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RESEARCH ARTICLE

The HK5 and HK6 cytokinin receptors mediate diverse developmental pathways in rice

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ABSTRACT

The phytohormone cytokinin regulates diverse aspects of plant growth and development. Our understanding of the metabolism and perception of cytokinin has made great strides in recent years, mostly from studies of the model dicot *Arabidopsis*. Here, we employed a CRISPR/Cas9-based approach to disrupt a subset of cytokinin histidine kinase (HK) receptors in rice (*Oryza sativa*) in order to explore the role of cytokinin in a monocot species. In *hk5* and *hk6* single mutants, the root growth, leaf width, inflorescence architecture and/or floral development were affected. The double *hk5 hk6* mutant showed more substantial defects, including severely reduced root and shoot growth, a smaller shoot apical meristem, and an enlarged root cap. Flowering was delayed in the *hk5 hk6* mutant and the panicle was significantly reduced in size and infertile due to multiple defects in floral development. The *hk5 hk6* mutant also exhibited a severely reduced cytokinin response, consistent with the developmental phenotypes arising from a defect in cytokinin signaling. These results indicate that HK5 and HK6 act as cytokinin receptors, with overlapping functions to regulate diverse aspects of rice growth and development.

KEY WORDS: Cell signaling, Cytokinin, Plant development, Plant hormones

INTRODUCTION

The monocotyledonous plant *Oryza sativa* (rice) is one of the most widely grown cereal crops, supplying approximately 23% of the calories consumed by humans (Muthayya et al., 2014). Appropriate modulation of the activity of phytohormones, which are crucial regulators of growth and development, can substantially contribute to increasing yield of this and other cereal crops. One such phytohormone, cytokinin, is a particularly promising target for improving crop species (Jameson and Song, 2016) as it regulates nearly all plant processes, many of which have agronomic relevance, including meristem activity, leaf senescence, nutrient uptake, various abiotic and biotic interactions, and multiple developmental pathways (Kieber and Schaller, 2014, 2018; Mok and Mok, 2001). For example, mutations in the *CYTOKININ OXIDASE2* gene increase the levels of cytokinin in developing rice panicles and elevate yields in *indica* rice varieties (Ashikari et al., 2005; Yeh et al., 2015).

The cytokinin signaling pathway in plants is comprised of sensor histidine kinases (HKs), histidine phosphotransfer proteins (AHPs), and response regulators (RRs, type-A and type-B) (Hwang et al., 2012; Kakimoto, 2003; Kieber and Schaller, 2014; Schaller et al., 2007). Cytokinin binding to the CHASE domain of the receptors activates autophosphorylation of the cytosolic histidine-kinase domain (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). This phosphate is then transferred to a conserved aspartate residue localized within the receiver domain of the HK receptor. The phosphate is subsequently transferred to a histidine residue on an AHP protein, and ultimately to a conserved aspartate residue on an RR protein. Phosphorylation of the type-B RRs activates these transcription factors, which then bind to their genomic targets to regulate the first wave of cytokinin-regulated transcriptional changes (Argyros et al., 2008; Mason et al., 2005; Zubo et al., 2017). One target of the type-B RRs are the type-A *RR* genes, which act as negative feedback regulators of the pathway (D'Agostino et al., 2000; To et al., 2004).

Although much of the understanding of cytokinin function comes from studies of *Arabidopsis* (Argueso et al., 2009; Kieber and Schaller, 2014), important insights have also come from analysis of rice and other monocot species. For example, disruption of the *LOG* gene in rice, which encodes an enzyme involved in cytokinin biosynthesis, was found to compromise maintenance of meristematic cells in inflorescence meristems, resulting in a smaller panicle (Kurakawa et al., 2007; Kuroha et al., 2009). Further, *log* mutants often produce spikelets with only a single stamen and no pistil, a result of premature floral meristem termination, suggesting that cytokinin functions in both the apical and axillary meristems in the rice inflorescence. Disruption of a type-A *RR* gene in maize, which acts as a negative regulator of cytokinin signaling, results in an altered pattern of phyllotaxy and an enlarged shoot apical meristem (Giulini et al., 2004). A few studies have examined alterations in cytokinin signaling elements in rice. Overexpression of *RR6*, encoding a type-A RR, in rice resulted in cytokinin hyposensitivity (as measured by a shoot regeneration assay), and a dwarf shoot with smaller, sterile panicles and reduced root system (Hirose et al., 2007). Disruption of expression of two rice *AHP* genes by RNAi resulted in decreased cytokinin sensitivity and various growth defects, including reduced internode lengths, enhanced lateral root growth, premature leaf senescence, fewer tillers and reduced fertility (Sun et al., 2014). A recent study of a subset of type-B RRs found that disruption of three type-B RRs resulted in multiple developmental defects, including reduced root and shoot growth, a smaller root apical meristem, reduced panicle

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development, reduced trichrome and stigma brush development and reduced fertility (Worthen et al., 2019). Disruption of *RR24*, encoding a distinct type-B RR, resulted in infertility as a result of defective anther development and a lack of pollen production (Worthen et al., 2019; Zhao et al., 2018).

The cytokinin HK receptors are generally present as a small gene family in plants, with the different cytokinin receptors exhibiting different affinities for various cytokinin species (Choi et al., 2012; Inoue et al., 2001; Romanov et al., 2006, 2005; Spíchal et al., 2004; Stolz et al., 2011; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). In *Arabidopsis*, there are three cytokinin receptors that have overlapping roles in regulating development, with AHK2 and AHK3 playing predominant roles in shoot development (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). In rice, there are four genes encoding CHASE domain-containing HKs (*HK3*-*HK6*), as well as one encoding a CHASE domain fused to a serine/threonine kinase (*CRL4*) (Ito and Kurata, 2006). Rice HK3, HK4 and HK6 receptors respond to various cytokinin species with different affinities in an *Arabidopsis* protoplast assay, with HK6 responding strongly to isopentenyladenine, but less so to *cis*-zeatin, *trans*-zeatin, or dihydrozeatin (Choi et al., 2012). An ethyl methane sulfonate (EMS)-induced missense mutation was recently identified in the rice *HK6* gene that reduced sensitivity to exogenous cytokinin, although this mutation had no substantial effects on rice growth and development (Ding et al., 2017). Overexpression of HK6 in rice calli resulted in a hypersensitivity to cytokinin in a shoot regeneration assay (Choi et al., 2012). Rice HK6 localized to the endoplasmic reticulum when expressed in onion epidermal cells (Ding et al., 2017), similar to its *Arabidopsis* and maize counterparts (Caesar et al., 2011; Lomin et al., 2011; Wulfetange et al., 2011).

Here, we characterize the effects of disruption of two of the four cytokinin HK receptors in rice. Our results indicate that *HK5* and *HK6* functionally overlap to mediate the effects of cytokinin on a diverse array of growth and developmental processes, a subset of which are distinct from those observed in comparable *Arabidopsis ahk* receptor mutant lines.

RESULTS

HK5 and *HK6* mediate cytokinin responses

The CHASE domain-containing family of histidine kinases in rice consists of four members that form distinct clades with their *Arabidopsis* counterparts: HK5 is most similar to AHK2 from *Arabidopsis*; HK3 clades with AHK3; and HK4 and HK6 belong to a clade expanded in monocots that includes four maize HKs as well as AHK4/WOL/CRE from *Arabidopsis* (Fig. S1A). All four of the rice *HK* genes are expressed highly in roots, shoots and the panicle apical meristem (Fig. S1B) (Tsai et al., 2012; Yamburenko et al., 2017).

To explore the role of cytokinin in a monocot species, we employed a CRISPR/Cas9-based approach to disrupt *HK5* and *HK6*, which are the *HK* genes that are most highly expressed in the panicle meristem (Fig. S1B). The CRISPR guide used was a tandem array targeting unique sequences within the second exon of *HK5* and *HK6* (Fig. S1C). We identified a rice line heterozygous for five- and four-base deletions in *HK5* and *HK6*, respectively, and isolated both the single and the double mutants based on these alleles (*hk5-1*, *hk6-1* and *hk5-1 hk6-1*) (Fig. S1D). We also identified a second double mutant with four- and seven-base pair deletions in *HK5* and *HK6*, respectively (Fig. S1D), from a second, independent T_0 line. Unfortunately, we were unable to obtain seeds from these *hk5-2 hk6-2* plants, most likely because it became locked into homozygosity early during the CRISPR editing event, and, as described below, the double mutant was sterile. Thus, unless specified otherwise, the *hk5-1* and *hk6-1* alleles were used for subsequent analyses. Because all these deletions shift the reading frame of the cognate gene early in the coding region, they most likely represent null alleles. In all cases, the mutations resulted in an in-frame stop codon within 25 codons after the mutation (Fig. S1D). Analysis of potential secondary targets for either the *HK5* or *HK6* guide sequence (<5 mismatches to the guide) in the coding portion of the rice genome by this CRISPR guide revealed no off-target editing in the *hk5-1 hk6-1* line (Table S1).

We determined whether disruption of these *HK* genes altered cytokinin responsiveness using a variety of assays. First, we analyzed the growth of wild-type and mutant seedlings in the presence and absence of the cytokinin 6-benzylaminopurine (BA) (Fig. 1A). The inhibition of seminal root growth, lateral root formation and shoot growth in response to cytokinin was comparable in wild-type and *hk5* mutant seedlings (Fig. 1A-D). In contrast, the *hk6* mutant displayed significant hyposensitivity to cytokinin in all three of these responses. The double *hk5 hk6* mutant was insensitive to cytokinin in shoot elongation, but a severe developmental defect in root growth precluded assessment of cytokinin responsiveness in root growth assays.

We also assessed the response to cytokinin using a dark-induced leaf senescence assay. We used the chlorophyll fluorescence decrease ratio (the vitality index; RFd) as an early measure of the cessation of photosynthesis (Sobieszczuk-Nowicka et al., 2018). Application of cytokinin significantly blocked the reduction in RFd that was observed in wild-type leaf segments transferred to the dark for 3 days (Fig. 1E,F). Both *hk5* and *hk6* single mutants showed a reduced response to cytokinin in this assay. In the absence of exogenous cytokinin, the double *hk5 hk6* mutant showed a substantially larger decrease in RFd after 3 days in the dark compared with wild-type leaves and also showed a significantly muted response to exogenous cytokinin.

We examined the molecular response to cytokinin in mutant roots by analyzing the induction of expression of type-A *RR*s, which are cytokinin primary response genes (Brandstatter and Kieber, 1998) that are highly induced in response to cytokinin in multiple plant species, including rice (Tsai et al., 2012). Treatment of wild-type roots with exogenous cytokinin resulted in significant induction of multiple type-A *RR* genes (Fig. 1G). The *hk5* mutant showed comparable induction of all three of the tested type-A *RR*s in response to cytokinin, consistent with the root growth response assays. In the *hk6* mutant, *RR2* and *RR4* induction was significantly reduced, suggesting that disruption of *HK6* compromised the molecular response to cytokinin. In contrast, *RR6* induction was comparable in *hk6* to that observed in wild-type roots, indicating that HK6 does not mediate the totality of the effects of cytokinin on gene expression. In the *hk5 hk6* double mutant, the expression of all three *RR* genes was nearly nonresponsive to exogenous cytokinin.

Together, these results suggest that HK5 and HK6 functionally overlap to regulate multiple cytokinin responses in roots and shoots, with HK6 in general playing a more prominent role in the response to exogenous cytokinin.

Disruption of *HK5* and *HK6* alters root growth and development

Cytokinin affects multiple aspects of root growth and development in *Arabidopsis*, including inhibition of primary root growth and lateral root formation, reduction in the size of the root apical meristem (RAM) and inhibition of xylem development (Kieber and Schaller, 2014). We examined the effect that disruption of *HK5* and

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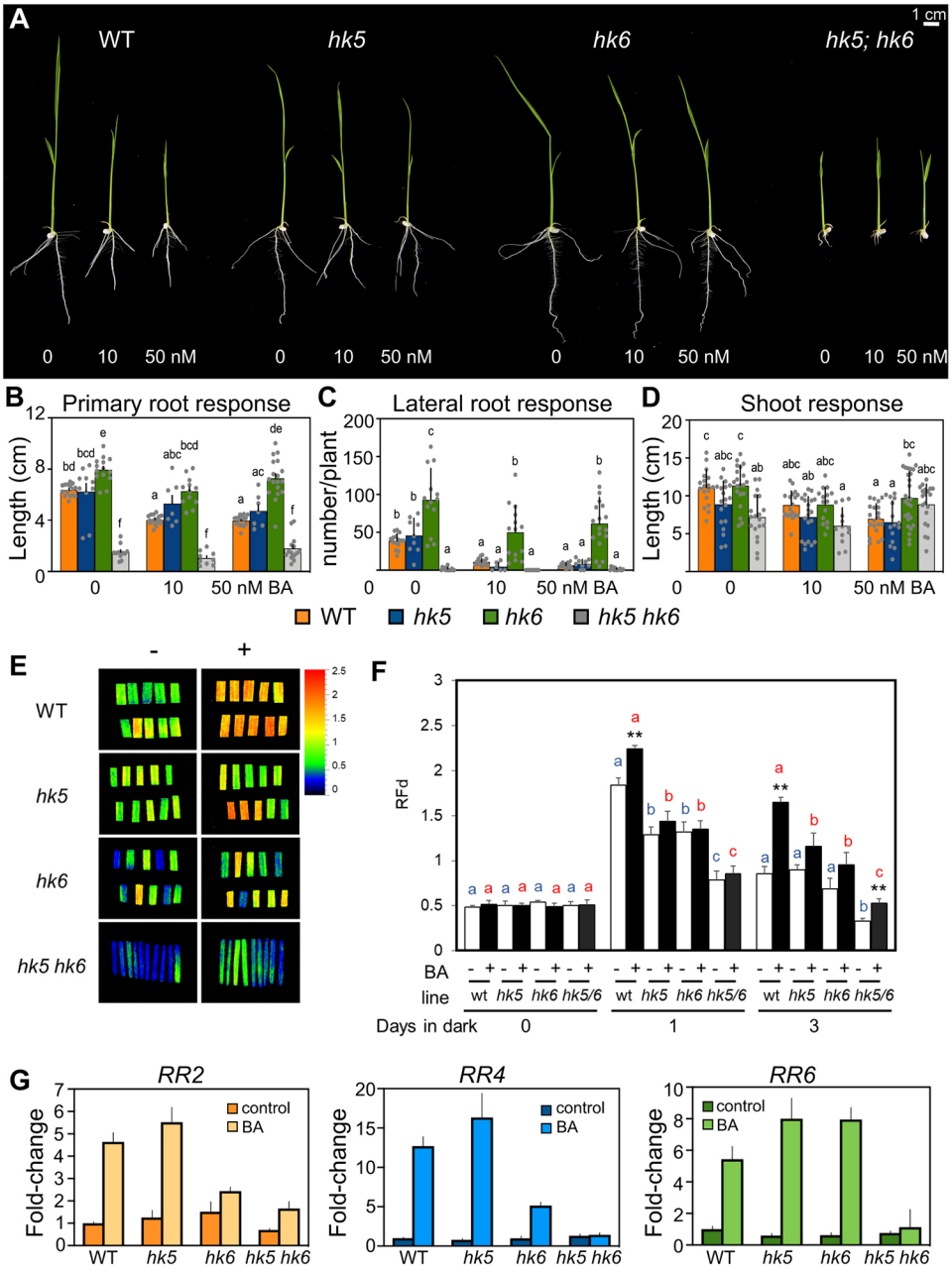

Fig. 1. Effect of disruption of *HK5* and *HK6* on cytokinin responses. (A) Morphology of rice seedlings grown in the presence and absence of cytokinin. Wild-type or the indicated *hk* mutants were grown on Kimura B nutrient solution (Ma et al., 2001) solidified with 1% (w/v) gellan gum for 7 days in the presence of a vehicle control (0), 10 nM BA or 50 nM BA (6-benzylaminopurine) and representative seedlings photographed. (B-D) Quantification of primary root length (B), the number of lateral roots (C) and shoot length (D) in seedlings grown as in A. Letters indicate differences at a $P<0.05$ significance level using ANOVA analysis with a Tukey post-hoc correction. $n\geq15$. (E,F) Fluorescence decline ratio (RFd) of wild-type (WT), *hk5*, *hk6* and *hk5 hk6* leaf sections in a dark-induced senescence assay. Dissected rice leaves were incubated for 3 days in the dark. RFd was measured before incubation (day 0) and 1 and 3 days after dark incubation. (E) Pseudo-coloring of leaves based on calculated RFd parameter at day 3 of the dark incubation. (F) RFd at day 0, 1 and 3 in the leaf sections from the indicated lines. Letters indicate differences at a $P<0.05$ significance level using ANOVA analysis with a Tukey post-hoc correction; lower-case blue letters (−BA samples) and red letters (+BA samples). ** indicates a significant difference ($P<0.05$) when samples without BA were compared to cognate +BA samples using a *t*-test. $n\geq9$. (G) Quantification of type-A *RR* expression in response to cytokinin. Roots from 7-day-old wild-type or *hk* mutant seedlings were treated with 5 µM BA or a vehicle control for 1 h. Expression of *RR2*, *RR4* and *RR6* was quantified using qRT-PCR. The expression values were normalized to an *ACT1* control gene and expressed as a fold-change relative to the vehicle control. Data represent the mean±s.e.m. of three biological replicates ($n=3$), each with three technical replicates.

HK6 had on the growth and development of rice roots (Fig. 2). The root system of the *hk5* mutant was indistinguishable from wild type, both in 7-day-old seedlings grown *in vitro* (Fig. 1A) and 9-week-old soil-grown plants (Fig. 2A). The *hk6* single mutant seedlings had a longer seminal root and an increased number of lateral roots when compared with 7-day-old wild-type seedlings (Fig. 1A-C), as well as a more extensive root system in 9-week-old soil-grown plants as compared to the wild type (Fig. 2A), which is similar to the increased root growth seen in rice plants that overexpress cytokinin dehydrogenase 4 (CKX4) (Gao et al., 2014). This is consistent with observations in *Arabidopsis* and other dicots that cytokinin negatively regulates root growth (Werner et al., 2001). In rice, exogenous cytokinin inhibits lateral root initiation, but promotes lateral root elongation (Rani Debi et al., 2005), suggesting that the increased number of lateral roots in the *hk6* mutants is the result of enhanced initiation. In contrast to the single *hk* mutants, disruption of both *HK* genes strongly reduced root growth; the double *hk5 hk6* mutants displayed reduced root elongation (Fig. 1A,B) with fewer lateral roots (Fig. 1C) as young seedlings and a substantially reduced root system in 9-week-old soil grown plants (Fig. 2A).

Root growth is controlled by a combination of cell proliferation, which occurs primarily in the RAM, and cell elongation. Prior studies indicate that, in *Arabidopsis*, cytokinin reduces root growth at least in part by promoting the transition from cell proliferation to cell elongation, thus reducing RAM size (Dello Ioio et al., 2007). We examined RAM size in the seminal root of the single and double rice *hk* mutants grown in liquid medium, measuring the distance from the quiescent center to the point where cells first elongate in root tips (Fig. 2B). The size of the RAM in the *hk5* mutant was comparable to that of wild-type seedlings. The *hk6* mutant RAM was slightly smaller (Fig. 2C), despite the longer length of the seminal root, suggesting that differences in cell elongation probably underlie the increased growth of the *hk6* seminal root. In the *hk5 hk6* double mutant, the RAM was substantially smaller than in wild-type

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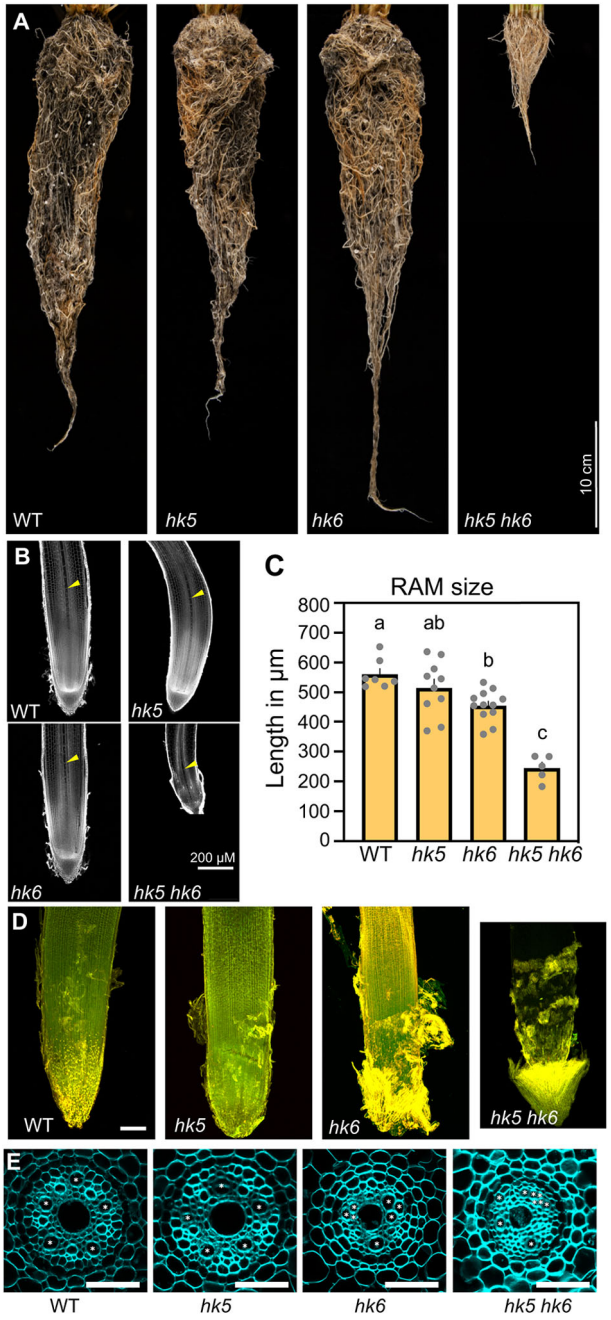

Fig. 2. Disruption of *HKs* alters root growth and development. (A) Adult root phenotypes of 9-week-old wild type and the indicated *hk* mutants grown in soil. The plants were removed from their pots, the soil gently washed off the roots and the plants photographed; representative images are shown. (B) Representative images of root apical meristems (RAMs) of seminal roots. Confocal microscopy images of fixed root tips from 14-day-old seedlings of the indicated genotypes grown in liquid Kimura B nutrient solution. The yellow arrows indicate the upper extent of the RAM in each root tip. (C) Quantification of RAM size for roots grown as in B. The RAM was measured from the quiescent center to the point where central xylem cells first elongate (yellow arrows in B). Values represent the mean±s.e.m. ($n \geq 5$). Individual data points are shown as gray circles. Letters indicate differences at a $P<0.05$ significance level using ANOVA analysis with a Tukey post-hoc correction. (D) Representative images of root tips of wild-type and the indicated *hk* mutants grown on Kimura B nutrient solution solidified with 1% (w/v) gellan gum. Roots were fixed and visualized via confocal microscopy. Scale bar: 100 µM. (E) Cross-sections of wild-type and *hk* mutant roots. Seedlings were grown for 5 days and the roots then embedded in agarose and stained with calcofluor white. Asterisks mark presumptive metaxylem cells. Scale bars: 50 µM.

roots, which probably contributes to the reduction in their root growth.

We examined root tips of seedlings grown *in vitro* in solid media by clearing with methyl salicylate followed by confocal microscopy imaging. Intriguingly, in *hk6* seedlings, and to a lesser extent *hk5*, there were excess cells loosely associated with the root tip and root cap, a phenotype that was strongly exacerbated in the *hk5 hk6* double mutant (Fig. 2D). In rice, the root cap maintains a constant size via a balance between cell generation from divisions in the overlying stem cells precisely coupled with separation and sloughing off of individual root cap cells (border cells) into the surrounding environment (Hawes et al., 2002). These border cells remain viable for days, are transcriptionally distinct from their parental root cells and are thought to play a role in plant defense and modulation of the root microbiota (Hawes et al., 2016, 2002). The root tips in the double *hk5 hk6* mutant displayed a range of phenotypes, but nearly all exhibited a substantially larger root cap and unreleased border cells (Fig. S2), suggesting that cytokinin plays a role in promoting the release of these cells.

We examined the cellular organization and vasculature of wild-type and *hk* mutant roots in cross section (Fig. 2E). The seminal roots of both the wild type and *hk5* had five to six xylem poles spaced equidistantly around the perimeter of the root, with each pole typically containing a single large metaxylem cell. In the *hk6* mutant and the *hk5 hk6* double mutant, this highly organized pattern was disrupted, resulting in fewer poles and frequent incidences where many metaxylem elements were present at each pole. Similarly, an increase in the number of xylem cells per pole has also been observed in double cytokinin receptor mutants in *Arabidopsis* (Mähönen et al., 2006). Although the single *hk5* and *hk6* mutants had a comparable number of cells around the perimeter of the stele, the *hk5 hk6* double mutant had significantly fewer, suggesting that cell proliferation is reduced in the double mutant root (Fig. S3).

Disruption of *HK5* and *HK6* alters shoot and panicle development

We examined shoot growth and development in wild-type and *hk* mutant rice plants. In 7-day-old plants grown in culture, the *hk5* and *hk6* mutant shoots were comparable in size to the wild type, and the *hk5 hk6* double mutant substantially stunted (Fig. 1A). In 7-week-old adult plants, just prior to flowering, the overall size of the single mutants was also comparable to the wild type, but that of the double mutant was severely diminished (Fig. S4). Likewise, after 12 weeks of growth, the overall size of the single mutants was again comparable to the wild type, but the *hk5 hk6* double mutant was much smaller (Fig. 3A). The reduced size of the adult shoot was similarly reduced in the double *hk5-2 hk6-2* mutant, derived from an independent CRISPR editing event (Fig. S5A). The width of leaves in both single *hk* mutants were slightly reduced relative to wild-type leaves (Fig. 3C); the double *hk5 hk6* mutant showed a strongly additive phenotype, with a ∼50% reduction in leaf width compared with the wild type. Wild-type and *hk6* single mutant plants had visible panicle exsertion at 9 weeks after germination [$\bar{X} = 63.4 \pm 2.0$ (s.d.) days for wild type (n=29); $\bar{X} = 64.2 \pm 2.6$ days for *hk6* (n=20)]; the *hk5* single mutant was slightly delayed ($\bar{X} = 67.1 \pm 3.1$ days, n=30); the double mutant was significantly delayed, showing visible panicle exsertion approximately 12 weeks after germination ($\bar{X} = 83.2 \pm 5.1$ days, n=19). The single *hk5* and *hk6* mutant had a comparable number of tillers to the wild type, but the double mutant showed significantly fewer (Fig. 3D). Although the single mutants had a similar number of panicles to the wild type, the double *hk5 hk6* mutant showed a

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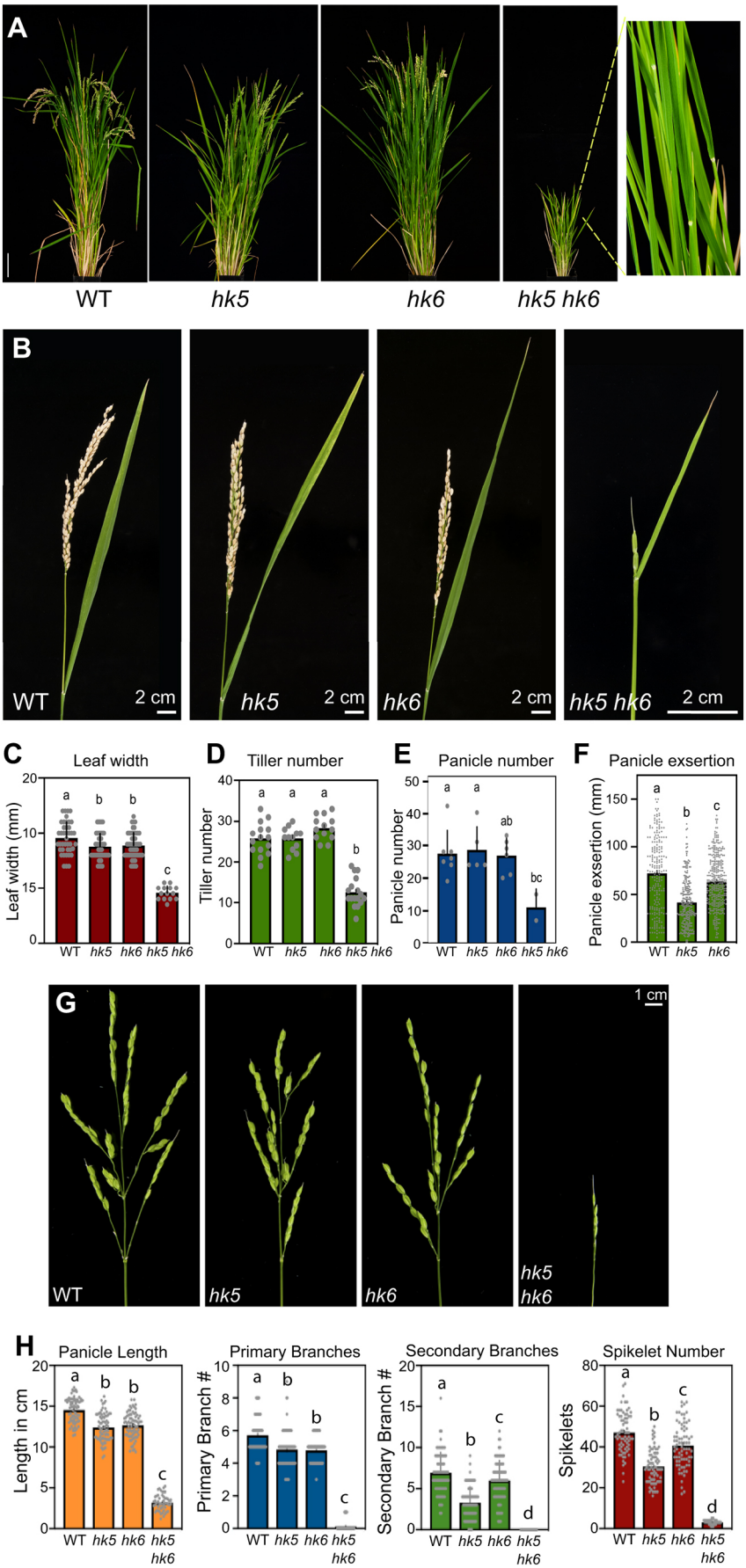

Fig. 3. Shoot and panicle phenotypes of *hk* mutants. (A) Representative images of adult plants. Wild-type and *hk* mutants were grown in soil for 12 weeks and representative plants photographed. The inset on the right is a close-up of *hk5 hk6* tillers lacking panicles. Scale bar: 10 cm. (B) Representative images of mature panicles with flag leaf from 20-week-old plants showing panicle exsertion. (C-F) Quantification of leaf width (C), tiller number (D), panicle numbers (E) and the length of panicle exsertion (F) in 20-week old wild-type and the indicated *hk* mutant lines. Values represent the mean±s.e.m. $n \geq 15$ (C); $n \geq 13$ (D,E); $n \geq 207$ (F). (G) Representative images of isolated immature panicles from wild type and the indicated *hk* mutants. (H) Quantification of various aspects of panicle morphology in wild type and the indicated *hk* mutants ($n \geq 51$). For C-F and H, individual data points are shown as gray circles. Letters indicate differences at a $P<0.05$ significance level using an ANOVA analysis with a Tukey post-hoc correction.

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severe reduction (Fig. 3E). Many tillers in the double mutant lacked a visible panicle altogether (Fig. 3A, inset). Both the *hk5* and *hk6* single mutants displayed reduced exsertion of the panicle as compared to the wild type; the double mutant showed a strongly additive phenotype, with the panicles never fully exserting from the sheath (Fig. 3B,F). Panicle exsertion is an agronomically important trait that has been previously linked to gibberellins (Gao et al., 2016).

The panicles of both the *hk5* and *hk6* single mutants had an array of phenotypes related to the panicle architecture, including reduced length, fewer primary and secondary branches and reduced spikelet number (Fig. 3G,H), similar to cytokinin-insensitive lines overexpressing *RR6*, encoding the rice type-A RR6 (Hirose et al., 2007). The *hk5* single mutant was more severely affected than the *hk6* mutant in this regard as it had significantly fewer secondary branches and fewer spikelets (Fig. 3G,H). These genes functionally overlap to regulate panicle development as the double mutant produces ∼65% fewer and smaller panicles (Fig. 3E), many lacking primary and secondary branches altogether and developing fewer than five incomplete spikelets (Fig. 3G,H). A similar defect in panicle development was observed in the *hk5-2 hk6-2* line (Fig. S5B). The reduced size of the *hk5 hk6* mutant panicles were similar, although more extreme, to those reported to be produced by the *log* mutants, which are compromised in cytokinin biosynthesis (Kurakawa et al., 2007).

In rice, many of the phenotypic defects in the *log* mutant have been traced to a reduction in the size of the vegetative and inflorescence meristems (Kurakawa et al., 2007). We thus examined the early reproductive panicle of the *hk5 hk6* double mutant in comparison to the wild type, because changes in the mature panicle architecture of the mutant probably arise from alterations in the meristematic activities that establish branching and flower development. Samples for sectioning were collected when stems began to elongate, a developmental time point that typically marks the beginning of inflorescence development. The majority of the wild-type reproductive panicles were at stage 5 or later, according to the stages described by Furutani et al. (2006), at which point primary and secondary branches have initiated and floret meristems have begun to emerge from the spikelets (Fig. 4A). In contrast, the majority of *hk5 hk6* samples had not transitioned to inflorescence development at this point (Fig. S6), consistent with many of the tillers lacking panicles altogether (Fig. 3A,E). The *hk5 hk6* panicles that did form were much smaller than their wild-type counterparts and displayed substantial developmental alterations. For example, as shown in Fig. 4A, both the wild-type and mutant panicle meristems were at stage 4, at which point they formed spikelet meristems (Fig. 4A, white triangles). The wild-type early panicle had established multiple primary and secondary branches, but the *hk5 hk6* early panicle only had a single primary branch on the primary axis. In addition, the two prominent spikelet meristems of the mutant were smaller than those of the wild type. Similar developmental defects were observed in other early panicles of the double mutant (Fig. S6), indicating an early role for *HK5* and *HK6* in establishing the inflorescence meristems that define the architecture of the mature panicle.

To understand the roles of the HK cytokinin receptors in early panicle development, we examined the expression of various meristem-specific genes in wild-type and *hk* mutant panicle meristems. Because the double mutant undergoes transition to reproductive development later than the other lines, we examined gene expression in the *hk5 hk6* lines using samples of the same age (*hk5 hk6*a) and at the same developmental stage (*hk5 hk6*b) as the wild-type and single *hk* mutants (Fig. 4B). The genes examined include a general marker for apical meristem identity (*ORYZA SATIVA HOMEOBOX1*; *OSH1*) (Sato et al., 1996; Sentoku et al., 1999), four genes implicated in the regulation of inflorescence development (*LAX PANICLE1*, *LAX1*; *WUSCHEL RELATED HOMEOBOX 3B/GLABROUS LEAF AND HULL1*, *WOX3B/GL1*; *TILLERS ABSENT1*, *TAB1*; *FRIZZY PANICLE*, *FZP1*) (Komatsu et al., 2003a,b; Tanaka et al., 2015; Zhang et al., 2012) and *RR11*, which encodes a type-A RR whose expression in the shoot apical meristem increases after the transition to inflorescence development (Yamburenko et al., 2017). All six genes exhibited significantly reduced expression in the *hk5 hk6* double mutant samples, whether the samples were isolated based on age or developmental status (Fig. 4B). The reduction in expression was particularly pronounced for the four genes specifically implicated in the regulation of inflorescence development (*LAX1*, *WOX3B/GL1*, *TAB1* and *FZP1*) as compared to the more general apical meristem marker *OSH1*.

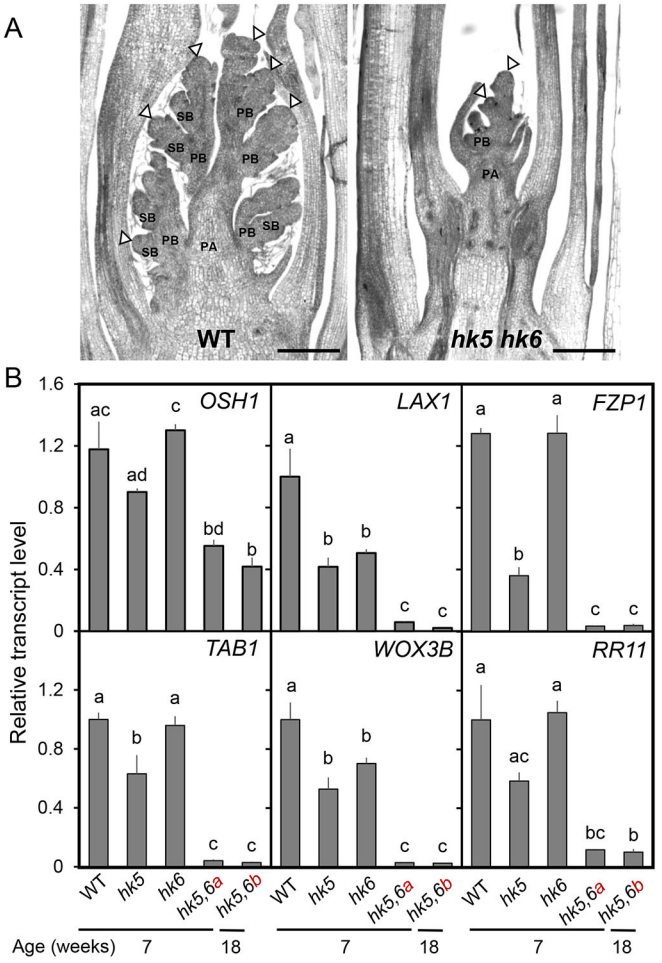

Fig. 4. The *hk5 hk6* mutants exhibit altered panicle meristematic activity. (A) Longitudinal sections of wild-type (WT) and *hk5 hk6* inflorescences collected at stage 4 of rice inflorescence development (Furutani et al., 2006). White triangles point to spikelet meristems (SMs). PA, primary axis (rachis); PB, primary branch; SB, secondary branch. Scale bars: 200 µm. (B) Expression of genes associated with panicle meristem development in 7-week-old wild-type and *hk* mutant plants, including both 7-week-old (*hk5 hk6*a) and 18-week-old (*hk5 hk6*b) *hk5 hk6* plants. Letters indicate differences in gene expression at a $P<0.05$ significance level using ANOVA analysis with a Tukey post-hoc correction. Data represent the mean±s.e.m. of three biological replicates ($n=3$), each with two technical replicates.

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These four genes are implicated in the regulation of branch meristem formation and spikelet initiation, and their decreased expression is thus consistent with the altered panicle architecture observed in the early and mature panicles. The single *hk5* and *hk6* mutants exhibited variable reductions in expression for these six genes, in no case exhibiting the striking reduction found in the *hk5 hk6* double mutant, consistent with *HK5* and *HK6* having overlapping function in the regulation of inflorescence development.

Disruption of *HK5* and *HK6* alters floral development

Prior studies have linked cytokinin to floral development in rice, including a reduced number of floral organs in the *log* mutant (Kurakawa et al., 2007) as well as decreased stigma brush development and defective anther development in various type-B RR mutants (Worthen et al., 2019; Zhao et al., 2018). Thus, we examined the role of *HK5* and *HK6* in floral development. Both single *hk* mutants showed a slight decrease in the number of stamens as well as significantly decreased fertility (Fig. 5A-C). The fertility defect was more severe in the *hk5* mutant, in which only ∼10% of the spikelets were fertile (Fig. 5C). The infertility of the *hk5* mutant was correlated to a defect in anther development and reduced pollen release (Fig. 5A), which is similar to the rice *rr24* mutant that disrupts a type-B RR (Worthen et al., 2019; Zhao et al., 2018). In contrast, the *hk6* mutant displayed significant reduction in the development of stigmatic brushes (Fig. 5F,G), probably accounting for its reduced fertility. Prior studies with the *rr21,22,23* triple type-B RR rice mutant also found a decrease in stigmatic brush development (Worthen et al., 2019). Interestingly, although the *hk6* mutant produced fewer seeds, the seeds were significantly larger than wild-type or *hk5* seeds (Fig. 5D,E). This is similar to the increased seed size previously reported in some cytokinin-insensitive *Arabidopsis* mutants, such as the *ahk2,3,4*, *ahp1,2,3,5* and *arr1,10,12* mutants (Argyros et al., 2008; Hutchison and Kieber, 2007; Riefler et al., 2006).

As with most of the other phenotypes, the floral phenotypes of the *hk5 hk6* double mutant were much more severe than those found in either single mutant. The few spikelets that do form in the double mutant showed severely altered morphology (Fig. 5A) and never resulted in the development of a seed (Fig. 5C). The double mutant spikelets were missing multiple organs; on average, they only produced a single stamen, which consisted of only a filament with no associated anther (Fig. 5A,B). The double mutants formed no structures that could be considered analogous to the female organs (Fig. 5A).

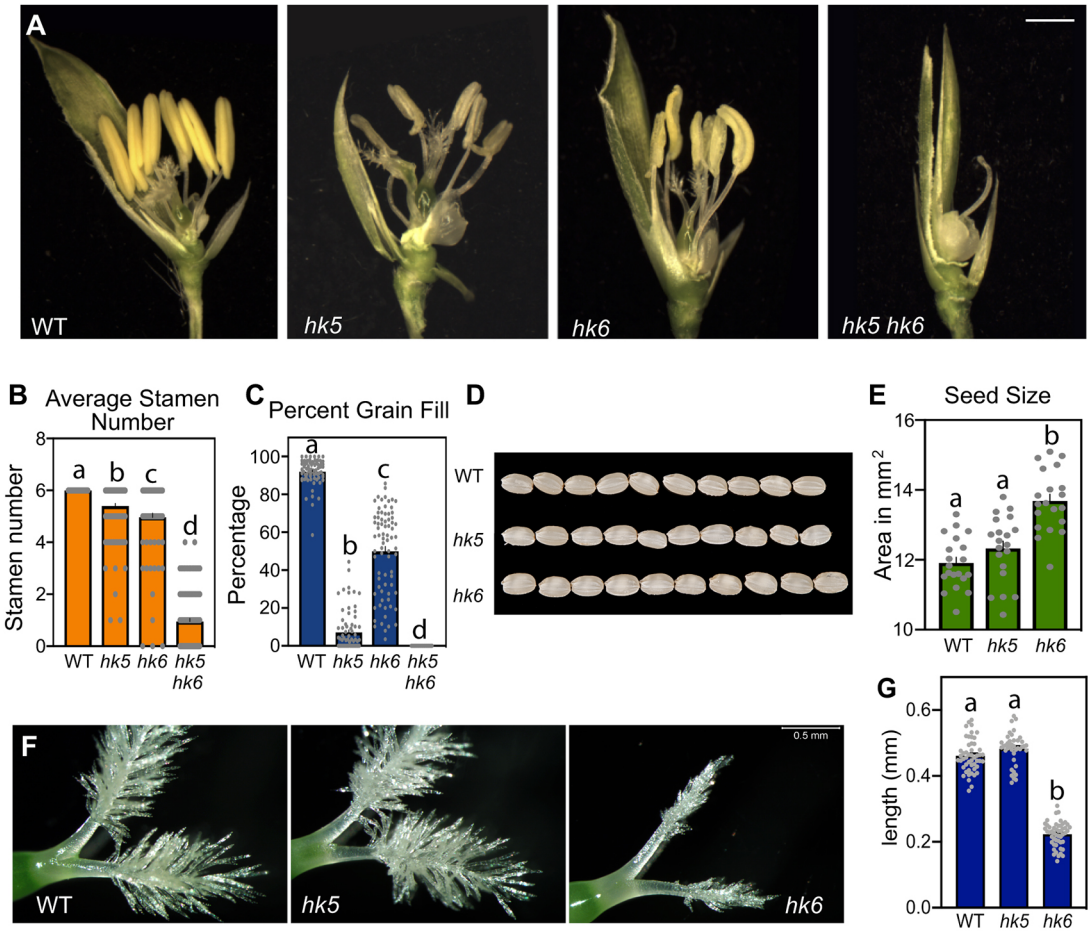

Fig. 5. Effects of *hk* mutations on floral and seed development. (A) Representative images of wild-type (WT) and the indicated *hk* mutant spikelets, all of which have had the palea removed in order to reveal inner whorl organs. Scale bar: 1 mm. (B) Quantification of the number of stamens per spikelet in wild-type and *hk* mutant spikelets ($n \geq 50$). (C) Quantification of the percentage of spikelets that were filled with grain in wild-type and *hk* mutants ($n \geq 50$). (D) Representative images of ten seeds from wild-type and the single *hk* mutants. (E) Quantification of the mean area of wild-type and *hk* mutant seeds ($n \geq 20$). (F) Representative images of wild-type and single *hk* mutant stigmas illustrating reduced brushes. (G) Quantification of the length of the brushes from wild-type and single *hk* mutant stigmas. $n \geq 40$. For B,C,E,G. letters indicate differences at a $P<0.05$ significance level using an ANOVA analysis with a Tukey post-hoc correction.

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DISCUSSION

We used the CRISPR/Cas9 system to generate indel mutations in two of the four cytokinin HK receptors in rice to define roles for this phytohormone in a model monocot species. The single *hk* mutants each show modest effects on vegetative growth and development, but the double *hk5 hk6* mutant exhibits severe disruption of multiple aspects of rice development. Some of the developmental alterations in the rice *hk* mutants are similar to phenotypes observed in the *Arabidopsis ahk* mutants, including enhanced root growth, reduced shoot growth, increased seed size and increased xylem cells per pole, but the reduced RAM size in rice *hk6* and *hk5 hk6* mutants is opposite to the reported phenotypes of the *Arabidopsis ahk* mutants (Dello Ioio et al., 2007). Moreover, other phenotypes of the rice *hk* mutants have not been reported or are more pronounced in rice as compared to *Arabidopsis*, such as the severely reduced floral organ number, severely delayed flowering and excess root cap cells. Thus, cytokinin may have been co-opted to regulate some distinct aspects of monocot development.

The phenotypes observed for these *hk* mutants are highly likely to reflect disruption of these genes and not be the result of off-target effects of the CRISPR editing system or other secondary mutations for the following reasons. First, a second *hk5 hk6* double mutant derived from an independent line transformed with the CRISPR guide shared the shoot and panicle phenotypes observed in *hk5-1 hk6-1*. Second, the closest potential off-targets of the employed CRISPR guide for both *HK5* and *HK6* did not show editing in the *hk5 hk6* line (Table S1). Third, nearly all of the phenotypes observed were only present or greatly enhanced in the double *hk5 hk6* mutant line, consistent with partial genetic redundancy between these two paralogs. Finally, most of the phenotypes we observed have been observed in other rice lines with reduced cytokinin function, such as the *log* single mutant, type-B *rr* multiple mutants or *AHP* RNAi lines (Kurakawa et al., 2007; Sun et al., 2014; Worthen et al., 2019). Often, the phenotypic effects in these lines are not as severe as in the *hk5 hk6* line, but they nevertheless support a link to reduced cytokinin function.

One difference in cytokinin receptor function between rice and *Arabidopsis* is the effect on flowering time and floral development. The various double *ahk* cytokinin receptor mutants (disrupting two of the three cytokinin receptors) in *Arabidopsis* have little (Nishimura et al., 2004) or no (Riefler et al., 2006; Nishimura et al., 2004) effect on flowering time or floral development. In contrast, disrupting only two of the four *HK* cytokinin receptor genes in rice severely delays flowering time and floral development. However, the differing effects of reduced cytokinin function on floral development between *Arabidopsis* and rice are probably linked to distinct effects on meristem function (Kurakawa et al., 2007) (see below). Despite minimal effect of the *ahk* mutations on flowering time in *Arabidopsis*, other studies indicate a clear link between cytokinin and induction of flowering (Bartrina et al., 2017; Besnard-Wibaut, 1981; D'Aloia et al., 2011; Werner et al., 2003). In the monocot barley, overexpression of a gene encoding a cytokinin oxidase prevents the transition to flowering (Mrízová et al., 2013), consistent with our results in rice with the *hk* mutants. Overall, the results suggest that cytokinin can promote flowering in both monocots and dicots.

Studies in *Arabidopsis* have delineated an important role for cytokinin in regulating cell division in the SAM and in establishing organization of the SAM, most notably in the positioning of the *WUS* expression domain (Gordon et al., 2009; Schaller et al., 2015), a homeodomain transcription factor that promotes stem cell activity in the SAM. Multiple studies have found that *WUS* is a direct target of *Arabidopsis* type-B ARRs (Meng et al., 2017; Wang et al., 2017; Zhang et al., 2017; Zubo et al., 2017), and hence of cytokinin, and WUS in turn regulates cytokinin responsiveness by repressing the expression of a subset of type-A *ARR*s (Leibfried et al., 2005). Consistent with a role for cytokinin in regulating the SAM in *Arabidopsis*, altered cytokinin levels affect SAM size as *ckx3 ckx5* double mutants form larger SAMs (Bartrina et al., 2011) and overexpression of *CKX* results in diminished activity of the vegetative and floral SAMs (Werner et al., 2003). Furthermore, the triple *ahk* mutant has a small SAM (Higuchi et al., 2004; Nishimura et al., 2004), although it is difficult to ascribe this solely to a primary effect of cytokinin as opposed to a secondary effect of drastically altered root and shoot development, including disrupted phloem development. The *Arabidopsis arr1/10/12* mutant, in which multiple type-B *ARR*s are disrupted, also has a small SAM (Argyros et al., 2008). Disruption of a negative regulator of cytokinin signaling, *AHP6/PHP1*, results in altered inflorescence architecture as a result of aberrant cytokinin activity in the SAM (Besnard et al., 2014).

The results in monocots suggest a prominent role for cytokinin in regulating meristematic activity in the shoot. The premature termination of the shoot meristem in the *log* mutants suggests a key role of cytokinin in maintaining panicle meristems in rice (Kurakawa et al., 2007). In maize, disruption of a single type-A RR gene, *ABERRANT PHYLLOTAXY 1* (*ABPH1*), results in an enlarged shoot meristem and altered leaf phylotaxy (Jackson and Hake, 1999). The majority of *hk5 hk6* tillers failed to produce a panicle, which may reflect early abortion of the reproductive meristems. Overall, these results suggest that the establishment of inflorescence architecture in rice is particularly sensitive to changes in cytokinin activity due to the prominent role cytokinin plays in regulating meristematic activity, potentially because rice produces a determinate, branched panicle-type inflorescence whereas *Arabidopsis* produces a simple indeterminate raceme-type inflorescence (Itoh et al., 2006; Prusinkiewicz et al., 2007).

The effects on panicle development in the *hk* mutants are correlated with reduced expression of genes necessary for meristem maintenance, with particularly strong effects on genes involved in branch and spikelet initiation and maintenance. For example, *LAX PANICLE1* (*LAX1*), which is important for the formation of axillary meristems and the initiation of primary and secondary branches and spikelet meristems (Komatsu et al., 2003a), shows significantly reduced expression in both *hk* single mutants, with an additive effect in the double mutant. This is consistent with the reduced number of primary branches, secondary branches and spikelets in the *hk* single mutants, as well as the additive phenotype of the double mutant. The expression of *TILLERS ABSENT1* (*TAB1*), a homeobox-containing rice ortholog of *Arabidopsis* WUS that regulates axillary meristem formation (Tanaka et al., 2015), is also reduced in the *hk* mutants. *FRIZZY PANICLE* (*FZP1*) is a floral meristem identity gene encoding an AP2/ERF transcription factor that is activated when panicle development switches from branching to spikelet initiation (Komatsu et al., 2003a). *FZP1* expression is reduced in *hk5* but not in *hk6* mutants, and the double mutant again shows substantially lower expression. Overall, the reduced expression of these genes, particularly in the *hk5 hk6* double mutant, suggests that cytokinin regulates panicle development through the canonical molecular regulators of meristem function in rice. Whether this is a direct effect of cytokinin on the expression of these genes or an indirect effect of altered meristem development remains to be elucidated.

An interesting novel phenotype of the rice *hk* mutants is the presence of unreleased border cells and a larger root cap as compared to wild-type rice. The root cap covers the root tip and acts

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as a protective tissue for the meristematic cells of the root apex and is also the site of perception of environmental signals, such as gravity (Barlow, 2002; Kumar and Iyer-Pascuzzi, 2020). The size of the root cap remains constant throughout the life of a plant, the result of the production of new cells being precisely balanced with turnover of mature cells via either programmed cell death or shedding (Kumpf and Nowack, 2015). One explanation for the excess cells observed in the *hk6* and *hk5 hk6* mutant root cap is a failure of the border cells to effectively separate from the parental root, perhaps due to defects in the production of the cell wall modifying enzymes involved in the release of these cells. Auxin is involved in the release of root cap cells in *Arabidopsis*, with cell separation occurring at auxin response minimum (Dubreuil et al., 2018). Auxin and cytokinin often act antagonistically to regulate numerous plant processes (Schaller et al., 2015), and this is consistent with cytokinin promotion of border cell release in rice. Alternatively, there may be excessive cell proliferation in the root cap cells in the double mutants. Nonetheless, this phenotype has not been described in *Arabidopsis* cytokinin mutants, which, like other *Brassicaceae*, do not release single border cells, but instead regulate root cap size by programmed cell death (in the lateral root cap) and sloughing off of entire cell layers in the distal root cap (Driouich et al., 2010).

Although it is clear that *HK5* and *HK6* have overlapping roles, as evidenced by the strongly additive phenotypes in the double mutant as compared to the parental singles, there is clearly some functional specification for each of the receptor genes. This is most apparent in their distinct effects on floral development, where *HK5* has a role in stamen development and *HK6* in stigma brush development, with both defects leading to reduced fertility. This subfunctionalization of floral development is similar to the roles of type-B *RR*s, in which genetic analysis demonstrated that *RR22* plays a predominant role in stigma brush development and that disruption of *RR24* specifically affects anther development (Worthen et al., 2019). This suggests that HK5 and RR24 act as the primary signaling modules to transduce the cytokinin signaling in anthers, and that HK6 and RR22 play a predominant role in the development of the sigma brush. There are additional differences in *HK5* and *HK6* function, including the contributions to seed size, root growth, root cap homeostasis and gene expression. The significant increase in grain size in the *hk6* single mutant is an especially interesting phenotype as this has clear implications for yield if the effects on fertility could be addressed. In rice, the spikelet hull restricts grain growth and plays a major role in determining the final grain size, and most of the genes that affect grain size act through regulation of the proliferation and expansion of the cells in the spikelet hull, although growth of the endosperm also influences grain size (Li and Li, 2016). Either of these factors could plausibly be impacted by altered cytokinin signaling. Alternatively, the increased seed size in the *hk6* mutant could be a result of the reduced seed set in this mutant, similar to *Arabidopsis* in which seed size is negatively correlated to overall seed number (Alonso-Blanco et al., 1999). It will be of interest to determine the roles of the other two HK cytokinin receptors in rice and how these overlap with those of *HK5* and *HK6.*

MATERIALS AND METHODS

Plant material and growth conditions

Seeds were sterilized in 50% bleach for 30 min, washed with sterile water five times and then germinated on moist Whatman filter paper overnight at 37°C. Seeds with emerged coleoptiles were moved to the indicated medium. For *in vitro* growth, seedlings were grown on Kimura B nutrient solution (Ma et al., 2001) solidified with 1% gellan gum (PhytoTech Labs) and grown at 10 h light (27.5°C)/14 h dark (23.5°C). Soil-grown plants were grown in a 50:50 mix of Pro-Mix BX and Profile Porous Ceramic Greens Grade (Profile) in 4×4×10 inch pots in the UNC greenhouse at 13 h light (28°C)/11 h dark (25°C) with supplemental lighting (450 W/m^2) as needed. The pots were continuously submersed in water and fertilized once per week with Peter's 15-5-15 (300 ppm) supplemented with Sprint Fe supplement (2.5 ppm).

CRISPR/Cas9-induced *hk5* and *hk6* mutations

The CRISPR targeting cassette consisted of a tandem array of the rice *U3* promoter and a sgRNA sequence specific to either *HK5* or *HK6* (Worthen et al., 2019). Target sequences to the *HK* genes were designed using the CRISPR-PLANT program (https://www.genome.arizona.edu/crispr/index.html) (Table S2). The CRISPR cassettes were assembled using In-Fusion (Takara) cloning and then moved into a Cas9 vector, pARS3_MUbCas9_MC (Worthen et al., 2019), transformed into the *Agrobacterium* strain EHA101 and finally transformed into Kitaake rice callus by the Iowa State University Plant Transformation Facility (https://www.biotech.iastate.edu/biotechnology-service-facilities/plant-transformation-facility/). CRISPR/Cas9 mutations were confirmed by DNA sequencing and the CRISPR/Cas9 cassette segregated away for the final lines. Primers for CRISPR cassette creation and editing assessment are listed in Table S2. Mutations in the *HK* genes were originally identified by size differences in the PCR products (as determined by PAGE) amplified with primers flanking the CRISPR target sites (Table S2) from DNA prepared from T_1 plants, and then confirmed by sequencing. The *hk5-1* and *hk6-1* alleles identified were both found as heterozygotes in a T_1 line, and the single and double mutants were identified as segregants from this *HK5hk5-1 HK6hk6-1* double heterozygous line. For seedling assays (Fig. 1A-D), all progeny from a *hk5-1hk5-1 HK6hk6-1* T_2 line were analyzed, the doubles identified afterwards and only their data included in the final analyses. For adult assays, the double mutants were identified as seedlings and grown to maturity. The second *hk* alleles were identified from an independently transformed line, but the CRISPR/Cas9 editing events in this case were homozygous for both *hk* mutations in the T_1 plants.

Cytokinin response assays

Seeds were sterilized, germinated overnight and then moved to Kimura B nutrient solution (Ma et al., 2001) solidified with 1% gellan gum (PhytoTech Labs). Seedlings were grown in 250 ml medium in Dart Solo (RTP16DBARE) cups capped with Dart Solo (DLW626) lids with the holes sealed using a sterile foam stopper for 8 days (10 h light 27.5°C/14 h dark 23.5°C) before data collection. The treatments were 10 nM or 50 nM 6-benzylaminopurine (BA; Sigma) dissolved in 1 N NaOH or a 1 N NaOH vehicle control. After 8 days of growth, the lengths of the shoots and primary roots were measured from images using ImageJ (Abramoff et al., 2004). The lateral roots emerging from the seminal root were counted using a stereoscope. At least 15 seedlings of each genotype per treatment were analyzed.

Measurement of fluorescence decline ratio

The second leaf from the top was collected from 8-week old soil-grown plants. Three 5 cm leaf sections were cut 5 cm down from the leaf tip and placed on a moist paper towel inside a Petri dish, with the leaf edges covered with wet strips of paper towel. Ten leaf samples were used for each genotype. For dark incubation and hormone treatments, the leaf sections were incubated in water in the absence or presence of 1 µM BA (BA was dissolved in 1 N NaOH and added as 1:10,000 of the final liquid volume) in covered Petri dishes in the dark at 30°C. The fluorescence decline ratio (RFd) was assessed at days 0, 1 and 3. PAM-mode (pulse-amplitude-modulated) quenching analysis was performed using FluorCam 800MF.

Measurement of meristem size

For the root apical meristem, the length was based on the length of the central metaxylem (cmx) cell file, starting from the quiescent center and ending at the lower border of where the cmx cells begin to rapidly elongate as described (Worthen et al., 2019). Meristem images of 5-13 roots for each genotype were analyzed.

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Imaging of root tips

Rice seedlings were grown on Kimura B nutrient solution (Ma et al., 2001) solidified with 1% gellan gum for 8 days. Root tips were isolated and fixed in cold FAA (2% formalin, 5% acetic acid and 60% ethanol) overnight with rocking at 4°C. Fixed roots were dehydrated in an ethanol series (2×70% ethanol for 30 min at 4°C; 80% ethanol for 30 min at 4°C; 95% ethanol for 30 min at 4°C; and 2×100% ethanol for 30 min at 4°C), cleared in 100% methyl salicylate overnight at room temperature and then imaged using a Zeiss LSM 710 confocal microscope. Stacks of images were collected through the root tips and a maximum projection of these images was created using ImageJ software.

Vascular cross sections

Rice seedlings were grown on 0.5× MS medium solidified with 1% agar for 14 days. Five roots were embedded in agarose in 3D printed molds (Atkinson and Wells, 2017). Sections were taken approximately 2 cm from the root tip using a vibrating microtome (Campden Instruments). Sections were removed from the vibratome bath and incubated in 0.3 mg/ml calcofluor white (Sigma-Aldrich) for 1 min, mounted in distilled water in a coverglass-bottomed cell chamber (Lab-Tek II, ThermoFisher) and imaged on a Lecia SP5 confocal microscope using a 405 nm laser.

Stigma brush imaging and hair length quantification

Fully developed stigmas were dissected from wild type, *hk5* and *hk6* plants and visualized using a Leica MZ16 microscope with Spot Idea software for image capture. To assess stigma brush size, the length of the five longest hairs was measured on ten stigmas for each genotype using ImageJ software (Abramoff et al., 2004).

Analysis of panicle meristems

Panicle tissue from mature rice plants was harvested at stage R1 (Counce et al., 2000) and fixed in 50% (v/v) ethanol, 5% (v/v) glacial acetic acid and 3.7% (v/v) formaldehyde, dehydrated in a graded ethanol series and then stained with 0.1% (w/v) Eosin Y for sectioning in 95% (v/v) ethanol for 16 h. Tissue was de-stained and cleared at room temperature with a graded series of CitriSolv:ethanol solutions. After embedding in paraffin (Paraplast Plus), 10 µm thick sections were cut with a microtome, fixed on poly-L-coated glass slides, stained with 0.05% (w/v) Toluidine Blue O (Sakai, 1973) and mounted into Permount media. Panicles were visualized on a Leica MZ16 light stereoscope.

Gene expression analysis

Seven-day-old seedlings grown in Kimura B nutrient solution solidified with 1% gellan gum (PhytoTech Labs) were removed and submerged in liquid Kimura B nutrient solution containing 5 µM BA or a vehicle control for 1 h. Total root RNA was extracted using RNAzol (Sigma, R4533). First-strand cDNA synthesis was performed with the ProtoScript II First Strand cDNA Synthesis Kit (NEB, E6560L) using poly-T primers. qRT-PCR was performed with PowerUp SYBR Green Master Mix (ThermoFisher, A25742) according to the manufacturer's instructions. qRT-PCR reactions were performed with three biological replicates and three technical replicates using the QuantStudio 6 Flex Real-Time system (Life Technologies).

Panicle meristems at stages 2-4 according to Furutani et al. (2006) were dissected from 7-week-old wild-type, *hk5*, *hk6* and *hk5 hk6* soil-grown plants as described (Yamburenko et al., 2017). Developmental timing of single *hk* mutants was similar to that of the wild type, but the *hk5 hk6* mutant had a delayed SAM transition to inflorescent meristem. Therefore, additional samples were collected from 18-week-old *hk5 hk6* plants, which were at a similar developmental stage as the wild type. In total, three biological replicates for each line were obtained from 9-18 plants, with 10 meristems per sample.

Expression of *ACT1* (LOC_Os03g0718100) was used for normalization in the qPCR. Relative gene expression was calculated as described (Pfaffl, 2001). Primers used for qRT-PCR are listed in Table S2.

Plant morphological analyses

Plants were grown in soil in the UNC greenhouse as described above. Pictures of the fully matured inflorescences were used to quantify grain filling, primary and secondary branch number, and panicle length. Images of flower organs were taken using 4-month-old plants. Floral organs were quantified using plants that were 15 weeks old. Seeds from genotyped plants were collected and allowed to completely dry. Seeds from each genotype were scanned and the area of each seed was autonomously calculated using the thresholding and analyze particles tools in Fiji (Schneider et al., 2012).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.A.B., G.E.S., J.J.K.; Methodology: C.A.B., M.V.Y., J.J.K.; Formal analysis: C.A.B., J.S., M.V.Y., J.J.K.; Investigation: C.A.B., J.S., M.V.Y., A.W., C.H., S.L.B., A.E., J.A., A.B., J.J.K.; Resources: C.H., Z.L.N., J.J.K.; Writing - original draft: J.J.K.; Writing - review & editing: C.A.B., J.S., M.V.Y., A.W., Z.L.N., A.B., G.E.S., J.J.K.; Supervision: Z.L.N., A.B., G.E.S., J.J.K.; Project administration: G.E.S., J.J.K.; Funding acquisition: Z.L.N., G.E.S., J.J.K.

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Supplementary information

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