we compared the transcriptomes of the developing fourth leaves of the selected accessions on the second day after emergence. The maximum number of differentially expressed genes (DEGs) was found between *O. sativa* cv. Nipponbare and *O. australiensis*, whereas a lower number of DEGs was found between cultivated accessions (Supplemental Table S3; Supplemental Dataset S1). Since the leaves of the wild rice *O. australiensis* were remarkably longer than IR64 and Nipponbare, we first compared the gene expression profiles

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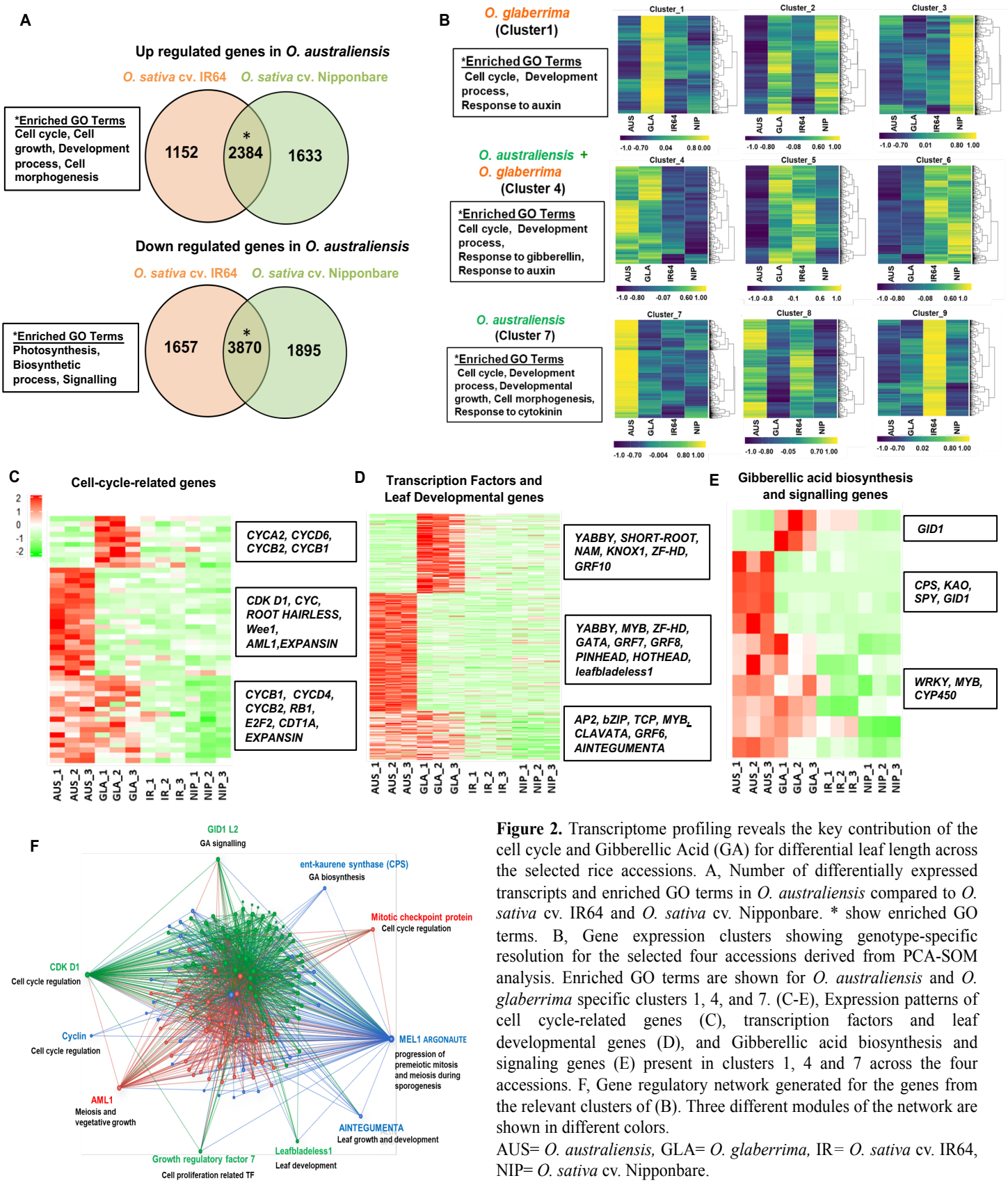


Figure 2. Transcriptome profiling reveals the key contribution of the cell cycle and Gibberellic Acid (GA) for differential leaf length across the selected rice accessions. A, Number of differentially expressed transcripts and enriched GO terms in *O. australiensis* compared to *O. sativa* cv. IR64 and *O. sativa* cv. Nipponbare. * show enriched GO terms. B, Gene expression clusters showing genotype-specific resolution for the selected four accessions derived from PCA-SOM analysis. Enriched GO terms are shown for *O. australiensis* and *O. glaberrima* specific clusters 1, 4, and 7. (C-E), Expression patterns of cell cycle-related genes (C), transcription factors and leaf developmental genes (D), and Gibberellic acid biosynthesis and signaling genes (E) present in clusters 1, 4 and 7 across the four accessions. F, Gene regulatory network generated for the genes from the relevant clusters of (B). Three different modules of the network are shown in different colors.
AUS= *O. australiensis,* GLA= *O. glaberrima,* IR= *O. sativa* cv. IR64, NIP= *O. sativa* cv. Nipponbare.

182 between the leaves of *O. australiensis* and two cultivated varieties of *O. sativa*. 2,384 genes
183 were upregulated and 3,870 genes were downregulated in growing leaves of *O. australiensis*

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compared to both IR64 and Nipponbare (Figure 2A). The upregulated genes showed enrichment of GO terms 'cell cycle', 'development process', 'cell growth', and 'cell morphogenesis' (Supplemental Dataset S2). The enriched GO terms for downregulated genes included 'photosynthesis', 'biosynthetic process', and 'signaling'. We also compared the gene expression differences between the significantly longer leaves of *O. glaberrima* and the two cultivated varieties of *O. sativa*. The 1,125 upregulated genes showed enrichment of GO terms 'cell cycle', 'development process', 'growth', and 'hormone transport', whereas 1,290 downregulated genes were enriched for the term 'metabolic process' (Supplemental Figure S2A; Supplemental Dataset S2). We then analyzed the differentially expressed genes in the wild rice *O. australiensis* compared to all three cultivated accessions. The 1,707 shared upregulated genes in the wild rice leaves showed enrichment of GO terms "development process", "cell cycle", and "growth", whereas the GO term "biosynthetic process" was enriched for downregulated genes (Supplemental Figure S2B; Supplemental Dataset S2).

We performed principal component analysis combined with a self-organizing map (PCA-SOM) to cluster the genes based on their expression pattern across the accessions. Principle component 1 (PC1), explaining 75% variation in the dataset, primarily explained the gene expression differences of the wild rice *O. australiensis* compared to the three cultivated accessions (Supplemental Figure S3, A and B). PC2, explaining 20% of the overall variation, explained the gene expression differences of *O. glaberrima* compared to the other three accessions. The clusters generated by PCA-SOM provided the species-specific resolution of the transcripts (Figure 2B; Supplemental Dataset S3). Since *O. australiensis* and *O. glaberrima* had significantly longer leaves compared to IR64 and Nipponbare, we analyzed the genes and enriched pathways in the clusters specific to either *O. australiensis* or *O. glaberrima* or both. The cluster 4 that had genes specific to both *O. australiensis* and *O. glaberrima* showed enrichment of terms 'cell cycle', 'development process', 'response to gibberellin', and 'response to auxin' (Figure 2B; Supplemental Dataset S4). The transcripts in *O. australiensis* specific cluster 7 and *O. glaberrima* specific cluster 1 were enriched for 'development process' and 'cell cycle'. In addition, we also found enrichment of terms related to phytohormones in clusters related to *O. glaberrima* and *O. australiensis*.

Since transcription factors, phytohormones, and cell division and expansion are crucial for leaf size regulation, we compared the expression of the related genes from the relevant clusters across the selected accessions (Supplemental Dataset S5). Several cell cycle genes, such as *CYCLINB, CYCLINA,* and *CDKD1*, as well cell cycle regulators, such as *E2F2* and *RB1*, were expressed at higher levels in *O. australiensis* and *O. glaberrima* than IR64 and

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Nipponbare (Figure 2C). Further, transcription factor genes known to regulate leaf growth and development, such as *GRF*, *TCP*, *YAABY*, *NAM*, and *ANT*, were upregulated in *O. australiensis* and *O. glaberrima* (Figure 2D). Among the genes related to phytohormones, GA biosynthesis and signaling genes that included *CPS, KAO* and *GID1* were expressed at higher levels in *O. australiensis* and *O. glaberrima* than IR64 and Nipponbare (Figure 2E). Moreover, differential expressions of auxin and cytokinin biosynthesis and signaling genes were also observed across the selected accessions (Supplemental Figure S3C).

The coexpression network of genes from the relevant clusters showed 3 major modules including more than 20 nodes (Figure 2F; Supplemental Dataset S6). The network showed significant connections for GA biosynthesis and signaling genes *CPS* and *GID1L2,* cell cycle-related genes *CYCLIN* and *CDKD1*, as well as *GRF* transcription factor. Investigation of the hub genes in module 2 showed *CDKD1* as one of the highly connected genes, which was connected to GA receptor *GID1L2,* and *GRF7* (Supplemental Figure S3D). Taken together, transcript profiling along with gene co-expression network highlighted cell cycle, GA, and transcription factors, such as GRFs, as key contributors of rice leaf length regulation.

Leaf kinematics to confirm the major role of cell division to regulate rice leaf length

We performed DAPI staining and kinematics analyses on the growing fourth leaves to investigate the differences in cell division and growth zones across the selected accessions. Wild rice *O. australiensis*, with longer leaves compared to the cultivated varieties, showed dividing cells for longer length from the leaf base, and thus longer division zone at different days of emergence (Supplemental Figure S4A). *O. glaberrima* also showed a longer division zone compared to IR64 and Nipponbare. Detailed kinematics analyses on the fourth leaves of each selected accession confirmed the differences in the division zone (Figure 3; Table 2; Supplemental Figure S4). All the four selected accessions showed a sigmoidal curve for epidermal cell length profile, consisting of a division zone at leaf base followed by elongation zone and maturation zone (Figure 3, A and B; Supplemental Figure S4, B and C). The cultivated varieties Nipponbare and IR64 showed division zone extending up to 7.8mm and 7.5mm from the leaf base, respectively (Figure 3, A and B, Supplemental Figure S4B). *O. australiensis*, with the longest leaves, showed a remarkably longer division zone that extended to 16.7mm from the leaf base (Figure 3, C and D). The longer division zone in *O. australiensis* leaves was accompanied by faster cell production rate and leaf elongation rate (Table 2). *O. glaberrima* also showed a longer division zone as well as a higher cell production rate compared to IR64 and Nipponbare (Supplemental Figure S4C). *O. australiensis* showed reasonably high overlap

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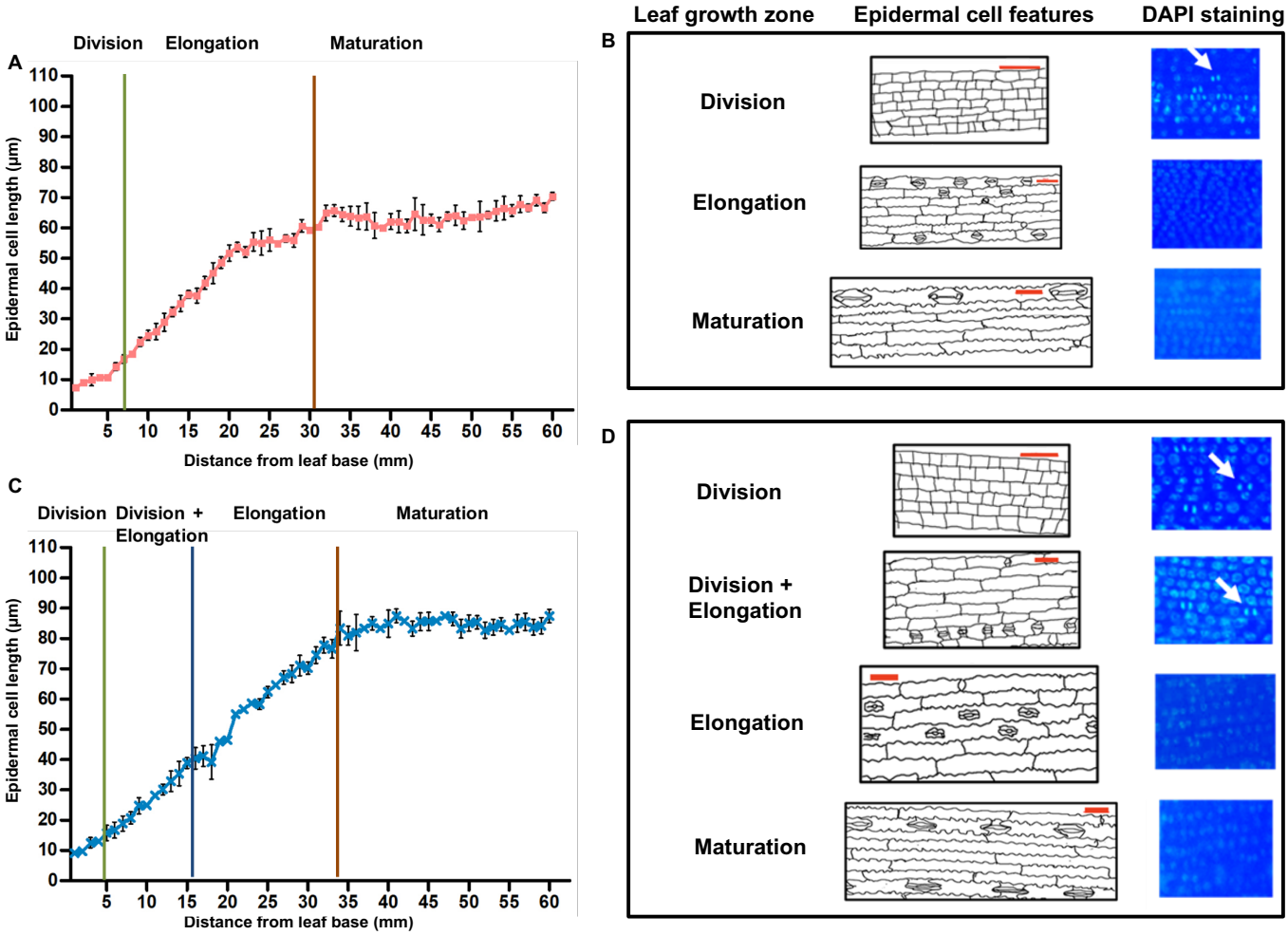


Figure 3. Leaf kinematics analyses confirm the cell division as a major regulator of rice leaf length. A, Different growth zones on leaf four of *O. sativa* cv. Nipponbare at the second day of emergence as quantified by epidermal cell length profiling and DAPI staining. B**,** Representative hand-drawn microscopic images of epidermal cells along with DAPI staining images for the different leaf-growth-zones of *O. sativa* cv. Nipponbare. C, Different growth zones on leaf four of *O. australiensis* at the second day of emergence. D, Representative hand-drawn microscopic images of epidermal cells along with DAPI staining images for the different leaf-growth-zones of *O. australiensis.* Values in A and C represent mean±SD (n=5). Vertical lines of different colors represent the separation of different leaf-growth zones. White arrows in B and D show DAPI-stained nuclei indicating dividing cells. Red scale bar = 20µm in B and D.

of the division zone with the elongation zone, which was not evident in the cultivated varieties (Figure 3; Supplemental Figure S4).

GA promoted cell division and the size of the division zone to increase the rice leaf length

Since transcript profiling indicated the possible involvement of GA for the leaf length differences across the accessions, we asked the question if GA could regulate rice leaf length by controlling cell proliferation activity. To this end, we examined the effects of exogenous treatment of GA_3 and GA-biosynthesis inhibitor Paclobutrazol (PAC) on leaf length and growth zones of *O. australiensis* and Nipponbare. Exogenous GA_3 treatment promoted the leaf length of both Nipponbare and *O. australiensis* (Figure 4, A and B; Supplemental Figure S5, A and

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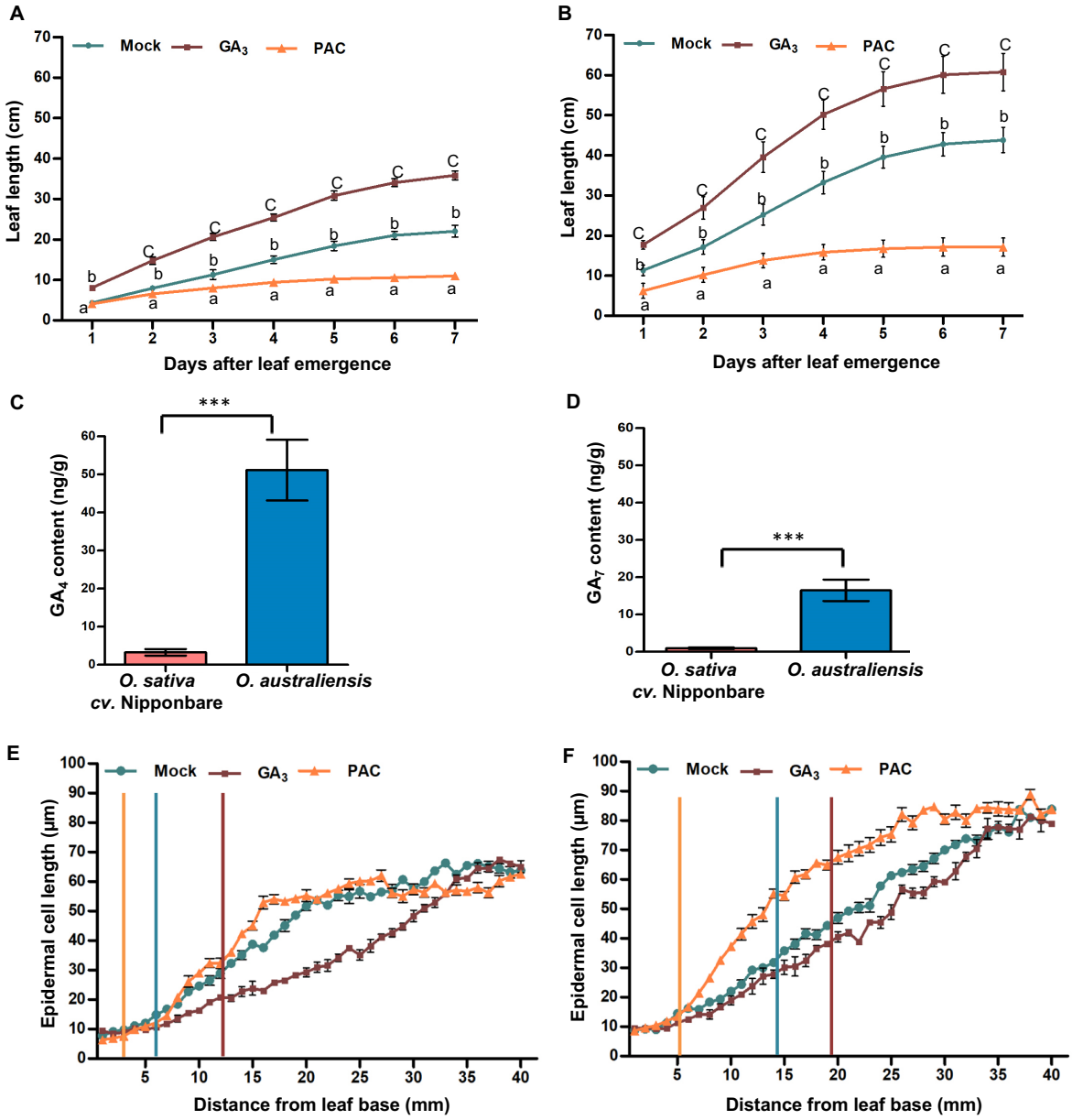


Figure 4. GA-mediated cell division control regulates the rice leaf length. (A, B), Effect of GA_3 (10μM) and Paclobutrazol (PAC, 1μM) treatments on leaf length of *O. sativa cv.* Nipponbare (A) and *O. australiensis* (B). Values represent mean±SD (n=15). Different letters indicate statistically significant differences at each day after emergence according to one-way ANOVA followed by post-hoc Tukey HSD calculation at $P < 0.05$. (C, D), GA quantification in leaf four of *O. sativa cv.* Nipponbare and *O. australiensis* at the second day of emergence. Data show mean ±SD (n=5),*** $P < 0.001$ by Student's t-test. (E, F), Different growth zones on leaf four of *O. sativa* cv. Nipponbare (E) and *O. australiensis* (F) in response to GA_3 and PAC treatments as quantified by epidermal cell length profiling and DAPI staining. Values represent mean ±SD (n=5). Vertical lines of different colors represent the size of division zones under different treatments.

B). The extent of increase in leaf length was more in Nipponbare (~1.6-fold) than *O. australiensis* (~1.3-fold). Exogenous treatment of PAC drastically reduced the leaf length of both the accessions. PAC inhibited the leaf length of Nipponbare by ~2-fold, and of *O. australiensis* by ~2.5-fold. The higher extent of increase in leaf length of Nipponbare by GA_3 treatment and higher extent of decrease in leaf length of *O. australiensis* under by PAC suggested that GA levels could be determining for the differences in the leaf length between

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the two accessions. Higher expression of GA-biosynthesis genes in *O. australiensis* leaves than Nipponbare, further, supported our hypothesis. To confirm this, we quantified GA in growing fourth leaves of Nipponbare and *O. australiensis* on the second day of emergence. Consistent with our hypothesis, we detected significantly higher levels of GA_4 and GA_7 in the leaves of *O. australiensis* compared to Nipponbare (Figure 4, C and D).

To check the effects of GA on cell proliferation and division zone, we performed leaf kinematics analysis on fourth leaves of *O. australiensis* and Nipponbare under GA3 and PAC treatment. We observed that the leaf length promotion in response to GA treatment was due to an increase in the size of the division zone in both the accessions (Figure 4, E and F; Supplemental Figure S5, C and D). However, the impact of GA on cell division was more striking in Nipponbare than *O. australiensis* in terms of the size of the division zone, cell production rate, and the number of cells in the meristem (Supplemental Table, S4 and S5). The inhibitory effect of PAC on the leaf length was also clearly due to the reduced size of the division zone in both the accessions (Figure 4, E and F; Supplemental Figure S5, C and D). The final epidermal cell length appeared to be similar for mock, GA treatment as well as PAC treatment for both the accessions, suggesting no major effects on cell elongation. Interestingly, the kinematics profile of GA-treated Nipponbare leaves resembled that of *O. australiensis* leaves under control conditions with an overlap of cell division and elongation zone (Figure, 4E and 3C). Together the exogeneous treatment experiments and GA quantification along with leaf kinematics profile in response to GA and PAC confirmed that the GA increased the rice leaf length by promoting cell division and the size of the division zone.

Growth-zone-specific expression analysis of GA-biosynthesis and signaling genes and cell-cycle-related genes

Next, we asked if zone-specific expressions of GA-related and cell cycle genes contribute to leaf size differences. To this end, we quantified the expression of GA biosynthesis genes in two leaf zones of Nipponbare and *O. australiensis*: zone I (leaf base – 0.8 cm; division zone in both accessions) and zone II (0.8 – 1.6cm; elongation zone in Nipponbare, where division extends in *O. australiensis*). The transcript levels of GA biosynthetic genes *OsGA20OX2* and *OsGA3OX2* were higher in the zone I of *O. australiensis*, which showed longer leaves and had higher GA levels in their leaves than Nipponbare (Figure 5, A and B). Furthermore, the expression level of the GA catabolic gene *OsGA2OX4* was higher in zone II of Nipponbare than *O. australiensis* (Supplemental Figure S6A). The transcript levels of *OsSLR1*, encoding a GA-signalling repressor DELLA protein, was also higher in both the

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302 regions of Nipponbare leaves (Supplemental Figure S6B). Taken together, higher expression
303 of GA biosynthetic genes *OsGA20OX2* and *OsGA3OX2* in the zone I, lower expression of GA
304 catabolic gene *OsGA2OX4* in zone II, and lower expression of GA-signalling repressor *OsSLR1*
305 in *O. australiensis* would promote higher GA effects in *O. australiensis* than Nipponabre.

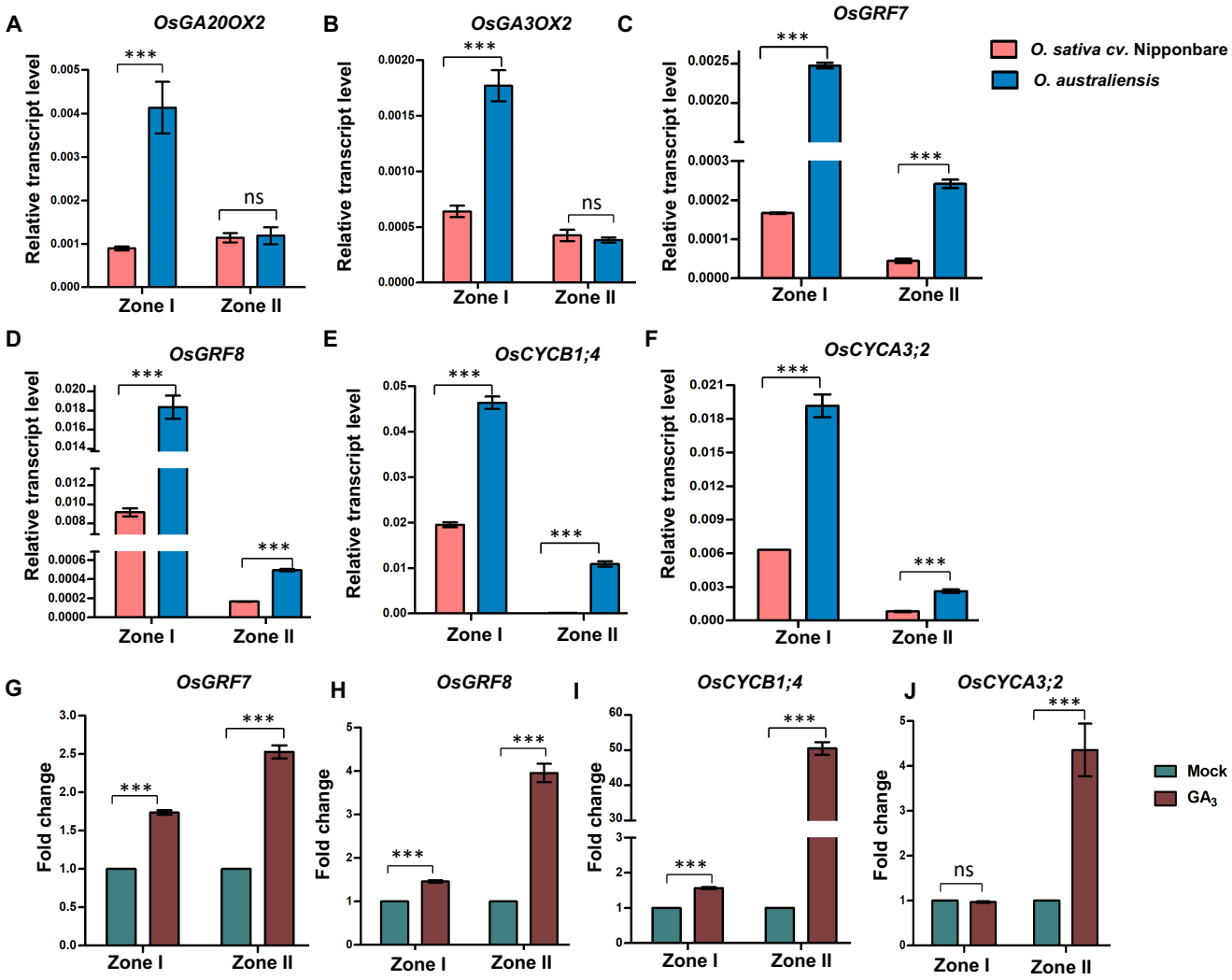


Figure 5. Zone-specific expression analysis of GA biosynthetic and signaling genes as well as cell cycle-related genes. (A-F), Expression pattern of GA biosynthesis genes *OsGA20OX2* and *OsGA3OX2* (A, B), genes encoding GRF transcription factors *OsGRF7* and *OsGRF8* (C, D), and cell cycle genes *OsCYCB1;4* and *OsCYCA3;2* (E, F) in two growth zones of *O. sativa cv.* Nipponbare and *O. australiensis*. Zone I represents the region from leaf base to 0.8cm and zone II represents 0.8cm to 1.6cm from the leaf base. (G-J), Effect of GA_3 treatment on the expression of *OsGRF7* (G), *OsGRF8* (H), *OsCYCB1;4* (I), and *OsCYCA3;2* (J) in the two growth zones of *O. sativa cv.* Nipponbare. Data shown are mean ±SD of three biological replicates, *** $P < 0.001$ by Student's t-test.

306 We checked the growth-zone-specific expressions of genes encoding GRF transcription
307 factors and cyclins. Similar to the expression of GA-biosynthesis and signaling genes, we
308 observed remarkably higher transcript levels of *OsGRF7* and *OsGRF8* in both zones I and II
309 of *O. australiensis* leaves compared to Nipponbare (Figure 5, C and D). The transcript levels
310 of *OsCYCB1;4* and *OsCYCA3;2* were also higher in both zone I and II of the *O. australiensis*

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than Nipponbare (Figure 5, E and F). Limited transcript levels of *OsCYCB1;4* and *OsCYCA3;2* in zone II of Nipponbare leaves matched with the negligible cell division activity beyond 0.8cm in the accession. The significantly lower expression level of cell cycle inhibitor *OsKRP4* and a higher level of cell cycle activator *OsE2F2* in basal regions of *O. australiensis* leaves than Nipponbare further reflected on the differences in cell cycle between the two accessions (Supplemental Figure S6, C and D). We then asked the question of whether a GA-mediated increase in the size of the division zone and leaf length of Nipponbare involved GRFs and cyclins. The exogenous GA treatment remarkably induced the expression levels of *OsGRF7* and *OsGRF8* as well as *OsCYCB1;4* and *OsCYCA3;2* in zone II of Nipponbare, leading to the increased size of the division zone (Figure 5, G-J). Treatment of GA-biosynthesis inhibitor PAC led to significant suppression of the expressions of *GRF*s and *CYCLIN*s in the division zone of Nipponbare (Supplemental Figure S6E). Together, gene expression analyses in different zones of the two contrasting accessions as well as in response to the exogenous GA treatment strongly supported our hypothesis of GA-mediated cell cycle control through GRFs to determine the rice leaf length.

Silencing of GA biosynthesis and signaling genes to confirm the GA-mediated regulation of rice leaf length

To confirm the roles of GA biosynthesis and signaling in rice leaf length regulation, we performed transient silencing of genes encoding a GA biosynthesis enzyme OsGA20OX2, a GA signaling repressor OsSLR1, and a GA-responsive transcription factor OsGRF7 in Nipponbare. These genes were selected based on transcriptomic comparisons as well as zone-specific quantification of expression levels. Silencing of *OsGA20OX2* led to a reduction in leaf length of Nipponbare, similar to exogenous PAC treatment (Figure 6, A and B; Supplemental Figure S5A). The reduction in leaf length of the silenced lines was associated with reduced size of the division zone as well as lower expression levels of *GRF*s and *CYCLIN*s than the control (Figure 6, B and C). In contrast, silencing of *OsSLR* led to a longer leaf length, increased size of division zone, and higher expression levels of *GRF*s and *CYCLIN*s compared to the control (Supplemental Figure S7, C and E). Similar to *OsGA20OX2*, silencing of *OsGRF7* also reduced the Nipponbare leaf length along with the smaller size of the division zone and reduced expression of downstream *CYCLIN* genes (Figure 6, D-F). Together, leaf phenotypes of these silencing lines confirmed the GA-mediated regulation of cell division zone through GRF transcription factors to determine the rice leaf length.

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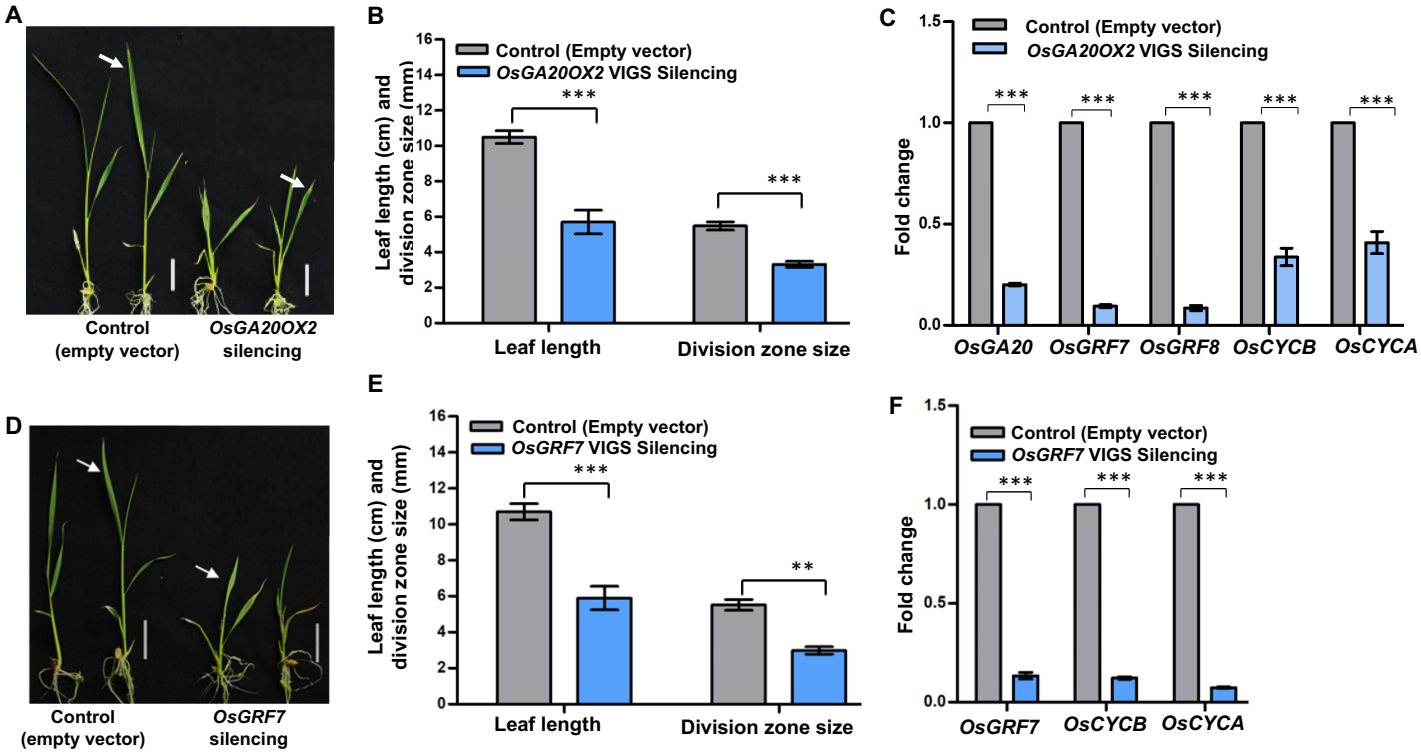


Figure 6. The silencing of GA-related genes confirms the GA-mediated regulation of rice leaf length. A, Shown are the leaf phenotypes of *OsGA20OX2* silencing in *O. sativa cv.* Nipponbare along with the control plants. B, Effect of *OsGA20OX2* silencing on leaf length and size of division zone**.** C, Expression levels of target GA biosynthetic gene, *GRFs*, and *CYCLINs* in *OsGA20OX2* silencing lines. D, Shown are the leaf phenotypes of *OsGRF7* silencing in *O. sativa cv.* Nipponbare along with the control plants. E, Effect of *OsGRF7* silencing on leaf length and size of division zone**.** F, Expression analysis of target *GRF7* and *CYCLINs* in *OsGRF7* silencing lines. Data represent mean ±SD of at least three biological replicates, *** $P < 0.001$ and ** $P < 0.01$ by Student's t-test.

Discussion

Detailed genetic understanding of the leaf size regulation is important for crop improvement programs, as leaf size strongly influences physiological processes, such as light capture efficiency, transpiration, and gas exchange (Zhu et al., 2010). Natural variation for the leaf size in a crop species and related wild accessions is an excellent resource for understanding the genetic and hormonal basis of leaf size regulation. Since crop plants, such as rice, have significantly lost the genetic variability for many desirable agronomic traits during the domestication, inclusion of wild relatives for a comparative study with cultivated crop varieties would provide a comprehensive genetic basis of leaf size changes (Huang et al., 2012; Chen et al., 2021). Studies have reported remarkable variations in the leaf morphological and anatomical features across cultivated and wild species of genus *Oryza*, suggesting a strong genetic control on leaf features of rice (Chatterjee et al., 2016; Mathan et al., 2021). We selected four rice accessions, based on detailed leaf size phenotyping of eleven accessions, that not only represented a significant genetic diversity but also covered the entire range of leaf length

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359 variations among the investigated accessions for detailed mechanistic studies. Consistent with
360 the genetic differences, the transcriptome profile of wild rice *O. australiensis* was significantly
361 different from the cultivated accessions, resulting in a higher number of differentially expressed
362 genes for the wild rice and cultivated rice comparisons than between the cultivated accessions.
363 Nonetheless, we detected basic leaf developmental, phytohormonal, and transcription factor
364 genes, though at different expression levels, suggesting the conserved nature of these genes
365 across the selected accessions.

366 Differences in the leaf length are attributed to basic cellular features and leaf
367 developmental programing (Gonzalez et al., 2012; Kierzkowski et al., 2019). Consistent with
368 this, cell cycle and development were the obvious enriched terms for the genes expressed at
369 higher levels in the accessions with longer leaves. Detailed leaf kinematics analyses for the
370 contrasting cultivated and wild rice accessions precisely established the predominant role of
371 cell division activity for defining the rice leaf length. Higher cell production rate and extension
372 of cell division to an extended length from leaf base drove the longer leaf phenotype in the wild
373 rice *O. asutraliensis* than the cultivated varieties (Figure 3; Table 2). Cell cycle duration and
374 cell division rate were comparable across the accessions, suggesting that the longer cell
375 division zone and increased cell production rate at the leaf base resulted in the production of a
376 higher number of cells that contributed to long-sized leaf in the wild rice. Higher expression
377 levels of genes encoding cyclins and cyclin-dependent kinases in the growing leaves of the *O.*
378 *asutraliensis* from the comparative transcriptome profiling along with zone-specific expression
379 analysis showing higher and expanded domain of *CYCLIN*s expression in *O. asutraliensis* than
380 the cultivated variety further supported the key importance of the cell division activity for the
381 rice leaf length regulation (Figure 2C; Figure 5, E and F). *O. asutraliensis*, besides showing
382 the expansion of the division zone, showed longer epidermal cell compared to cultivated
383 varieties, suggesting that the cell elongation also contributes to the longer leaf length of the
384 wild rice (Figure 3C).

385 Spatial and temporal regulation of cell division activity in plants is under complex
386 regulation of phytohormones and transcription factors (Nelissen et al., 2012; Takatsuka and
387 Umeda, 2014). We detected auxin-, cytokinin-, and GA-related genes among the differentially
388 expressed genes for the selected rice accessions. Besides, genes encoding transcription factors
389 known to regulate cell division, such as GRFs and TCPs, were also differentially expressed
390 between the contrasting rice accessions (Figure S3C; Vercruysse et al., 2020b). GA is shown
391 to control the cell division activity via the involvement of GRF transcription factors in
392 Arabidopsis and maize (Lantzouni et al., 2020; Fina et al., 2017). Our comparative

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transcriptome profiling showed higher expression of GA-biosynthesis and signaling genes as well as GRF transcription factors in the longer leaves of *O. australiensis* and *O. glaberrima* than IR64 and Nipponbare, suggesting the key functions of GA and GRF transcription factors to regulate cell division activity for differential leaf length across the selected accessions (Figure 2, D and E). Therefore, we hypothesized the GA-GRF-cell cycle as a key module not only as a general regulator of rice leaf length but also for attenuating the leaf size during the domestication. The differentially expressed auxin- and cytokinin-related genes attest to the fine-tuning of leaf size and other leaf features through the coordinated functions of multiple different phytohormones. Differential expression of many leaf developmental genes across the rice accessions would likely explain the extensive leaf patterning and differentiation differences across different cultivated and wild rice.

GA is reported to be involved in the maintenance of the division zone size and accumulates in the transition zone in maize (Nelissen et al., 2012). In support of the GA-GRF-cell cycle module to regulate rice leaf length, growing leaves of *O. australeinsis* had higher levels of bioactive GAs than Nipponbare that would promote the size of the division zone extending into the elongation zone, leading to a significantly enlarged transition zone in the wild rice (Figure 4, C and D). Higher expression levels of GA biosynthesis genes *OsGA20OX2* and *OsGA3OX2* in the basal division zone of *O. australeinsis* leaves than Nipponbare corroborated the higher GA content in the wild rice (Figure 5, A and B). In addition, lower expression of GA-catabolic gene *OsGA2OX4* in the transition zone of the wild rice than Nipponbare likely maintained higher GA levels in the zone. Consistent with these, exogenous GA-treatment increased the rice leaf length by extending the zone where cell division operates. GA is known to induce cell elongation response (Jones and Kaufman, 1983). Exogenous GA-treatment, however, increased the leaf length without altering the mature epidermal cell length, suggesting that GA primarily affects rice leaf length via regulation of cell division (Supplemental Table S4 and S5). The longer epidermal cells of *O. australeinsis*, thus, could likely be attributed to the functions of other phytohormones. Finally, reduction in rice leaf length by silencing *OsGA20OX2* that involved downregulation of *CYCLIN*s confirmed the central role of GA in rice leaf length regulation through cell division control (Figure 6, A-C).

GRF transcription factors promote cell division activity by directly binding to the promoters of *CYCA1;1* and *CDC20* (Li et al., 2018). Moreover, GRF7 regulation of GA-biosynthesis and auxin-signaling genes control the rice plant architecture (Chen et al., 2020). Higher *GRF7* and *GRF8* expression in growing leaves of rice accessions with longer leaves from RNAseq analysis along with more *GRF7* and *GRF8* transcripts in division and transition

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zones of *O. australeisnsis* than Nipponbare suggested that GRF7 and GRF8 may connect GA-levels and cell cycle for rice leaf size regulation (Figure 5, C and D). GA-treatment, increasing the leaf length by increasing the domain of cell division, induced the *GRF7* and *GRF8* expression in both division and transition zone, further, suggesting the GRF7 and GRF8 functions downstream to GA (Figure 5, G-J). Consistent with this, silencing of *GRF7* resulted in reduced rice leaf length with an associated decrease in the expression of the *CYCLIN*s (Figure 6, D-F). Silencing of *OsSLR1*, a DELLA protein that inhibits GRF functions to repress GA signaling, led to increased leaf length along with higher expression of *GRF7* and *GRF8*, further supporting the functions of GRF7 and GRF8 to integrate GA levels and leaf length regulation.

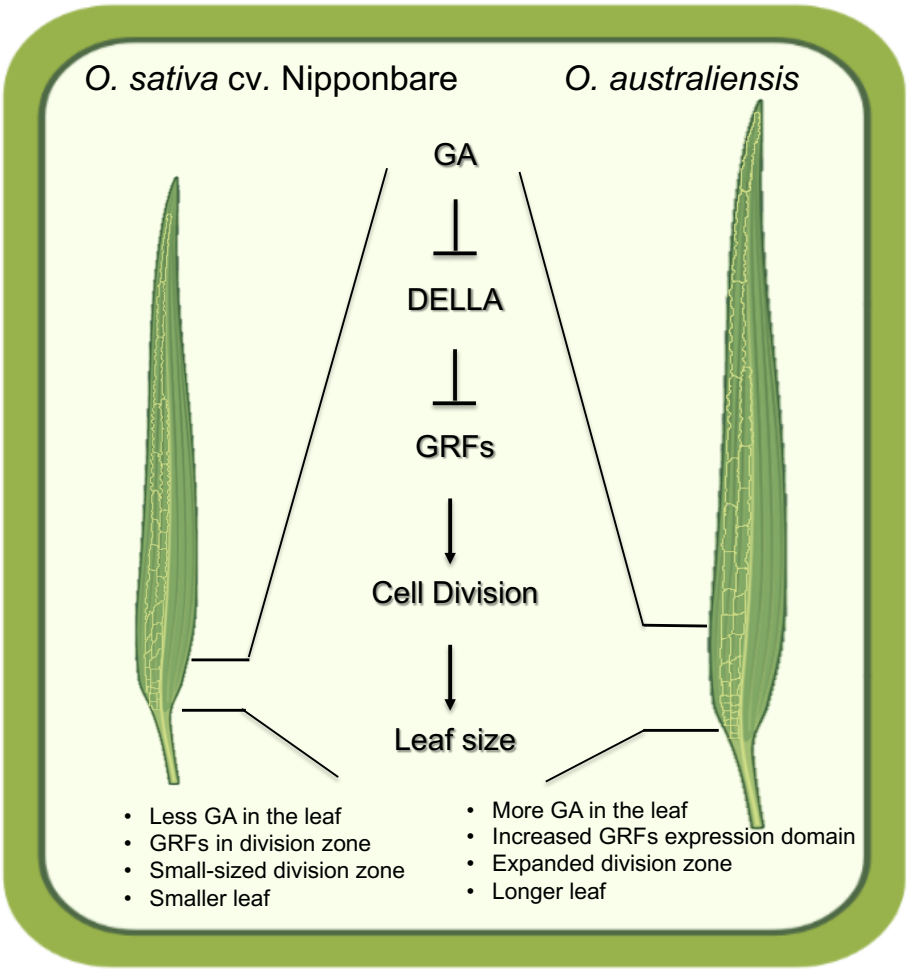


Figure 7. The GA-GRF-cell cycle module is a key regulator of the rice leaf length control. GA-mediated induction in the expression domain of GRF transcription factors expands the region with cyclin expression at the leaf base, thus promoting the longer division zone with a higher cell production rate leading to longer leaves in rice. Higher GA levels and downstream signaling resulting in increased cell division activity explained the longer leaves of the wild rice *O. australiensis* compared to the cultivated varieties.

In conclusion, we showed that the GA-regulated cell proliferation controls the final rice leaf length and explains the variation in leaf length across diverse rice accessions (Figure 7).

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Comparative transcriptomic profiling along with gene-coexpression networks suggested the key roles of GA, GRF transcription factors, and cell cycle for rice leaf length regulation. Leaf kinematics and GA experiments further strengthened the hypothesis of GA-mediated leaf length regulation via cell division control. Zone-specific expression analysis and silencing of candidate GA biosynthesis and signaling genes confirmed that the GA-mediated cell division control to regulate leaf length involves downstream GRFs. Taken together, higher GA levels and associated downstream components resulting in increased cell division activity explained the long leaves of the wild rice *O. australiensis* compared to the cultivated varieties (Figure 7). The established GA-GRF-cell cycle module could not only be a general regulator of rice leaf length but also have contributed to the optimization of leaf size during domestication. The module could, further, be a way for plants to achieve cellular plasticity in response to their growth environment.

Materials and Methods

Plant materials and growth conditions

The genotypes used for this study are summarized in Supplementary Table S1. Rice seeds were first germinated on Petri plates containing wet germination paper for 7 days, followed by transfer of germinated seedlings to a hydroponic solution containing one fourth MS Media (Himedia) with micro and macronutrients in standard concentrations. The nutrient solution was changed every third day during the seedling growth. Seedlings were grown in a greenhouse at 28 ºC day and 23 ºC night temperature with 14:10 hours of light and dark cycle. For exogenous GA and GA-biosynthesis inhibitor treatment experiments, 10µM of gibberellic acid (GA3), and 1µM of gibberellic acid biosynthesis inhibitor PAC (paclobutrazol) were directly added in hydroponic solution.

Phenotyping of leaf morphological traits and leaf growth analysis

The quantification of leaf length and width was performed on each individual fully-grown leaf from second to eight leaves of all the selected cultivated and wild rice accessions. Leaf length and width were quantified manually using a ruler. Leaf-sheath length was measured as a distance between the seedling base to ligule. The leaf-blade length was quantified as the distance between the ligule to the leaf tip. Leaf-blade width was measured in the central widest part of the fully expanded leaf blade. Total mature leaf length was calculated by adding leaf sheath and leaf blade length. The leaf growth analysis was done on leaf four, when the leaf emerged from the sheath of the third leaf, as explained by Sprangers et al. (2016). Leaf length

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was measured daily from the day of emergence up to the seventh day by considering the seedling base as the starting point of the leaf. The time of emergence of the fourth leaf varied from species to species (around 10 days in *O. sativa* cv. IR64 and Nipponbare, 12 days in African cultivated rice *O. glabberrima*, and 18-19 days in the wild rice *O. australiensis* after germination). Leaf elongation rate (LER) was calculated as the leaf growth in one day divided by the time interval. For this, the leaf length was quantified at specific time points on each day. All the quantifications were performed using at least 15 individual plants of each accession.

RNA-seq library preparation and sequencing

Leaf four of the selected accessions were collected at the same developmental stage (48 hrs. after leaf emergence when the leaves are growing at a steady growth rate) for RNA-seq library preparation. Libraries were prepared in three biological replicates using YourSeq Full Transcript RNAseq Library Kit (AMARYLLIS NUCLEICS) as per the manufacturer's protocol. These RNA-seq libraries were sequenced on the Illumina HiSeq platform, and reads were generated in 150bp paired-end format.

Quality filtering, mapping of reads, and differential gene expression analysis

The reads were quality filtered using the NGSQC toolkit that involved removal of adapter sequences and low-quality reads (Phred score <30) (Patel et al., 2012). The quality-filtered reads were then mapped to reference coding DNA sequences (CDS) of rice from the Rice Genome Annotation Project database (RGAP 7) using default parameters of bowtie2 (Langmead and Salzberg, 2012). SAMtools were used to generate bam alignment files that were used to tabulate raw counts for each gene (Li et al., 2009). edgeR was used for differential gene expression analysis (Robinson et al., 2010). Genes for which there were 2 or fewer cpm in less than three replicates were not considered further. glm approach with quasi-likelihood F-test was used for differential gene expression analysis. Significant differences in gene expression levels for different pair-wise comparisons were determined based on FDR cut-off < 0.05 and logFC value >1 and <-1.

Principle Component Analysis with Self Organizing Map (PCA-SOM) clustering

Normalized read counts (Supplemental Data Set S7) were used for a gene expression clustering method (Chitwood et al., 2013). For clustering, the genes in the upper 50% quartile of coefficient of variation for expression across different species were used. The scaled expression values were used to generate species-specific gene clusters (Wehrens and Buydens, 2007).

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Three by three hexagonal SOM clusters and 100 training interactions were used during clustering. The SOM clusters were visualized in PCA space, where PC values were calculated based on gene expression across the species with the help of the prcomp R package. The heatmaps were generated for each cluster using superheat R package.

GO Enrichment Analysis

GO enrichment analysis of the differentially expressed genes as well as for genes present in each PCA-SOM cluster was performed using the Plant Transcriptional Regulatory Map GO enrichment tool of Plant Transcription Factor Database (Tian et al., 2019).

Gene regulatory network analysis

An unsigned correlation network was constructed using the weighted gene correlation network (WGCNA) package version 1.68 (Langfelder et al., 2008). Data were z-score normalized (R scale function). The soft-thresholding power was chosen based on a scale-free topology with a fit index of 0.8. An adjacency matrix with the selected soft-thresholding power was calculated and transformed into a topological overlap matrix (TOM). Using the TOM, network properties such as strength were calculated and the network was visualized using the igraph package version 1.2.5 (Csardi et al., 2006) and visNetwork 2.0.9 (https://datastorm-open.github.io/visNetwork/). A Fast-Greedy modularity optimization algorithm was selected to define modules in the integrated network.

Leaf kinematics analysis

Leaf kinematics analysis was performed as described by Sprangers et al. (2016). To estimate the size of division zone size, the basal 4 cm segment of leaf four was collected from each accession at different days of emergence up to day five. Samples were placed in 3:1 (v/v) absolute ethanol: acetic acid for fixation of cell walls and clearing of chlorophyll, rinsed for 30 min in a buffer containing 50 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl, pH 7, and nuclei were stained by incubating the samples in the dark for 2 min in the same buffer solution containing 49,6-diamino-phenylindole (DAPI) at a concentration of 1 mg/ml. Fluorescent nuclei were observed with a microscope equipped with an epi-fluorescent condenser (Nikon 80i-epifluorescence Microscope), and the images obtained were used to measure the size of the division zone as the distance between the base of the leaf and the most distal mitotic cell. To determine cell length profiles, leaf four was collected on the second day after leaf emergence at the steady-state growth, and prepared for microscopy. The collected leaves were initially

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kept in absolute ethanol for 48 h (ethanol changed once after 24 h), and then transferred to lactic acid and stored at 4°C. Samples were mounted on microscope slides using glycerol as a mounting medium, and then successive images were taken using a bright field microscope (LMI, UK). Leaf kinematics analyses for GA- or PAC- treated seedlings were also performed as explained above. The various growth parameters from the leaf kinematics experiments were quantified using formulae explained in Sprangers et al. (2016).

Gibberellic acid (GA) quantification

200-250 mg of fresh leaves tissue at the second day of emergence at steady-state growth, when leaf kinematics and transcriptomics were also performed, were collected and finely ground in liquid nitrogen. The samples were extracted in ice-cold buffer (MeOH: H2O: HCOOH, 15:4:0.1) on a shaker for overnight at 4°C. Then internal standard was added in the extract and vortexed for 10 min. The extract was centrifuged for 15 min at 16,000 rcf at 4°C. Then the supernatant was passed onto a pre-conditioned (MeOH and 0.1% of HCOOH) C18 RP SPE column. The column was washed with 0.1% HCOOH and 5% MeOH. Finally, elution was done with ice-cold 0.1% HCOOH in Acetonitrile. After evaporation of the solvent, the pellet was re-suspended in 5% MeOH. GA content was then analyzed using LC-MS/MS.

RNA isolation, cDNA synthesis, and qRT-PCR analysis

After defining growth zones on leaf 4, samples for expression profiling were collected according to the length of a specific zone. Total RNA was isolated using Trizol reagent (Invitrogen) as described by Longeman et al. (1987) and treated with RNase-free DNase I (Thermo Fisher Scientific). 1.5 µg of total RNA was reverse transcribed to first-strand cDNA using RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) following the manufacturer's instructions. The quantitative real-time-PCR (qRT-PCR) analysis was performed on the CFX Connect Real-Time System (Bio-rad) using PowerUp™ SYBR® Green fluorescence dye (Thermo Fisher Scientific). The primer pairs for qRT-PCR were designed based on gene sequences obtained from RGAP 7 (Rice Genome Annotation Project database) (Supplemental Table S6). The primer efficiency analyzed using a standard curve method. From each species five different cDNA concentration (25,50,100,200 and 400ng) used to generate standard curve and primer efficiency was calculated using the formula E = (10[-1/slope] 257 - 1) *100. The primer specificity of amplified gene products across the selected rice genotypes was viewed through dissociation curve analysis. Relative expressions of the target genes were analyzed using actin (LOC_Os03g50885.1) as the internal control applying the $2^{-\Delta\Delta Ct}$ method

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for fold change and $2^{-\Delta Ct}$ method for relative transcript levels quantification (Livak and Schmittgen 2001). The expression pattern of each gene was confirmed by the independent biological experiments.

Transient gene silencing line generation

Virus-induced gene silencing (VIGS) mediated transient silencing line for *OsGA20OX2, OsGRF7,* and *OsSLR1* genes were generated using the mVIGS vector as previously described by Kant et al. (2017). In short, recombinant vector constructs were transformed into Agrobacterium strain EHA105, and a single Agrobacterium colony was used to initiate a primary culture in LB medium supplemented with kanamycin and rifamycin antibiotics. After overnight incubation at 28 ºC, a secondary culture was grown to an OD600 of 0.6 to 0.8 with 200 µM acetosyringone. Cells were harvested after centrifugation and resuspended in 10 mM MES, 10 mM MgCl2, and 200 µM acetosyringone. 6-day old rice seedlings were injected with 50 µL of bacterial suspension into the meristematic region located at the basal region of seedlings. After Agro-inoculation seedlings were transferred onto sterile Whatman No. 1 filter paper immersed in MS medium placed on a solid support with its ends dipped into a reservoir containing the nutrient medium. To avoid drying, plant roots were covered with moist tissue paper and transferred to tubes containing MS medium 24 h post-inoculation and were maintained at 28°C under conditions described above. Transgenic seedlings were screened based on expression analysis of target genes as well as based on leaf morphological differences. As a positive control, we generated a transient silencing line for chlorophyll biosynthesis gene (*ChlH*) and confirmed positive transgenic plants by observing leaves chlorosis phenotype and gene expression analysis (Supplemental Figure S7, A and B).

Accession Numbers

The quality-filtered reads, which were used to get the normalized read counts and for DE analysis, were deposited to the NCBI Short Read Archive under accessions SRRXXXXXXX to SRRXXXXXXX.

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609 **Supplemental Data**

610 **Supplemental Figure S1.** Leaf length and width phenotyping of the four selected accessions.

611

612 **Supplemental Figure S2.** Differentially expressed transcripts and enriched GO terms in *O.*
613 *glaberrima* and *O. australiensis*.

614

615 **Supplemental Figure S3.** PCA-SOM clustering of gene expression across the selected rice
616 accessions.

617

618 **Supplemental Figure S4.** Cellular basis of leaf size differences across the selected rice
619 accessions.

620

621 **Supplemental Figure S5.** Effects of GA and paclobutrazol treatments on leaf length and
622 division zone size.

623

624 **Supplemental Figure S6.** Zone-specific expression analysis of GA- and cell cycle-related
625 genes.

626

627 **Supplemental Figure S7.** Silencing of positive control *OsChlH* and *OsSLR1,* a GA-signalling
628 repressor.

629

630 **Supplemental Table S1.** List of cultivated and wild rice accessions used in the present study

631

632 **Supplemental Table S2.** Quantification of maximum leaf width of fully-developed leaves of
633 the seven cultivated and four wild rice accessions.

634

635 **Supplemental Table S3.** The total number of differentially expressed genes along with
636 upregulated and downregulated genes for each pair-wise comparison.

637

638 **Supplemental Table S4.** Quantification of leaf kinematics parameters of the growing leaf
639 four of *O. sativa* cv. Nipponbare under exogenous GA_3 and paclobutrazol treatments.

640

641 **Supplemental Table S5.** Quantification of leaf kinematics parameters of the growing leaf
642 four of *O. australiensis* under exogenous GA_3 and paclobutrazol treatments.

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643

644 **Supplemental Table S6.** List of primers used for gene expression analysis and transient
645 silencing experiments.

646

647 **Supplemental Dataset S1**. List of statistically significant differentially expressed genes for
648 each pair-wise comparison.

649

650 **Supplemental Dataset S2.** List of statistically significant GO-terms enriched for the
651 upregulated and downregulated genes in Figure 2 and Supplemental Figure S3.

652

653 **Supplemental Dataset S3.** List of genes present in each cluster derived from PCA-SOM
654 analysis.

655

656 **Supplemental Dataset S4.** List of statistically significant GO-terms enriched for the genes
657 present in the PCA-SOM clusters.

658

659 **Supplemental Dataset S5.** List of cell cycle-related genes, transcription factors and leaf
660 developmental genes, and phytohormone-related genes present in clusters 1, 4, and 7 that were
661 used for generating heatmaps in figure 2, C-E and figure S3C.

662

663 **Supplemental Dataset S6.** List of genes present in the expression network shown in figure 2F
664 with their network properties and module belongings.

665

666 **Supplemental Dataset S7.** Normalized read counts used for generating gene coexpression
667 network.

668

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Tables

Table 1. Quantification of leaf length (cm) of fully-developed leaf two to leaf eight of the seven cultivated and four wild rice accessions. Each value represents mean ± SD, where n = 15 data points from different plants. Different letters indicate statistically significant differences according to one-way ANOVA followed by post-hoc Tukey HSD calculation at $P < 0.05$.

Oryza species	Cultivar/ accession number	Leaf number						
		Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
Oryza sativa ssp. japonica	Nipponbare	3.94 ± 0.35 (a)	11.8 ± 1.15 (a)	21.96 ± 1.53 (a)	26.92 ± 2 (a)	38.11 ± 1.73 (a)	44.37 ± 1.4 (a)	48.71 ± 2.84 (a)
Oryza sativa ssp. indica	IR 64	5.6 ± 0.22(b)	11.75 ± 0.85 (a)	20.95 ± 1.91 (a)	26.25 ± 1.72 (a)	34.05 ± 1.07 (a)	41.56 ± 1.48 (b)	44.85 ± 2.75 (b)
Oryza sativa ssp. indica	Vandana	4.54 ± 0.62 (a)	11.5 ± 1.43 (a)	21.12 ± 1.55 (a)	29.06 ± 1.34 (a)	32.59 ± 1.71 (b)	37.36 ± 1.77 (c)	45.59 ± 1.74 (ab)
Oryza sativa ssp. indica	Swarna	4.7 ± 0.44 (a)	14.93 ± 1.24 (b)	27.92 ± 2.52 (b)	39.88 ± 2.65 (b)	46.9 ± 1.87 (c)	52.76 ± 2.86 (d)	58.72 ± 3.03 (c)
Oryza sativa ssp. indica	Pusa Basmati1	6.25 ± 0.54(b)	14.66 ± 2.36 (b)	28.48 ± 3.11 (b)	37.11 ± 2.01 (c)	42.98 ± 1.76 (d)	48.3 ± 2.26 (a)	55.17 ± 1.94 (d)
Oryza sativa ssp. indica	Nagina 22	4.21 ± 0.38 (a)	14.55 ± 1.52 (b)	27.98 ± 1.3 (b)	38.5 ± 2.29 (bc)	44.56 ± 2.14 (de)	53.41 ± 2.59 (d)	62.77 ± 1.79 (e)
Oryza glaberrima	IR 102925	6 ± 0.3(b)	14.95 ± 1.65 (bc)	26.9 ± 1.18 (b)	38.2 ± 1.19 (c)	45.9 ± 2.42 (ce)	53.7 ± 2.4 (d)	59.5 ± 2.82 (c)
Oryza glumaepatula	IR104151	8.01 ± 0.56 (c)	23.24 ± 1.68 (e)	37.26 ± 3.1 (c)	43.3 ± 1.3 (d)	46.76 ± 1.51 (ce)	53.35 ± 2.05 (d)	63.91 ± 3.07 (e)
Oryza punctata	IRGC105137	6.08 ± 0.63(b)	17.67 ± 1.19 (cd)	34.38 ± 1.27 (cd)	47.52 ± 1.39 (e)	51.92 ± 1.31 (f)	58.26 ± 1.23 (e)	70.67 ± 2.04 (f)
Oryza latifolia	IRGC 99596	10.15 ± 0.62(d)	18.32 ± 1.42 (d)	31.52 ± 2.05 (d)	46.13 ± 1.19 (e)	53.12 ± 1.1 (f)	60.63 ± 2.26 (e)	70.93 ± 1.68 (f)
Oryza australiensis	IRGC 105272	20.57 ± 2.68 (e)	34.19 ± 3.44 (f)	50.66 ± 4.55 (e)	56.57 ± 2.6 (f)	64.74 ± 4.36 (g)	69.5 ± 0.71 (f)	87.8 ± 5.27 (g)

Table 2. Quantification of leaf kinematics parameters of the growing leaf four of selected cultivated and wild rice accessions. Each value represents mean ± SD (n = 15 for mature leaf length and leaf elongation rate, n = 5 for all other parameters). Different letters indicate statistically significant differences according to one-way ANOVA followed by post-hoc Tukey HSD calculation at $P < 0.05$.

Growth parameters	***O. sativa* cv. IR 64**	***O. sativa* cv. Nipponbare**	***O. glaberrima***	***O. australiensis***
Mature leaf length (cm)	20.06 ± 1.38(a)	21.46 ± 0.81(a)	26.98 ± 1.01(b)	41.38 ± 4.25(c)
Leaf elongation rate (mm/h)	1.32 ± 0.01(a)	1.34 ± 0.01(a)	2.01 ± 0(b)	2.41 ± 0.01(b)
Meristem length (mm)	7.52 ± 0.14(a)	7.81 ± 0.34(a)	12.32 ± 0.34(b)	16.66 ± 0.64(c)
Length of the growth zone (mm)	24 ± 1(a)	31.33 ± 0.58(b)	24.67 ± 1.53(a)	34.33 ± 0.58(c)
Mature cell length (µm)	70.61 ± 0.15(a)	64.26 ± 1.41(b)	66 ± 1.07(b)	84.57 ± 1.32(c)
Cell production rate (cells/h)	18.76 ± 0.12(a)	20.88 ± 0.4(b)	30.44 ± 0.51(c)	28.49 ± 0.42(d)
Number of cells in meristem	738.29 ± 27.5(a)	715.92 ± 17.05(a)	842.73 ± 31.5(b)	904.75 ± 52.9(b)
Number of cells in growth zone	1193.63 ± 70.37(a)	1328.8 ± 26.33(b)	1100.53 ± 43.44(ac)	1211.09 ± 38.48(abc)
Number of cells in elongation zone	455.35 ± 68.5(a)	612.88 ± 41.5(b)	257.8 ± 38.3(c)	306.34 ± 19.5(c)
Average cell division rate (cell. $cell^{-1}.h^{-1}$)	0.03 ± 0(a)	0.03 ± 0(ab)	0.04 ± 0(bc)	0.03 ± 0(ab)
Cell cycle duration (h)	27.29 ± 1.15(a)	23.78 ± 0.99(b)	19.2 ± 1.03(c)	22 ± 0.98(b)
Time in elongation zone (h)	24.26 ± 3.53(a)	29.34 ± 1.62(a)	8.47 ± 1.29(b)	10.76 ± 0.79(b)
Time in division zone (h)	260.01 ± 12.4(a)	225.54 ± 10.1(b)	186.66 ± 11.05(c)	216.13 ± 11.45(bc)
Length of cells leaving meristem (µm)	15.15 ± 0.37(a)	17.79 ± 0.41(a)	31.33 ± 1.41(b)	39.93 ± 3.36(c)
Average cell expansion rates (µm. $\mu m^{-1}.h^{-1}$)	0.06 ± 0.01(ac)	0.04 ± 0(a)	0.09 ± 0.01(bc)	0.07 ± 0.01(ac)

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Figure Legends

Figure 1. Wild rice *Oryza australiensis* grows longer leaf compared to the selected cultivated rice accessions. A, Representative photographs of the selected cultivated and wild rice accessions showing leaf size variations. B, Quantification of mature leaf length of leaf two to leaf ten. C, Representative leaf four images at the second day of emergence. D, Quantification of leaf four lengths at the different day of emergence. E, Leaf elongation rate of the selected rice accessions. Values in graphs represent mean±SD (n=15). Different letters indicate statistically significant differences according to one-way ANOVA followed by post-hoc Tukey HSD at $P < 0.05$.

Figure 2. Transcriptome profiling reveals the key contribution of the cell cycle and Gibberellic Acid (GA) for differential leaf length across the selected rice accessions. A, Number of differentially expressed transcripts and enriched GO terms in *O. australiensis* compared to *O. sativa* cv. IR64 and *O. sativa* cv. Nipponbare. * show enriched GO terms. B, Gene expression clusters showing genotype-specific resolution for the selected four accessions derived from PCA-SOM analysis. Enriched GO terms are shown for *O. australiensis* and *O. glaberrima* specific clusters 1, 4, and 7. (C-E), Expression patterns of cell cycle-related genes (C), transcription factors and leaf developmental genes (D), and Gibberellic acid biosynthesis and signaling genes (E) present in clusters 1, 4 and 7 across the four accessions. F, Gene regulatory network generated for the genes from the relevant clusters of (B). Three different modules of the network are shown in different colors.
AUS= *O. australiensis,* GLA= *O. glaberrima,* IR= *O. sativa* cv. IR64, NIP= *O. sativa* cv. Nipponbare.

Figure 3. Leaf kinematics analyses confirm the cell division as a major regulator of rice leaf length. A, Different growth zones on leaf four of *O. sativa* cv. Nipponbare at the second day of emergence as quantified by epidermal cell length profiling and DAPI staining. B**,** Representative hand-drawn microscopic images of epidermal cells along with DAPI staining images for the different leaf-growth-zones of *O. sativa* cv. Nipponbare. C, Different growth zones on leaf four of *O. australiensis* at the second day of emergence. D, Representative hand-drawn microscopic images of epidermal cells along with DAPI staining images for the different

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leaf-growth-zones of *O. australiensis.* Values in A and C represent mean±SD (n=5). Vertical lines of different colors represent the separation of different leaf-growth zones. White arrows in B and D show DAPI-stained nuclei indicating dividing cells. Red scale bar = 20μm in B and D.

Figure 4. GA-mediated cell division control regulates the rice leaf length. (A, B), Effect of GA3 (10μM) and Paclobutrazol (PAC, 1μM) treatments on leaf length of *O. sativa cv.* Nipponbare (A) and *O. australiensis* (B). Values represent mean±SD (n=15). Different letters indicate statistically significant differences at each day after emergence according to one-way ANOVA followed by post-hoc Tukey HSD calculation at $P < 0.05$. (C, D), GA quantification in leaf four of *O. sativa cv.* Nipponbare and *O. australiensis* at the second day of emergence. Data show mean ±SD (n=5),*** $P < 0.001$ by Student's t-test. (E, F), Different growth zones on leaf four of *O. sativa* cv. Nipponbare (E) and *O. australiensis* (F) in response to GA3 and PAC treatments as quantified by epidermal cell length profiling and DAPI staining. Values represent mean ±SD (n=5). Vertical lines of different colors represent the size of division zones under different treatments.

Figure 5. Zone-specific expression analysis of GA biosynthetic and signaling genes as well as cell cycle-related genes. (A-F), Expression pattern of GA biosynthesis genes *OsGA20OX2* and *OsGA3OX2* (A, B), genes encoding GRF transcription factors *OsGRF7* and *OsGRF8* (C, D), and cell cycle genes *OsCYCB1;4* and *OsCYCA3;2* (E, F) in two growth zones of *O. sativa cv.* Nipponbare and *O. australiensis.* Zone I represents the region from leaf base to 0.8cm and zone II represents 0.8cm to 1.6cm from the leaf base. (G-J), Effect of GA_3 treatment on the expression of *OsGRF7* (G), *OsGRF8* (H), *OsCYCB1;4* (I), and *OsCYCA3;2* (J) in the two growth zones of *O. sativa cv.* Nipponbare. Data shown are mean ±SD of three biological replicates, *** $P < 0.001$ by Student's t-test.

Figure 6. The silencing of GA-related genes confirms the GA-mediated regulation of rice leaf length. A, Shown are the leaf phenotypes of *OsGA20OX2* silencing in *O. sativa cv.* Nipponbare along with the control plants. B, Effect of *OsGA20OX2* silencing on leaf length

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and size of division zone**.** C, Expression levels of target GA biosynthetic gene, *GRFs*, and *CYCLINs* in *OsGA20OX2* silencing lines. D, Shown are the leaf phenotypes of *OsGRF7* silencing in *O. sativa cv.* Nipponbare along with the control plants. E, Effect of *OsGRF7* silencing on leaf length and size of division zone**.** F, Expression analysis of target *GRF7* and *CYCLINs* in *OsGRF7* silencing lines. Data represent mean ±SD of at least three biological replicates, *** $P < 0.001$ and ** $P < 0.01$ by Student's t-test.

Figure 7. The GA-GRF-cell cycle module is a key regulator of the rice leaf length control. GA-mediated induction in the expression domain of GRF transcription factors expands the region with cyclin expression at the leaf base, thus promoting the longer division zone with a higher cell production rate leading to longer leaves in rice. Higher GA levels and downstream signaling resulting in increased cell division activity explained the longer leaves of the wild rice *O. australiensis* compared to the cultivated varieties.

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