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Published in:
Nature Plants

DOI:
10.1038/s41477-021-00957-3

Publication date:
2021

Document Version
Peer reviewed version

Title:

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Abstract

Temperature stresses affect plant phenotypic diversity. The developmental stability of the inflorescence, required for reproductive success, is tightly regulated by the interplay of genetic and environmental factors. However, the mechanism(s) underpinning whether and how plant inflorescence architecture has responded to temperature are largely unknown. We demonstrate that the barley SEPALLATA MADS-box protein HvMADS1 is responsible for maintaining an unbranched spike architecture at high temperatures, while the loss-of-function mutant forms a branched inflorescence-like structure. HvMADS1 exhibits increased binding to target promoters via A-tract CArG-box motifs, which change conformation with temperature. Target genes for high-temperature dependent HvMADS1 activation are predominantly associated with inflorescence differentiation and phytohormone signalling. HvMADS1 directly regulates the cytokinin-degrading enzyme *HvCKX3* to integrate temperature response and cytokinin homeostasis, which is required to repress meristem cell cycle/division. Our findings reveal a novel mechanism by which genetic factors direct plant thermomorphogenesis, extending the recognised role of plant MADS-box proteins in floral development.

Keywords

Inflorescence branching; Temperature response; Meristem identity; MADS-box protein; Cell division; Gene expression; Cytokinin homeostasis; Barley

Introduction

As sessile organisms, plants have undergone long-term phenological and morphological adaptations to elevated temperatures[1–3]. These adaptive mechanisms respond to gradual increases in temperature by modifying a suite of developmental traits, such as flowering time, stem elongation, and seed germination[4,5]. In *Arabidopsis thaliana*, several key sensors/regulators have been reported to be involved in thermal adaptation. Histone variant H2A.Z contributes to chromatin remodelling in response to temperature changes[6] and both PHYTOCHROMES (PHYs) and the basic helix-loop-helix (bHLH) transcription factor PHYTOCHROME INTERACTING FACTOR4 (PIF4) function in temperature regulatory responses and thermomorphogenesis that lead to developmental and architectural variations[7,8]. However, the factors that establish and maintain phenology and organ identity in response to temperature changes remain unknown.

Plant inflorescence morphology is determined by the arrangement of its branches and florets, developmental processes that are governed by a range of genetic and environmental factors[2,3,9–11]. Fitness landscape model predictions of inflorescence architecture and the Watson and Dallwitz database of flowering plants reveal higher frequencies of cyme-plants (flowers terminating the axis) in temperate compared with tropical climes, possibly because there is a benefit associated with the sequential maturity of flowers in cymes, and the opposite for highly branched panicles[11]. Plants with unbranched raceme-type inflorescences are also shown to be more frequent in temperate than tropical conditions, but not significantly[11], suggesting a complex interaction between inflorescence development and environmental adaptation. Triticeae crops, like wheat (*Triticum spp.*) and barley (*Hordeum vulgare*), have unbranched inflorescences (spikes) where the flower-producing spikelets are attached directly to the main axis of the inflorescence. An increase in ambient temperature (e.g., > 25 °C) leads to delayed inflorescence meristem development, and reduced the number of spikelet primordia both in wheat and barley[12,13], indicating that high temperatures inhibit both inflorescence and spikelet development during early reproductive stages of Triticeae crops. With climate change likely to drive changes in global plant phenology[2,14], the adaptation of fitness-related traits is becoming paramount in plants, including many crops[1,2,12,15]. Extreme temperature changes are speculated to impact reproductive growth in particular[16], which is potentially devastating for grain yield in cereal crops[17,18]. However, the mechanism(s) by which plants alter inflorescence structure under changing environmental temperatures is poorly understood.

SEPALLATA (SEP) proteins are plant E-class MADS-box family transcription factors that regulate a wide variety of reproductive events, including flowering time, inflorescence architecture, flower organ development, and fruit ripening[19–23]. Like other MADS-box proteins, SEPs bind a canonical CArG-box DNA motif [CC(A/T)$_6$GG or C(A/T)$_8$G] to regulate target gene expression[24,25]. Strikingly, the curvature of DNA regions containing A-tract elements that minimize the incidence of TpA sites in the (A/T)$_n$ core strongly depends on temperature, which may affect *in vivo* binding by transcription factors[25–28]. However, whether or how MADS-box proteins regulate *in vivo* thermal gene expression and plant thermomorphogenesis remains unknown.

Here, we examine the role of SEPs in directing inflorescence architecture in barley. Our results demonstrate that one SEP, HvMADS1, is critical in maintaining an unbranched barley spike under high ambient temperatures by modulating cytokinin homeostasis, providing insights into inflorescence thermomorphogenesis.

Results

HvMADS1 regulates inflorescence plasticity in response to high temperature

In barley, there are five *SEP* genes in two clades: one *LOFSEP* clade with three members: *HvMADS1*, *HvMADS5* and *HvMADS34*; and the other clade with two *SEP3*-like genes, *HvMADS7* and *HvMADS8*[29]. These five genes are known to be highly expressed in developing inflorescences based on our previous RNA-seq data[30]. *LOFSEP*-like family members, and their orthologs are associated with inflorescence architecture regulation in *Arabidopsis*[21], rice (*Oryza sativa*)[22], and tomato (*Solanum lycopersicum*)[23]. To explore the genetic basis of barley inflorescence development, we used CRISPR/Cas9[31] in the UK barley variety Golden Promise (GP) to create individual loss-of-function mutants and double mutants of three *LOFSEP* MADS-box genes, i.e., *HvMADS1*, *HvMADS5,* and *HvMADS34* (Extended Data Fig. 1a–e). Under control growing conditions (15 °C day/10 °C night), none of the mutants displayed any visible changes in spike architecture or organ morphology in either the central or lateral spikelets, except that spikes of *Hvmads1* single and double (*Hvmads1/5* and *Hvmads1/34*) mutants produced shorter awns compared with wild-type plants (Fig. 1a and Extended Data Figs. 2 and 3). Surprisingly, under heat stress conditions (28 °C day/23 °C night), spikes of *Hvmads1* single and double mutants produced ectopic spike/spikelet-like organs, while *Hvmads5*, *Hvmads34*, and *Hvmads5/34* mutants exhibited wild-type spike architecture (Fig. 1a,b and Extended Data Fig. 2), indicating that HvMADS1 acts a key regulator involved in

directing spike architecture in response to temperature. Detailed phenotypic analysis revealed that the heat-induced ectopic organs in *Hvmads1* spikes emerged from the base of central spikelet, attached to the joint between the central spikelet and main spike axis (rachis) (Fig. 1b and Extended Data Fig. 4a–c). Some young branch-like meristems were formed at late stages of main spike development, suggesting that high temperature continually induced the initiation of ectopic organs in the mutant (Extended Data Fig. 4b). Morphologically, these new organs could be classified as ectopic inflorescence-like (EI) or spikelet-like (ES) structures (Fig. 1b and Extended Data Fig. 4d–f), which emerged more often at the basal end of the main spike (Extended Data Fig. 4g). More EI and ES developed with increasing temperature, with *Hvmads1* spikes producing twice as many spikelets (including ectopic spikelet/meristems from EI or ES) as wild-type spikes at 28 °C (Extended Data Fig. 4h,i). Compared with the wild-type, the central spikelet of the *Hvmads1* mutants at 28 °C lost the rachilla (a secondary rachis at the germ end of the ventral crease of the barley spikelet), but developed normal glumes, lemmas, paleas, and lateral spikelets (Fig. 1c), suggesting that some EIs or ESs are likely reverted from the rachilla of the central spikelet. Reversion of the rachilla has also been reported in other barley branched inflorescence mutants, such as *Hvcom1* (*compositum 1*), *Hvcom2*, and *Hvvrs4* (*six-rowed spike 4*)[32–34]. The *Hvmads1* ES produced at 28 °C showed a normal flower organ structure (Fig. 1c), but the *Hvmads1* EI exhibited defective flower morphology with multiple lemmas, paleas, or carpels in spike-like structures. Occasional secondary EI with a long, rachis-like axis were observed within these defective spikelets (Fig. 1d).

To confirm that the phenotype is associated with high temperature, we created *Hvmads1* mutants in two Australian barley varieties, Vlamingh (Vla, Western Australia) and WI4330 (WI, South Australia), adapted to a warmer climate than GP (Extended Data Fig. 1a–c). The *Hvmads1*/Vla and *Hvmads1*/WI spikes also produced shorter awns at control temperatures (Fig. 1a and Extended Data Fig. 3a,b), and grew new ectopic organs at high temperatures (Fig. 1a and Extended Data Fig. 4j,k), although less frequently than *Hvmads1* spikes in the GP background (Fig. 1e,f). As growth temperature increased, the number of EI and ES also increased (Fig. 1e and Extended Data Fig. 4h), as did the proportion of EI compared with ES in all three backgrounds (Fig. 1f). Taken together, our observations indicate that HvMADS1 regulates barley inflorescence architecture in response to temperature.

High ambient temperatures induce branching events in *Hvmads1*

To further assess the role of HvMADS1 in temperature-associated spike meristem development, wild-type (GP) and mutant plants were grown at five different temperatures, including control (15 °C), high ambient (20 °C, 23 °C and 25 °C), and heat stress (28 °C) conditions, from Waddington stage W1 (initiation of inflorescence meristem) to W7 (completion of spike morphogenesis)[35] (Fig. 2a). Overall, more ectopic organs/branch-like structures were produced as temperature increased (Fig. 2b–d), indicating a dosage-dependent, rather than threshold, effect of temperature on *Hvmads1* phenotypes. Similar to the phenotype of *Hvmads1* in a GP background, more branching events were observed in *Hvmads1* inflorescences in Vla or WI backgrounds at 28 °C than at 23 °C (Fig. 2c).

After high temperature treatments, plants were transferred back into control temperature conditions (15 °C). EI further developed into two types: short EI with a few spikelets; or elongated EI producing normal spikelet-like structures (Fig. 2d,e and Extended Data Fig. 4l,m). Most of the spikelets from short EI had abnormal organs, including extra glumes, lemmas, paleas, carpels, and secondary EI (Fig. 1d and Extended Data Fig. 4l). We observed a higher proportion of short EI at lower temperatures (20 °C, up to 90%) than at 28 °C (decreased to ~60%). Elongated EI showed the converse trend (Extended Data Fig. 4n). While central spikelets from ES and EI could produce grains at all elevated temperatures, the overall fertility of ES and EI reduced as temperature increased (Extended Data Fig. 4o–q). This indicates that the ectopic spikelets have normal seed-setting capacity that is impacted by temperature.

Scanning electron microscopy revealed no morphological difference between wild-type (GP) and *Hvmads1* inflorescence development at 15 °C, other than retarded awn elongation in the mutant (Extended Data Fig. 5a,b). At 28 °C, *Hvmads1* spikes showed three types of changes to meristem identity: determinate central spikelet meristems (CSM) likely converted into indeterminate inflorescence meristems; the rachilla meristem possibly converted into spikelet/inflorescence-like meristems; and the ectopic meristems initiated at the base of CSMs (Fig. 2f,g and Extended Data Fig. 5c,d). Some CSMs at the base of the main spike appeared to be transformed into inflorescence meristems, which were frequently seen at early stages of spike development (W2.5–W4.0), indicating that the lost determinacy of CSMs may lead to the initiation of branch meristems in the *Hvmads1* mutant under high temperatures. Spikelet/inflorescence-like meristems possibly reverted from rachilla meristems were observed from W2.5. However, the appearance of ectopic meristems was more frequently seen in the middle section of spike later in development (W5), even though the main central spikelets were

completely formed (Fig. 2g and Extended Data Fig. 5d). Hence, these extra meristems leading to ES/EI appear not to be generated from main central spikelets (Fig. 1b and Extended Data Fig. 4d,e). These data are consistent with our observations that ectopic organs are continually induced under high temperatures (Extended Data Fig. 4b). Thus, inflorescence meristems possibly reverted from rachilla or CSM, and the ectopic meristems around CSM, all likely contribute to the branching phenotype of *Hvmads1* spikes under high temperatures.

Heat stress conditions led to reduced meristem determinacy and the production of ectopic meristems that develop into ES or EI. Similar developmental defects were observed in *Hvmads1* mutants at intermediately high ambient temperatures (20–25 °C) (Extended Data Fig. 5e). Moreover, we observed that in all three backgrounds of barley varieties, the *Hvmads1* mutation delayed inflorescence development, including spikelet meristem formation and differentiation, compared with wild-type (Extended Data Fig. 5f,g). This suggests that *HvMADS1* may repress the spike branching, and mutations in *HvMADS1* caused the developmental delays in meristems assuming spikelet identity. The indeterminate branch meristems initiated from CSMs in *Hvmads1* spikes under high ambient temperatures continued to grow, forming branch-like structures (Fig. 2g and Extended Data Fig. 5d). Taken together, HvMADS1 maintains the unbranched inflorescence shape at high temperatures by controlling meristem identity and development.

HvMADS1 represses ectopic cell division activity of meristems at high temperature

Meristem determinacy directs tissues to undergo programmed cell division and differentiation to maintain proper inflorescence architecture[9,10]. To investigate how HvMADS1 affects cell division, we used 5-ethynyl-2′-deoxyuridine (EdU) to label S-phase nuclei during mitosis (Extended Data Fig. 6a). Young (W2.5) wild-type and *Hvmads1* (GP) spikes showed similar levels of cell division at control (15 °C) and high ambient temperatures (25 °C) (Extended Data Fig. 6b,c). As inflorescence development progressed, *Hvmads1* spikes at 25 °C exhibited additional mitotic activity at the rachilla position of CSM or in the rachis (Fig. 3a), showing ectopic clusters of cell division in non-floret meristem regions of the rachis (Extended Data Fig. 6c), consistent with the observed position of ectopic organs. Thus, the EdU tracking assays revealed changes in the location of cell division in the meristems of the *Hvmads1* spikes under high temperature, likely associated with the phenotype of inflorescence-like meristems converting from rachilla, CSM, and/or ectopic meristems. Additionally, the cell division marker gene, *HvHistone4*, was expressed more broadly at the base of the CSM and axis regions in *Hvmads1* at high temperatures, compared with tightly controlled expression at the CSM tips

in wild-type tissues (Fig. 3b). Thus, high ambient temperatures induce ectopic cell division activity in *Hvmads1* CSM. Moreover, *HvMADS1* mRNA in wild-type spikes was expressed throughout the spike at W2.5, accumulating later in spikelet primordia and floral organs (Fig. 3c,d). Expression of the HvMADS1 protein in *pro::HvMADS1-eGFP* transgenic lines showed a similar pattern, found throughout the young inflorescence (W2.25–W3.5) (Fig. 3e), and accumulating later in central spikelets (W5) and floral organs, including lemma, palea, anther and lodicule (Fig. 3f and Extended Data Fig. 7a). Thus, HvMADS1 appears to maintain inflorescence shape by repressing cell division, thereby stabilising the meristem determinacy of cells from the central spikelet stimulated by high ambient temperatures; in *Hvmads1* spikes, meristem determinacy is reduced, and ectopic meristems develop into branches.

HvMADS1 coordinates thermal transcriptome programming of inflorescence meristems

To further probe how *HvMADS1* regulates barley inflorescence thermomorphogenesis, we examined the impact of temperatures on HvMADS1 expression. Neither HvMADS1 mRNA nor protein levels were affected by temperature (Fig. 3g and Extended Data Fig. 7b–d), leading us to speculate that *HvMADS1* maintains barley inflorescence morphogenesis in response to high ambient temperatures by modulating the expression of downstream genes. To test this possibility, we performed transcriptome analysis (RNA-seq) using inflorescence meristems at stages W2.5 (triple mound) and W3.5 (awn primordium) from wild-type (GP) and *Hvmads1* plants grown at 15 °C and 25 °C, respectively. Consistent with the obvious inflorescence defects of *Hvmads1* under high temperature, principal component analysis revealed that wild-type and mutant global transcriptional patterns diverged more at 25 °C at both developmental stages, and that PC1, which diverged more at high ambient temperature, explained 39.1% of the differences (Fig. 4a), highlighting an essential role of HvMADS1 in gene expression at the higher temperature. Correlation analysis confirmed significant dysregulation of thermal response genes and spike developmental genes at high ambient temperature in *Hvmads1* (Fig. 4b and Extended Data Fig. 8a). Gene transcription was generally inhibited in *Hvmads1* spikes (slope < 1), suggesting that HvMADS1 activates thermal response genes.

To investigate the molecular consequences of the loss of *HvMADS1* function in barley inflorescence development and temperature response, we performed global transcriptional comparisons to evaluate the possible correlation of gene expression with developmental phase, temperature, and/or genotype, and their interactions. In total, 9,434 differentially expressed genes (DEGs) were identified (Fig. 4c, Extended Data Fig. 8b and Dataset 1). Of 3,194 DEGs

affected by *HvMADS1* expression (genotype), 2,568 (80.4%) were also co-regulated by temperature (Fig. 4c). To investigate the interaction between *HvMADS1* genotype and temperature, we compared the *Hvmads1* transcriptome at 25 °C with the *Hvmads1* transcriptome at 15 °C and the wild-type transcriptome at 25 °C by Venn diagram analysis. We found that 266 DEGs in W2.5 and 476 DEGs in W3.5 spikes, respectively, are likely to affect the mutant branching phenotype (Extended Data Fig. 8b). In agreement with phenotypic observations, expression of transcripts affected by *HvMADS1* at 25 °C was more distinct than at 15 °C. Co-expression cluster analysis based on developmental phase, temperature, and genotype revealed 22 unsupervised groups of transcripts (Extended Data Fig. 8c and Dataset 2). Clusters 6–13 contained meristem-associated and thermal response genes that were affected by all three variables. Genes in Clusters 1–5 and 15–19, encoding e.g., cell cycle/division and plant hormone pathway components, were co-regulated by temperature and *HvMADS1*. Clusters 14 and 22 included genes encoding receptor proteins and transcription factors that were affected only by *HvMADS1* (Extended Data Fig. 8d and Dataset 2). Gene ontology (GO) enrichment analysis indicated that DEGs at 15 °C were mainly involved with cellular component biosynthesis and nucleotide metabolism, whereas DEGs at 25 °C were largely associated with inflorescence development and regulation, meristem activity, cell cycle, stimulus response, and gene expression (Fig. 4d and Datasets 3 and 4), suggesting that HvMADS1 has different regulatory functions at 15 °C and 25 °C. We therefore conclude that HvMADS1 plays dominant roles in thermal transcriptome programming during barley inflorescence development. Due to the stable expression of HvMADS1 under control and high temperatures, the stability of spike architecture at high temperature is likely achieved by changes in the targets of HvMADS1, but not HvMADS1 itself. Other characteristics of HvMADS1, such as protein folding or binding affinity, may be affected by high temperature to regulate its downstream gene expression.

The most prominent changes in expression occurred in W3.5 *Hvmads1* spikes at 25 °C, for genes encoding meristem identity and transition regulators, i.e., WUSCHEL-like, CLAVATA-like, TAWAWA-like, MADS-box, TEOSINTE BRANCHED (TCP transcription factors), and KNOX transcription factors (Extended Data Fig. 9a and Dataset 5). These genes generally exhibited lower levels of transcription in *Hvmads1* spikes at 25 °C, confirmed by qRT-PCR (Extended Data Fig. 9b), which supports observations of delayed development of meristems in the mutant. TCP transcription factors play key roles in barley inflorescence development, i.e., regulation by HvVRS5/int-C (INTERMEDIUM-C) of row-type[36], and HvCOM1 / BDI1

(BRANCHED AND INDETERMINATE SPIKELET 1) in inflorescence architecture and meristem identity[32,37]. The expression of several *TCP* family genes was down-regulated in *Hvmads1* spikes at 25 °C (Extended Data Fig. 9a), implying that genes of this family may be involved in barley inflorescence thermomorphogenesis. However, the expression of other genes encoding key barley inflorescence regulators, such as *HvCOM2* and the five *HvVRS* genes[33,34,36,38–40], was not significantly affected by *HvMADS1* expression (Extended Data Fig. 9c), suggesting that HvMADS1-mediated inflorescence development is independent of known RAMOSA or conserved BD1 (Branched silkless1, maize)/FZP (FRIZZY PANICLE, rice) pathways[41–43]. Moreover, DEGs governing cell cycle progression, including *Cyclins*, *Histones*, *Cyclin-Dependent Protein Kinases* (*CDKs*) and *E2F* factors[44], figured prominently in the GO analysis at 25 °C, but not at 15 °C (Fig. 4d), and showed opposite expression patterns in *Hvmads1* spikes at the two temperatures (Fig. 4e and Dataset 5), consistent with observed defects of meristem determinacy and cell cycle/division activity. DEGs associated with auxin, gibberellic acid, and cytokinin (CK) biosynthesis, metabolism and signalling also showed significant changes in *Hvmads1* at 25 °C compared with our other transcriptomes (Fig. 4f and Dataset 5), consistent with the critical role of these three hormones in barley inflorescence development[39].

The barley homologs of *Arabidopsis PHYB* and *PIF4* with reported functions in perceiving temperature and thermomorphogenesis[4,5,7,8] showed reduced expression levels in *Hvmads1* spikes, particularly at 25 °C (Extended Data Fig. 9a,d and Dataset 5). Moreover, changes in *Histone* gene expression in *Hvmads1* spikes between 15 °C and 25 °C imply that HvMADS1 likely involves chromatin remodelling to control thermal transcription (Fig. 4e). In *Arabidopsis*, Histone variant H2A.Z has been reported to regulate nucleosome occupancy of thermal transcription[4–6]. It is noteworthy that a large number of *Heat Shock Protein* genes[4] and heat tolerance players (i.e., *ERECTA* and *Thermo-Tolerance 1*)[45,46] showed large variability of transcription in *Hvmads1* spikes at 25 °C compared with wild-type (Extended Data Fig. 9a,d and Dataset 5), consistent with the dysregulation of global thermal responsive gene expression in *Hvmads1* (Fig. 4b and Extended Data Fig. 8a).

HvMADS1 binds to the CArG-box to regulate gene transcription in response to temperature

SEP proteins regulate transcription of target genes by binding to A-tract-rich CArG-box motifs[24,25], which change conformation with temperature *in vitro*[26–28]. In *Arabidopsis thaliana*,

the *in vitro* binding affinity of SEP3 to A-tract CArG-boxes increases with temperature but binding to non-A-tract CArG-boxes remains temperature-independent[25]. To test whether A-tract sequences affect temperature-dependent transcriptional activation by HvMADS1, we made artificial promoters carrying A-tract or non-A-tract CArG-boxes for *in vivo* dual-luciferase assays and *in vitro* electrophoretic mobility shift assays (EMSA) under different temperature conditions (Fig. 5a). HvMADS3, the homolog of *Arabidopsis* AGAMOUS known to regulate gene expression via CArG-boxes independent of temperature, was used as a control[25]. HvMADS1 exhibited temperature-dependent gene activation via A-tract CArG-boxes only; activation of A-tract CArG-boxes by HvMADS3 at both low and high temperatures demonstrated that temperature-dependent transcription activity of HvMADS1 is not shared with other non-SEP MADS-box proteins, such as HvMADS3 (Fig. 5b,c). EMSAs demonstrated increased binding of A-tract CArG-boxes by homodimeric and monomeric HvMADS1 at elevated temperatures (Fig. 5d), and *in vivo* co-immunoprecipitation assays showed that HvMADS1 can form homodimers in tobacco cells (Fig. 5e). Chromatin immunoprecipitation (ChIP)-PCR analysis of four putative thermal and developmental regulators *HvPIF4*[8], *HvRPK4* (*RECEPTOR-LIKE PROTEIN KINASE 4*), *HvTFL1-like* (*TERMINAL FLOWER 1-like*), and *HvTB1L* (*TEOSINTE BRANCHED 1-like*) with promoters containing A-tract CArG-boxes revealed that HvMADS1 binding increased with temperature (Fig. 5f), consistent with decreased expression of these genes in *Hvmads1* spikes at high ambient temperature (Extended Data Fig. 9a,b,d). Thus, we have demonstrated an *in-planta* mechanism by which a MADS-box protein regulates gene expression, by temperature-dependent binding to A-tract CArG-boxes to promote transcription of downstream response genes.

HvMADS1 integrates cytokinin homeostasis and temperature response to regulate barley inflorescence branching

Plant hormones, including auxin, gibberellic acid, and CK, are implicated in the control of plant architecture and inflorescence meristem activity[9,10,39]. Notably, of the hormone-related DEGs in our transcriptomic data (Fig. 4f and Dataset 5), two-component signalling *response regulators* (*RRs*) of CK[47], type A and type B, had opposite responses to high ambient temperature in *Hvmads1* spikes (Fig. 6a). Genes encoding type A *RRs* (repressed by the CK response) were downregulated in *Hvmads1* spikes at 25 °C, whereas genes encoding type B *RRs* (activated by the CK response) were upregulated, suggesting an enhanced CK response in *Hvmads1* plants at high temperature.

To examine how CK affects barley inflorescence development, wild-type, and mutant spikes from W1–W5 were treated with the cytokinin analogue benzylaminopurine (BAP). At 15 °C, *Hvmads1* spikes produced several EI meristems (~10 in *Hvmads1*) (Fig. 6b,c), which phenocopied *Hvmads1* inflorescences at high temperatures in the absence of BAP (Fig. 2b). At 28 °C, BAP also induced ES meristems from the lemma-side of the central spikelet in both wild-type and *Hvmads1* spikes, and EI in the central spikelet from the palea-side of *Hvmads1* spikes only (Fig. 6d,e). Thus, we propose that altered CK homeostasis may contribute to the abnormal ectopic branching phenotype of *Hvmads1* spikes at high temperature.

Measurement of endogenous CK levels by liquid chromatography–tandem mass spectrometry revealed that active CK forms (i.e., isopentenyladenine and trans-zeatin) and CK metabolites were significantly increased in *Hvmads1* spikes, with higher levels at 28 °C than at 15 °C (Fig. 6f). To monitor *in vivo* CK levels, we introduced a synthetic CK biosensor construct (*pTCS::YFPn*) into wild-type (WI) and *Hvmads1*/WI plants. At 15 °C, the CK-responsive YFP signal accumulated in spikelet meristems, while at 28 °C, a higher CK response was observed in the inflorescence main axis, which was both wider and earlier in *Hvmads1* spikes (Fig. 6g). Importantly, an ectopic CK response was observed at the base of central spikelet and at the main axis at 28 °C (Fig. 6g), which may contribute to indeterminacy in the adjacent meristems, mimicking ectopic branch meristem formation and cell cycle/division activity in *Hvmads1* spikes (Fig. 3a,b). These findings suggest that HvMADS1 promotes the decay of CK molecules to maintain hormone homeostasis that inhibits ectopic meristem activity in barley inflorescence at high ambient temperatures.

HvMADS1 directs *HvCKX3* to regulate spike determinacy under high temperatures

CYTOKININ OXIDASE/DEHYDROGENASE (CKX) proteins degrade CK to maintain hormonal homeostasis in response to environmental and developmental cues[46]. The changed flow of CK metabolites, e.g., isopentenyladenine N-glucoside and trans-zeatin O-glucoside (Fig. 6f), and enhanced CK response in *Hvmads1* plants are consistent with findings of altered CK homeostasis from rice and *Arabidopsis* plants with altered *CKX* expression[48,49]. Three *HvCKX* genes were also identified as DEGs in *Hvmads1* spikes (Dataset 5). One of them, *HvCKX3* (HORVU1Hr1G042360), is predominantly expressed in early spike (W2–3.5) development[30].

ChIP-PCR analysis targeting the CArG-boxes in the promoter and intron regions of *HvCKX3* using *pro::HvMADS1-eGFP* transgenic plants confirmed that HvMADS1 bound all *HvCKX3*

promoter fragments containing CArG-box sequences *in vivo*, but only binding of A-tract CArG-boxes improved with high temperatures (Fig. 7a). EMSAs confirmed the result *in vitro* (Fig. 7b), and *in vivo* dual-luciferase assays showed that improved binding of *HvCKX3* promoter by HvMADS1 at high ambient temperature and heat stress conditions led to increased reporter gene transcription (Fig. 7c, Extended Data Fig. 10), consistent with artificial A-tract CArG-boxes assays (Fig. 5a–d). Accordingly, the expression level of *HvCKX3* was lower in *Hvmads1* spikes, and increased with temperature in wild-type, but not in *Hvmads1*, spikes (Fig. 7d). *In situ* hybridisation assays also demonstrated that temperature-induced accumulation of *HvCKX3* mRNA occurred at the tip and base of CSMs and the joints between the CSM and main axis in wild-type spikes (Fig. 7e). Further, an *Hvckx3* mutant created by CRISPR/Cas9 consistently showed EI formation and reduced meristem determinacy at high ambient temperature (25 °C) and heat stress (28 °C) conditions (Fig. 7f,g), mimicking the defects of *Hvmads1*. Similar to the *Hvmads1* mutant, the *Hvckx3* spike did not show ectopic branches or spikelets at 15 °C (Fig. 7g), suggesting that HvMADS1 affects local excessive CK flow and metabolism during spike development by activating the expression of *HvCKX3* only under high temperature conditions (Fig. 7d,e). These results demonstrate that HvMADS1 controls CK homeostasis at high temperature to stabilize barley spike morphogenesis via HvCKX3.

Conserved *HvMADS1* sequence in barley varieties

To assess the natural variation of *HvMADS1*, we investigated the sequence of *HvMADS1* encoding and regulatory regions in selected barley cultivators. Analysis of exome sequencing data across 267 barley genotypes identified only three synonymous and one nonsynonymous single nucleotide polymorphisms (SNPs) in the C-terminal domain, and no SNPs in any other domains, of HvMADS1 (Extended Data Fig. 11 and Dataset 6)[50]. Further sequencing of 101 wild and cultivated barley varieties from different countries showed no further SNPs in the *HvMADS1* coding region and first intron (Dataset 7), revealing strong conservation of *HvMADS1* sequence during domestication.

Discussion

Global warming has been affecting numerous plant species, including their distribution, phenology, and biodiversity[1,2,14]. The developmental plasticity of the inflorescence is also likely regulated by temperature conditions[2,3,11–13]. Here, we have shown that a barley SEP protein, HvMADS1, maintains branchless inflorescence development under high ambient temperatures via control of cytokinin homeostasis (Fig. 7h). Development of ectopic meristems

391 in the *Hvmads1* mutant is regulated by a genotype × environment interaction, a phenomenon
392 not previously observed in reports of plant thermomorphogenesis. Further, our data reveal
393 that HvMADS1 fulfils its pivotal function in thermal response by controlling transcriptional
394 changes that regulate meristem identity and development, likely through improved binding to
395 promoters via A-tract CArG-boxes, whose physical conformations change with temperature[25–
396 28]. These findings reveal a novel role for MADS-box proteins in directing inflorescence
397 architecture through temperature-sensitive regulation of a myriad of regulatory and cellular
398 functions, which extends the recognised function of plant MADS-box genes as determinants
399 of floret identity[19,20].

400 E-class *SEP* genes are broadly involved in specifying all whorls of floral organs and for floral
401 determinacy[9,10,20]. Our genetic and phenotypic analyses revealed that loss of *HvMADS1* only
402 affected awn elongation at control temperatures, suggesting functional redundancy of
403 HvMADS1 with other SEP/MADS proteins. However, in other grasses, such as rice, the
404 *Osmads1* mutant exhibits elongated leafy paleas and lemmas, and defective inner organs[29,51,52],
405 which is different to the barley *Hvmads1* mutant, indicating functional diversity of *HvMADS1*
406 compared *MADS1* orthologs in other grasses. SEP proteins play a redundant role in regulating
407 plant inflorescence architecture by forming multimeric protein complexes as reported in
408 *Arabidopsis*, rice, and tomato[21,23]. Notably, barley *lofsep* double mutants (*Hvmads1/5* and
409 *Hvmads1/34*) did not show obvious inflorescence phenotypic differences compared with
410 *Hvmads1* single mutants in response to high temperatures. Future work on the generation and
411 analysis of the triple mutant of *HvMADS1*, *HvMADS5* and *HvMADS34* may elucidate *LOFSEP*
412 functional redundancy. Beside the *LOFSEPs*, two barley *SEP3*-like genes, *HvMADS7* and
413 *HvMADS8*, are highly expressed in developing inflorescences[30], and their orthologues in
414 *Arabidopsis* and rice are required for proper floret organ identity[19,20,53]. Further investigation
415 of barley *SEP3*-like genes in inflorescence development under different temperature conditions
416 will provide a mechanistic picture of *SEP* gene function in inflorescence
417 thermomorphogenesis.

418 Our work reveals that HvMADS1 regulates the thermal transcriptome to repress cell
419 cycle/division activity and maintain cytokinin homeostasis at high ambient temperatures.
420 Application of a cytokinin analogue induced ectopic organ formation in *Hvmads1* spikes at
421 control temperatures (Fig. 6b,c), leading to identification of *HvCKX3* as an HvMADS1 target.
422 In the absence of HvMADS1, insufficient levels of HvCKX3 cannot maintain local cytokinin
423 homeostasis at high temperatures, leading to reduced meristem determinacy and changed

meristem identity, ectopic cell division for branch meristems, and ultimately, development of a branched inflorescence (Fig. 7h). At high temperatures, an *Hvckx3* mutant phenocopied *Hvmads1* (Fig. 7g), further supporting our conclusion that HvMADS1 integrates thermal response and cytokinin homeostasis to maintain inflorescence architecture via HvCKX3. At control temperatures, HvMADS1 also regulates *HvCKX3* expression; challenging of barley spikes with ectopic cytokinin produced a branched inflorescence-like phenotype only in the *Hvmads1* mutant. In *Arabidopsis*, high temperatures affect developmental plasticity, including promotion of hypocotyl elongation and flowering time regulated mainly by the PHYB-PIF4 pathway[4,5,7,8]. In barley, however, thermally induced inflorescence branching does not occur in wild-type plants due to the regulatory effect of HvMADS1. Loss of *HvMADS1* led to a change in the morphogenesis of the spike, altered expression of barley homologs of *PHYB* and *PIF4*, and induced a large number of *Heat Shock Protein* and *Histone* genes under the high temperature. Details of the HvMADS1 association with the PHYB-PIF4 regulatory network, heat stress response, and chromatin remodelling-mediated thermal transcription in barley must await future investigation.

Seasonal temperature changes affect plant growth, flowering time, and phenotypic plasticity[2,3,11,15]. A severe consequence of climate change is the projected increase in temperature, posing a significant challenge for maintaining agricultural crop yield and quality[17,18]. Better understanding of the mechanisms underpinning desirable plant traits in response to temperature can therefore offer insights into breeding climate-smart plants to sustain productivity[1,11,15.] The branches of *Hvmads1*, which developed from ectopic meristems initiated after the formation of spikelet meristems, are not comparable to panicle-like inflorescences in grasses such as rice, because the branch meristems in rice are initiated before the spikelet meristems[9,10], suggesting possible diverse mechanisms regulating branching vs non-branching inflorescence in the grasses. The variability of MADS1 in directing temperature response in different grass crops remains to be investigated. Temperature-dependent binding of SEPs, and possibly other MADS-box proteins, to promoters is likely to regulate plant thermomorphogenesis, representing a novel biological control tool as yet unexploited in crop plants. Our findings provide mechanistic insights into the development of diverse grass inflorescence architectures in response to climate, which reveal new avenues for breeding of climate-smart plants to overcome the traditional compromise between heat tolerance and high yield.

Methods

Plant materials and generation of transgenic plants

Wild-type barley (*Hordeum vulgare*) varieties used included Golden Promise (GP, UK), WI4330 (WI, South Australia) and Vlamingh (Vla, Western Australia) for this study. A monocot-specific robust CRISPR/Cas9 system was used to create barley mutants[31]. Two target sequences for each *HvMADS* gene were selected within the MADS domain. A Blast search (https://webblast.ipk-gatersleben.de/barley_ibsc/) of the target sequences (including PAM, protospacer adjacent motif, NGG) was performed to confirm their targeting specificity in the barley genome[54]. The target sites of three *SEPALLATA* genes (*HvMADS1*, *HvMADS5*, *HvMADS34*) and *HvCKX3* were sequenced in GP, and *HvMADS1* target sites were also sequenced in WI and Vla, all showing 100% identity with reference (Morex) genome[54]. sgRNA-T1 was driven by rice promoter *OsU6a* and sgRNA-T2 was driven by rice promoter *OsU6b*. The sgRNA expression cassettes of *OsU6a-sgRNA-T1* and *OsU6b-sgRNA-T2* were amplified from pYLsgRNA-OsU6a and pYLsgRNA-OsU6b templates using the Phusion High-Fidelity DNA Polymerase (New England BioLabs) and cloned into a binary vector, pYLCRISPR/Cas9P_{ubi}-H using *Bsa*I as described[31]. sgRNA-T1 of *HvMADS1* and sgRNA-T2 of *HvMADS5* were used for *HvMADS1/5* construction to create the double mutant; sgRNA-T1 of *HvMADS1* and sgRNA-T2 of *HvMADS34* were used for *HvMADS1/34* constructs; sgRNA-T1 of *HvMADS5* and sgRNA-T2 of *HvMADS34* were used for *HvMADS5/34* constructs (Extended Data Fig. 1). All constructs were used for *A. tumefaciens* AGL1-mediated transformation of immature barley embryos as previously described[55]: *HvMADS1* into GP, Vla and WI varieties, *HvMADS5*, *HvMADS34*, *HvMADS1/5*, *HvMADS1/34*, *HvMADS5/34*, and *HvCKX3* into GP only. Independent T_0 plants carrying biallelic and homozygous mutations were identified by genotyping using a Phire Plant Direct PCR Kit (Thermo Fisher Scientific) and Sanger sequencing (AGRF, Australia). Editing efficiency of biallelic mutation, heterozygous and homozygous for three *SEPALLATA* genes in single and double mutants is listed in Supplementary Table 1. All primers used for CRISPR/Cas9 constructs are listed in Supplementary Table 2.

To analyse HvMADS1 protein accumulation and regulation, the *pro::HvMADS1-eGFP* construct was created by inserting the 2,489 bp *HvMADS1* promoter and full length *HvMADS1* cDNA fused with *eGFP* (enhanced Green Fluorescent Protein) into the *Kpn*I and *Bst*EII sites of pCAMBIA1301, using In-Fusion (Takara) cloning technology. The vector was transformed

into barley variety GP using *A. tumefaciens* AGL1-mediated transformation as described above. At least three independent lines were used for analysis. Primers are listed in Supplementary Table 3.

A cytokinin biosensor (*pTCS::YFPn*) was designed using a 3×*YFP* (*Yellow Fluorescent Protein*) reporter with a nuclear localisation sequence (*n*) driven by an artificial cytokinin-responsive two-component system promoter (*pTCS*) combined with a 35S minimal promoter. The *pTCSn-35Smin* sequence was synthesised in pUC57 by Genscript (Piscataway, NJ, USA), based on the *pTCSn1::GFP-ER* vector[56]. The synthesised fragment was flanked by 5′-*Hind*III and 3′-*Kpn*I restriction sites, allowing it to be cloned into the Gateway-compatible pMDC32 vector in place of the double 35S promoter. A 2.6 kb *3×YFPn* gene, optimised for use in barley, was transferred into the *pTCSn1::pMDC32* vector using LR clonase II (Thermo Fisher Scientific) as described[57,58]. The resulting *pTCS::YFPn* vector was transformed into barley variety WI using *A. tumefaciens* AGL1 as described above. 18 T_0 plants were identified with ideal YFP signals. Three independent lines were crossed with the *Hvmads1*/WI lines. T_3 offspring carrying both the *Hvmads1* mutation and YFP CK sensor were used for further CK response analysis.

Plant growth and temperature treatments

Barley grains were set in cocopeat soil, germinated, and grown at 15 °C light, 10 °C dark conditions (control temperatures) with a 16 h photoperiod at 50% humidity in growth chambers (The Plant Accelerator, Waite Campus, The University of Adelaide, Australia). In all experiments, night (dark) temperature was 5 °C below the day (light) temperature. For temperature treatments of wild-type, *Hvmads1*, *Hvckx3*, *pro::HvMADS1-eGFP* and *pTCS::YFPn* plants, plants were germinated and grown at control temperatures (15 °C/10 °C, light/dark) conditions to W1 (Waddington stage)[35], and then moved to different day temperatures (20 °C, 23 °C, 25 °C, 28 °C) for phenotype or fluorescent signal investigation. For examination of dosage effects of temperature on inflorescence development (Fig. 2a–c), plants were moved back to 15 °C day temperature at W7 for spike observation, but for all other experiments, plants were grown to maturity at experimental temperature conditions.

Nicotiana benthamiana plants were grown in a greenhouse at 23 °C with a 16 h period. Plants were grown until they had six leaves, when the youngest leaves > 1 cm long were infiltrated with *Agrobacterium tumefaciens*. Transformed plants were maintained in growth chambers at 10 °C, 15 °C, 20 °C, 25 °C or 28 °C for the duration of the experiment.

Plant phenotyping and scanning electron microscopy

Inflorescence development in wild-type and *Hvmads1* spikes were photographed using a stereomicroscope with digital camera (Leica, MZ FLIII). Barley spikes were photographed using a Nikon D5600 digital camera. Different Waddington stages of immature spike tissues from various temperatures were used for scanning electron microscopy (Philips, XL30 FEG) as previously described[43], and photographs were taken with an optical microscope (Ni-E, Nikon).

Microscopy and image processing

All confocal fluorescent images were recorded with a digital camera mounted to an A1R Laser Scanning Confocal Microscope (Nikon) using a FITC (fluorescein isothiocyanate)-specific filter (EdU and eGFP, excitation 488 nm, emission 505–520 nm), a PI filter (excitation 561 nm, emission 590–640 nm), a YFP filter (excitation 514 nm, emission 520–535 nm), or a DIC (differential interference contrast) filter. Dissection of *pTCS::YFPn* inflorescences was performed as previously described[59]. Images were extracted with a NIS-Elements Viewer 4.20 (Nikon).

EdU (5-ethynyl-2′-deoxyuridine) labelling

EdU staining was performed as described[60,61], with modifications as follow (Extended Data Fig. 6a). Briefly, leaves and sheaths were removed carefully before treatment. Intact inflorescences were incubated with 10 μM EdU for 30 min in the growth chamber and fixed with 0.5 mL fixative (100% ethanol, 0.1% [v/v] Triton X-100) for 30 min at room temperature. Samples were washed with 1× PBS (3×10 min), incubated in EdU detection cocktail (Alexa Fluor Azide reaction, Life Technologies) for 30 min in the dark, and washed again with 1× PBS (3×10 min). Samples were incubated with propidium iodide (PI) solution (20 μg/mL PI in PBS) for 30 min at room temperature and washed in 1× PBS (3×10 min) before observation by confocal microscope.

RNA extraction and qRT-PCR

Total RNA was isolated from barley tissues and *N. benthamiana* leaf samples using TRIzol reagent (Life Technologies). 2 μg of total RNA was incubated with 1 U of DNaseI (Fermentas) in a total volume of 10 μL at 37 °C for 15 min. cDNA was generated using 200 U of M-MLV (Moloney murine leukaemia virus) reverse transcriptase (ThermoFisher) and 2.5 μM oligo-dT primer, according to manufacturer's instructions. Diluted cDNA was used as template for real-

time qRT-PCR with a 384-well QuantStudio Flex 6 (Thermo Fisher Scientific) machine as previously described[62]. The qRT-PCR data for each target gene are presented as average expression levels from at least three biological replicates, each with three technical replicates. Gene expression is normalised to expression levels of housekeeping genes: *HvActin7* and *HvEF2* for barley, or the *REN* gene for *N. benthamiana* leaf samples. All primers used for qRT-PCR are listed in Supplementary Table 4.

Immunoblotting of HvMADS1

Total protein from W3.5 spikes collected from three independent *pro::HvMADS1-eGFP* lines were extracted using 1× Passive Lysis buffer (Promega). Protein samples were separated by SDS–PAGE on 12% acrylamide midi-gels (Bio-Rad) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The membrane was incubated with monoclonal anti-GFP (1:1,000 dilution; ABclonal, catalogue number, AE012) or anti-tubulin (1:2,000 dilution; Merck, catalogue number, 05-661) primary antibodies and secondary antibody conjugated to horseradish peroxidase (1:5,000 dilution; Cell Signaling Technology, catalogue number, #0706) as previously described[62]. Detection was performed with the Chemidoc MP Imaging System (Bio-Rad) using SuperSignal West Pico chemiluminescent substrate (Pierce).

***In situ* mRNA hybridisation analysis**

Spikes collected at early stages were prepared for *in situ* hybridisation as previously described[63]. Probe templates of 315 bp from *HvMADS1* cDNA (205–540 bp), 302 bp from *HvCKX3* cDNA (1,113–1,415 bp) and full length CDS of *HvHistone4* were amplified by PCR using specific primers fused with the T7 promoter (primers are listed in Supplementary Table 5). Digoxigenin (DIG)-labelled antisense and sense probes were synthesised using primers incorporating the T7 polymerase binding site at the 5′ end using an *in vitro* transcription kit (Roche) according to the manufacturer's instructions. Hybridisation with 2.5 ng/μL DIG-labelled RNA probes, post-hybridisation washes and immunodetection were performed automatically using an InsituPro VSi robot (Intavis). Slides were incubated with diluted (1:1,000) antibody conjugate (anti-DIG-AP, Roche, catalogue number, 11093274910) in BSA wash solution, then washed in BSA wash solution (3×15 min). Images were obtained using an optical microscope (Ni-E, Nikon). Empty slide background was colour matched in Photoshop (Adobe) to compare between separate slides.

RNA-seq library preparation

Inflorescence samples of wild-type (GP) and *Hvmads1* plants grown at 15 °C/10 °C (day/night) and 25 °C/20 °C were collected at W2.5 and W3.5. Total RNA was extracted from 15–20 spikes for each of three biological replicates using TRIzol (Invitrogen) and purified using a RNeasy Micro Kit (Qiagen, Germany) following manufacturer's instructions. RNA quality and integrity were assessed on the Agilent 2200 TapeStation. Library preparation was performed using 1 μg of high integrity total RNA (RIN > 8) using the TruSeq RNA Library Preparation Kit v2 (Illumina, RS-122-2101 and RS-122-2001), following manufacturer's instructions. The libraries were sequenced using paired-end sequencing of 250–300 bp fragments on a HiSeq4000 at Novogene (Beijing, China).

Analysis of RNA-seq data

The quality of raw sequencing reads for all samples were examined using FastQC (version 0.11.4; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)[64]. Reads with adaptors and of low quality (> 20% bases with quality score < 15) were removed using Trimmomatic software (version 0.38)[65], and reads composed of > 5% unknown bases (labelled N) were discarded. Fragments per kilobase per million (FPKM) were normalised by genome-wide coverage. Clean reads were mapped to the barley reference genome (2017 Morex genome, http://webblast.ipk-gatersleben.de/barley_ibsc/) using HISAT2 aligner (version 2.0.0)[54,66]. Read counts per kilobase per million (RPKM) was normalised using HTSeq[67].

Analysis was conducted on 39,734 high confidence genes detected. Principal component analysis was performed using the regularised-logarithm transformation (rlog) for read count data using custom R scripts. Scatter plots were generated using $\log_2$ fold change (from 25 °C to 15 °C), and linear relationships were calculated using custom R script[7,68].

Differentially expressed genes (DEGs) were identified using the R package DESeq2 (version 3.11)[69], following the model: ~genotype × temperature × phase to account for the genotype (wild-type or *Hvmads1*), the temperature at harvesting (15 °C or 25 °C), and the developmental stage (W2.5 or W3.5), as well as their interaction. The model enabled the identification of the contribution of (1) the genotype variable, (2) the temperature variable, (3) the phase variable and (4) their interaction term; that is, whenever a change in one of the variables has a direct effect on the other. Raw data counts were normalised and transformed to estimate the mean and variance[7,67]. Results for pairwise comparisons of any two variables were extracted to investigate the effects of genes. The Benjamini-Hochberg (BH) adjustment was implemented

to compute adjusted *P* values, and false discovery rate (FDR)-adjusted *P* values were used to assess significance; a common threshold of 1% was used throughout. In total, 9,434 DEGs were identified (Dataset 1) and annotated based on BLASTx alignments against protein databases of *Arabidopsis* (TAIR10_peptide; http://www.arabidopsis.org/) and rice (MSU7_peptide; http://rice.plantbiology.msu.edu/). Clustering was performed as described (http://research.microsoft.com/apps/pubs/default.aspx?id=67239)[7], to model clusters without prior restrictions. Using the coseq Bioconductor package (version 1.0.1)[70], a Gaussian mixture model was fit to the arcsine-transformed normalised profiles of differentially expressed contigs for k = 2, …, 100 clusters. Based on the Integrated Completed Likelihood (ICL) criterion for model selection[7], the model with k = 22 clusters was selected for unsupervised assembly (Dataset 2).

Venn diagrams were created from DEGs described above. Gene ontology (GO) analysis was performed using Barley gene-to-GO associations captured by a Python script from *Arabidopsis* GO annotations (http://www.arabidopsis.org/download/index.jsp). R package '*clusterProfiler*' was applied to GO enrichment analysis for DEGs[30]. Hypergeometric tests with Yekutieli as a multi-test adjusted method were performed using the default parameters to adjust the *P* value. AgriGO2 (http://amigo.geneontology.org/visualize?mode=client_amigo) was used for the GO classification analysis and the identification of pathways of stage-specific genes[66]. A corrected false discovery rate (FDR) of < 0.05 was considered to be significantly enriched. REVIGO was applied to summarize and visualize the GO term results as treemaps (Datasets 3 and 4)[71]. Using SimRel semantic similarity measures, terms were clustered at a specified similarity cut-off and were further manually modified to clarify the meaning of representative terms at low and high temperature conditions[71]. Genes related to temperature response, meristem transition and development, phytohormone pathways, cell cycle, and cell division from DEGs for hierarchical clustering analysis were selected manually (Dataset 5) and performed using package in R[68].

Chromatin immunoprecipitation ChIP-PCR

W3.5 inflorescences (~1 g) from *pro::HvMADS1-eGFP* plants grown at 15 °C/10 °C and 25 °C/20 °C were collected and fixed in buffer (10 mM Tris-HCl pH 8, 0.4 M sucrose, 0.1 mM phenylmethanesulfonyl fluoride, 5 mM β-mercaptoethanol, 1% [v/v] formaldehyde) under vacuum for 15 min. Fixation was stopped by adding glycine to a final concentration of 125 mM for 5 min under vacuum. Samples were washed and frozen in liquid nitrogen. Chromatin immunoprecipitation experiments were performed as previously described[72], with

modifications. Briefly, each sample was ground and resuspended in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% [v/v] Triton X-100, 0.1% [w/v] deoxycholate, 0.1% [w/v] SDS, 1 mM PMSF, 10 mM sodium butyrate, 1 μg/mL aprotinin, 1 μg/mL pepstatin A) to extract nuclei. DNA was sheared into ~250–750 bp fragments by sonication. After centrifugation (20 min at 13,000 rpm), supernatants were precleared with 60 μL salmon sperm (SS) DNA/Protein A agarose (Thermo Fisher Scientific) for 1 h at 4 °C. After 2 min of centrifugation at 1,000 rpm, supernatant was transferred to a siliconised tube, to which was added 10 μL of the anti-eGFP antibody (ABclonal). After shaking incubation overnight at 4 °C, 60 μL SS DNA/Protein A agarose was added and incubation continued for 2 h. The agarose beads were collected and washed with each of the following: 2× Low Salt buffer, 2× High Salt buffer, 2× LiCl wash buffer, and 2× TE as previously described[73]. The immunocomplexes were eluted from the beads with elution buffer (1% [w/v] SDS, 0.1 M $NaHCO_3$). Sodium chloride was added to a final concentration of 0.2 M, and crosslinks were reversed by incubation at 65 °C overnight. Residual protein was degraded by the addition of 20 μg Proteinase K in 10 mM EDTA and 40 mM Tris, pH 8, at 45 °C for 1 h, followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Pellets were resuspended in 50 μL 1× TE. DNA was diluted 1:5 and approximately 1–2 μL used for quantitative PCR by a 384-well QuantStudio Flex 6 (Thermo Fisher Scientific) equipment. Each immunoprecipitation was performed at least three times, and control precipitations without antibodies (No Ab) were conducted at the same time. PCR was done as 6 independent replicates, with final relative values calculated by normalising against the fraction of input. *HvACT7* gene was used as a negative control, which is not an HvMADS1 target gene. All primers are listed in Supplementary Table 6.

Dual-luciferase assays

The dual-luciferase (Dual-LUC) method was modified from a previously described protocol using *N. benthamiana* plants[62]. Effector plasmids were prepared by cloning full-length cDNAs of *HvMADS1*, and *HvMADS3* into the *Hind*III — *BamH*I site of the pGreenII-0000 vector that contains the 35S promoter. Full-length *HvCKX3* promoter (*proHvCKX3*) and truncated *proHvCKX3ΔI-ΔVI* promoters (Extended Data Fig. 10f) were amplified from the barley genome and cloned upstream of the *LUC* reported gene in pGreenII-0800-LUC using the *Hind*III site by means of an infusion kit (Clontech). Two CArG-box promoters fused to the minimum 35S promoter were synthesised by Generay Biotech (Shanghai, China): *proCArG-wt* contained three wild-type A-tract boxes, and *proCArG-mu* had its A-tract CArG boxes

replaced by non A-tract CArG boxes. These promoters were cloned into the *Hind*III — *BamH*I site of pUC19, and then recombined into the binary vector pGreenII-0800-LUC. The sequences of the recombinant CArG-box promoters are included in Supplementary Data 1. Primer sequences for all of the constructs are listed in Supplementary Table 7. All effectors (including empty vector, pGreenII-0000) and reporter constructs were transformed into *A. tumefaciens* GV3101 cells containing the helper plasmid, pSoup-P19, which encodes a repressor of co-suppression.

Overnight *Agrobacterium* cultures were collected by centrifugation, re-suspended in MS (Murashige and Skoog) liquid medium (pH 5.8) to OD_{600} 0.6, and incubated at room temperature for 2–3 h after adding MES, pH 5.6 (2-[*N*-morpholino] ethanesulfonic acid, to a final concentration of 10 mM) and acetosyringone (to a final concentration of 200 μM). The reporter strain was either incubated with empty vector or as a mixture with the effector strain (at a reporter:effector ratio of 1:4). The mixture was infiltrated into a young *N. benthamiana* leaf, and the plants were grown for about 48 h. Leaf samples were collected for the Dual-LUC assay using commercial reagents, according to the manufacturer's instruction (Promega). Briefly, infiltrated leaf discs (~1–2 cm diameter) were excised, ground in liquid nitrogen, and homogenised in 100 μL of the 1× Passive Lysis buffer (Promega). 20 μL of the crude extract was mixed with 100 μL of Luciferase Assay buffer (Promega), and the LUC activity was measured. LUC was quenched and the REN reaction initiated by the addition of 100 μL Stop and Glow buffer (Promega), using a refurbished GloMax-96 Microplate Luminometer (Promega). At least five biological repeats were measured for each sample. The LUC/REN activity obtained from a co-transfection with an empty effector and reporter construct was set to one for normalisation.

EMSA (electrophoretic mobility shift assay)

Selected promoter regions of *HvCKX3* were amplified by PCR from barley genomic DNA and artificial *CArG-wt/mu* oligonucleotides were amplified by PCR from Dual-LUC reporter vectors as mentioned above, using specific primer pairs combined with a universal sequence 5′-AGCCAGTGGCGATAAG-3′. DNA fragments were purified by a DNA purification kit (Thermo Fisher Scientific), and labelled via PCR using the universal primer sequence containing Cy5 at the 5′-end (Generay Biotech, Shanghai, China). The PCR conditions for Cy5 labelling were 94 °C / 3 min; 35 cycles of 94 °C / 25 s, 55 °C / 25 s and 72 °C / 30 s; and an extension at 72 °C / 5 min.

The CDS of *HvMADS1* was fused with a T7 promoter sequence (5'–TAATACGACTCACTATAGG–3') by PCR, which were used for protein translation. Proteins were synthesised using TNT T7 Quick Coupled Transcription/Translation System (Promega) according to manufacturer's instructions in a total volume of 10 μL. The binding reaction mixture was prepared as described previously[74], and contained 1.2 mM EDTA pH 8.0, 0.25 mg/mL BSA, 7.2 mM HEPES pH 7.3, 0.7 mM DTT, 60 μg/mL SS DNA, 1.3 mM spermidine, 2.5% (v/v) CHAPS, 8% (v/v) glycerol, 5 nmol/mL Cy5-labeled DNA, and 3 μL of *in vitro* synthesised protein. Protein-DNA binding was performed at 4 °C, 15 °C, 25 °C and 30 °C for 30 min before loading on a 5% polyacrylamide gel. Electrophoresis was performed at low voltage (75 V/6.8 cm gel) to avoid temperature changes. DNA bands were visualised by fluorescence imaging using the Cy5 channel of ChemiDoc MP imaging system (Bio-Rad). All primers used for EMSA are listed in Supplementary Table 8.

Co-immunoprecipitation

Co-immunoprecipitation analysis was performed with extracts from 4-week-old tobacco leaves, as previously described[62]. To create the Flag-tagged and HA-tagged HvMADS1 constructs for *in vivo* protein expression, the full-length coding region of HvMADS1 fused with 3× Flag tag or 6× HA tag was cloned into the *Hind*III — *BamH*I site of the pGreenII-0000 vector that contains the 35S promoter (primers are listed in Supplementary Table 9). The fusion proteins HvMADS1-Flag and HvMADS1-HA were transiently expressed in tobacco leaves as described above (Dual-luciferase assays). Proteins were extracted with ice-cold buffer containing 20 mM HEPES-KOH at pH 7.5, 40 mM KCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM PMSF, 10 mM sodium butyrate, 1 μg/mL aprotinin, and 1 μg/mL pepstatin A. After centrifugation at 16,000 rpm for 10 min, the supernatant was incubated with anti-HA antibody (Abcam, catalogue number, ab137838) and IgG-bound to Protein A Sepharose beads (Thermo Fisher Scientific) for 2 h at 4 °C, and the beads were washed five times with wash buffer (20 mM Hepes-KOH at pH 7.5, 40 mM KCl, 0.1% [v/v] Triton X-100). Proteins were eluted by boiling the beads in 2× SDS sample buffer and separated on SDS-PAGE before immunoblotting using anti-Flag (1:1,000 dilution, ThermoFisher, catalogue number, MA1-91878) or anti-HA (1:1,000 dilution) antibodies.

Cytokinin treatment and endogenous cytokinin measurement

CK treatments of barley inflorescence were performed using a modified method as previously described[39]. Wild-type and *Hvmads1* plants grown at low and high temperatures were injected

with 0 mM (mock), 1 mM and 5 mM benzylaminopurine (BAP, Sigma). The treatments were applied every two days starting from spike Waddington stage W1 (2–3 leaf stage) and stopped at stage W5 (6 leaf stage)[35].

For endogenous CK level measurement, three replicates of 200–300 mg W3.5 spikes were collected from wild-type and *Hvmads1* plants grown at low and high temperatures. CKs were extracted and measured as previously described[75,76]. Briefly, fresh plant tissues were frozen in liquid nitrogen and homogenised to fine powder using a ball mill Retsch MM 400 (Retsch, Newtown, PA, USA) at a frequency of 30 Hz for 1 min. The ground powder was extracted for 24 h in solvent (15:4:4 methanol:water:formic acid, v/v/v) with 400 pg of internal standards (iP, iPR, IP9G, tZ, tZR, tZ9G and tZOG). Crude extracts were further purified by loading onto the Oasis MCX cartridge (500 mg/6 mL; Waters, Milford, MA, USA) preconditioned with solvent. The cartridge was sequentially washed with formic acid/methanol solution. Fractions containing CK nucleobases, nucleosides and glucosides were eluted using ammonia/methanol solutions, and analysed on a LC–tandem MS/MS comprising an Acquity UPLC (Waters) and Qtrap 5500 system (AB Sciex, Shinagawa-ku, Tokyo, Japan) equipped with Electron Spray Ionisation source as described[75]. All active cytokinins, nucleobases, nucleosides, and glucosides were measured at the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (Beijing, China). Endogenous concentrations of CK were calculated as previously described[76].

Variation of HvMADS1

For SNP analysis of *HvMADS1*, the exome-sequencing data of 276 barley varieties was analysed (https://www.ebi.ac.uk/ena/data/view/PRJEB8044)[50]. Morex_contig_202661 containing the *HvMADS1* gene was used for SNP investigation compared with the reference genome (Dataset 6)[54]. SNP calling was performed manually by visual inspection of sequences. 101 barley varieties with diverse inflorescence architectures (Dataset 7) were grown in a growth chamber. The CDS region from and the first intron of *HvMADS1* were amplified from spike cDNA and genomic DNA by PCR using a Phire Plant Direct PCR Kit (Thermo Fisher Scientific) and sequenced by Sanger sequencing (AGRF, Australia), respectively. All primers used for *HvMADS1* SNP sequencing are listed in Supplementary Table 10.

Quantification and statistical analysis

All experiments were conducted with technical and biological replicates at an appropriate sample size estimated based on our previous experience. No statistical methods were used to

predetermine sample size. The experiments were not randomised, and investigators were not blinded to allocation during experiments and outcome assessment.

For quantification of EMSA band intensity, multiple exposures of Cy5 channel with different times were performed to avoid signal saturation, and a mildly exposed image was always selected for signal quantification with ImageJ. A constant-sized rectangle was drawn in ImageJ to enclose the band and the intensity inside it was measured. For each gel lane, the measured values were normalised to the average intensity of all the measurements, to remove systematic variability.

All experiments were replicated independently at least once, as indicated in each figure. Dot plots were routinely used to show individual data points for each experimental observation, and bar graphs contain individual data points for each experimental replicate. Statistical analyses of all box plots and bar graphs were performed using GraphPad Prism 8 or Microsoft Excel. One- or two-way analysis of variance (ANOVA) was used to evaluate significant variations between genotypes or temperatures, as appropriate, using GraphPad Prism 8. Tukey *post hoc* test was used to assess the statistical difference in comparisons after a one- or two-way ANOVA. Values of $P < 0.05$ were considered statistically significant. Details about the statistical approaches used can be found in the figures or figure legends. The data are presented as mean ± s.d., '*n*' represents the number of sample size.

Data Availability

The raw data files for the RNA-seq analysis reported in this paper have been deposited in the GEO database (accession no. GSE156526). The data supporting the findings of this study are available within the paper and its Supplementary Information files. Source gel data Fig. 5 and Extended Data Fig. 7 are provided in Supplementary Fig. 1; source data (graphs) for Figs. 1–3, 5–7 and Extended Data Figs. 3–7, 9, 10 are also provided and are available with the online version of the paper. Additional data, such a raw image files, that support the findings of this study, are available from the corresponding author upon request.

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Acknowledgments

We thank Dr Ludovico Dreni (Instituto de Biología Molecular y Celular de Plantas, Spain), Prof. Martin Kater (University of Milan, Italy) and Prof. Malcolm Bennett (The University of Nottingham, UK) for the discussion on Australian Research Council Discovery Project (DP170103352); Prof. Yaoguang Liu (South China Agricultural University) for providing vectors pYLsgRNA-OsU6a, pYLsgRNA-OsU6b and pYLCRISPR/Cas9P$_{ubi}$-H for CRISPR/Cas9 editing. We thank Dr Jinfang Chu (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China) for help with CK measurements, Dr Gwenda Mayo (Adelaide Microscopy) for assistance in the microscopy work, A/Prof. Dan Peet (The University of Adelaide) for guidance with dual-luciferase measurement, Ms Ann Chieng and Ms Hui Zhou (The University of Adelaide) for managing the plant materials, and Prof. George Coupland (Max Planck Institute for Plant Breeding Research, Germany), Prof. Elizabeth A. Kellogg (Donald Danforth Plant Science Center, USA), Prof. David Smyth (Monash University, Australia), Prof. Christine Beveridge (University of Queensland, Australia), A/Prof. Mary Byrne (The University of Sydney, Australia), and Dr Scott Boden (The University of Adelaide, Australia) for comments on the manuscript. This work was financially supported by the Australian Research Council (DP170103352); an Australia-China Science and Research Fund Joint Research Centre grant ACSRF48187; the Waite Research Institute (WRI) of the University of Adelaide; the National Key Technologies Research and Development Program of China, Ministry of Science and Technology (grant nos.

2016YFD0100804 and 2016YFE0101000) and the National Natural Science Foundation of China (grant nos. 31970803 and 31861163002) and the Science and Research grant of Southwest University of Science and Technology (19zx7146).

Author Contributions

D.Z. and R.B. initiated the project; G.L. and D.Z. conceived the project and designed the experiments. G.L. carried out most of the molecular and regulatory experiments; G.L. and H.K created the barley *sepallata* mutant lines; H.K. and X.Y. conducted scanning electron microscopy work, EdU labelling and RNA *in situ* hybridisation; X.Y. carried out *pro::HvMADS1-eGFP* transformation; H.L. and J.S analysed RNA-seq data under the guidance of G.L. and W.L.; X.Y., C.S. and M.T. conducted the cytokinin biosensor line and BAP treatment; G.L., H.K., X.Y., M.T., R.W., R.B. and D.Z. analysed the results and discussion; G.L., N.B. and D.Z. wrote the manuscript with input from all authors.

Declaration of Interests

The authors declare no competing interests.

Supplemental Information

Supplementary Dataset 1 DEGs across temperature, genotype, and developmental phase. (Excel format)

Supplementary Dataset 2 Co-expression clusters of DEGs from RNA-seq of 8 barley spike samples. (Excel format)

Supplementary Dataset 3 GO analysis of DEGs in wild-type and *Hvmads1* spikes at 15 °C. (Excel format)

Supplementary Dataset 4 GO analysis of DEGs in wild-type and *Hvmads1* spikes at 25 °C. (Excel format)

Supplementary Dataset 5 Curated list for genes of inflorescence development, temperature response, cell cycle/division and plant hormone pathways. (Excel format)

Supplementary Dataset 6 SNPs of *HvMADS1* exons in 267 barley varieties. (Excel format)

Supplementary Dataset 7 *HvMADS1* sequence variation in barley varieties. (Excel format)

Supplementary Data 1 Synthetic CArG-box promoter sequence.

Supplementary Figure 1 Source gel data of this study.

Supplementary Table 1 Gene editing efficiency of *SEPALLATA* genes in three barley varieties.

Supplementary Table 2 Primers used for CRISPR/Cas9 constructs.

Supplementary Table 3 Primers used for the *pro::HvMADS1-eGFP* construct.

Supplementary Table 4 Primers were used for qRT-PCR.

Supplementary Table 5 Primers used for *in situ* hybridisation.

Supplementary Table 6 Primers used for ChIP-PCR.

Supplementary Table 7 Primers used for dual-luciferase assays.

Supplementary Table 8 Primers used for EMSA.

Supplementary Table 9 Primers used for co-immunoprecipitation.

Supplementary Table 10 Primers used for *HvMADS1* variational sequencing.

Main Figures and Legends

Fig. 1 HvMADS1 regulates inflorescence plasticity in response to high temperature. **a**, Inflorescence phenotypes of wild-type (WT) plants and *hvm1* (*Hvmads1*) mutants in Golden Promise (GP), Vlamingh (Vla) and WI4330 (WI) backgrounds, under control (15 °C/10 °C, day/night) and heat stress (28 °C/23 °C, day/night) conditions. Red arrows indicate the ectopic organs. Bars = 1 cm. **b**, High temperature conditions induce formation of ectopic spikelets (ES) and ectopic inflorescences (EI) in *hvm1*, but not in WT (GP), spikes. CS, central spikelet. LS, lateral spikelet. Bars = 1 cm. The schematic of *hvm1* inflorescence architecture shows ES (blue) and EI (red). **c**, Morphology of a central spikelet (CS) and lateral spikelet (LS) in WT and *hvm1*, and a CS and LS from ectopic spikelet (ECS and ELS) in *hvm1* at 28 °C. gl, glume; ra, rachilla (red boundary); le, lemma; pa, palea; st, stamen; ca, carpel. Blue cycle indicates the initiated position of ES and EI. Bars = 0.2 cm. **d**, Phenotype of an ectopic inflorescence (EI) and its floret organ from *hvm1* at 28 °C. s-EI, secondary EI branch. Bars = 0.2 cm. **e**, The average number of ectopic organs (ES and EI) per spike in *hvm1* mutants (three genotypic backgrounds) at three temperatures. Data shown as mean ± s.d. (standard deviation). *P* values indicate results from indicated pairwise comparisons of one-way ANOVA tests. **f**, The proportion of EI:ES produced in response to high ambient temperatures. *P* values indicate results from indicated pairwise comparisons of two-way ANOVA tests. All experiments with treatment were repeated four times with similar results.

Fig. 2 High ambient temperatures induce branching events in *Hvmads1*. a, Temperature treatment programs. W1 and W7 indicate Waddington stages of spike development (Waddington et al., 1983). **b**, Dosage-dependent high ambient temperatures induce ectopic organs (ES and EI) in *hvm1*, but not in WT (GP), spikes. Bars = 1 cm. Schematic of *hvm1* inflorescence architecture indicate ES (blue) and EI (red). **c**, Quantification of branching events of *hvm1* spikes (three genetic backgrounds) at high temperature conditions. **d**, EI branches of the *hvm1* (GP) at high temperatures. Bar = 1 cm. **e**, Average spikelet number per EI at higher temperatures. Data shown as mean ± s.d. **f**,**g**, Scanning electron microscopy of WT (GP) (**f**) and *hvm1* (**g**) spike morphology at 28 °C. cs, central spikelet; ls, lateral spikelet; gl, glume; st, stamen; pi, pistil; le, lemma; pink shading indicates cs, blue shading indicates ls, green shading and green asterisks indicate the indeterminate inflorescence meristem possibly converted from the central spikelet meristems, yellow shading and yellow asterisks indicate the ectopic initiated meristems or inflorescence/spikelet meristems which may be reverted from rachilla. EI/ES meristems. Bars = 200 µm. All experiments were repeated three times independently with similar phenotype.

Fig. 3 HvMADS1 represses ectopic cell division activity of meristems at high temperature. **a**, Cell division activity in WT and *hvm1* spikes W3 and W3.5 at 25 °C. EdU, 5-ethynyl-2'-deoxyuridine (green signals); PI, propidium iodide (red signals). White arrows indicate high levels of cell division activity in ES/EI meristems. Bars = 100 µm. **b**, *In situ* hybridisation showing expression of the cell division gene, *HvHistone4*, in WT and *hvm1* spikes at 15 °C, 25 °C and 28 °C. Bars = 100 µm. **c**, Relative *HvMADS1* mRNA level (qRT-PCR) in different organs and stages of spike development in WT (GP) compared with control genes *HvActin7* and *HvEF2*. Data shown as mean ± s.d., *n* = 3. **d**, *In situ* hybridisation of *HvMADS1* in longitudinal sections of developing WT spikes at stages W2.5–W4. The sense probe served as a negative control. Bars = 100 µm. **e**,**f**, Accumulation of the HvMADS1 protein in spikes from W2.25 (triple mound stage) to W3.5 (**e**), and developing spikelet at W5 (**f**) in *pro::HvMADS1-eGFP* transgenic lines at 15 °C. IM, inflorescence meristem; BF, bright field. Bars = 100 µm. **g**, Accumulation of HvMADS1 protein in W3.5 *pro::HvMADS1-eGFP* transgenic spikes grown at 15 °C and 25 °C. Bars = 100 µm. The experiments in **a–d** were repeated three times independently with similar results.

Fig. 4 HvMADS1 coordinates thermal transcriptome programming of inflorescence meristems. a, Principal component analysis (PCA) on the expression-filtered transcriptomes from W2.5 and W3.5 spikes of WT (GP) and *hvm1* plants grown at 15 °C and 25 °C. **b**, Correlation analysis showing mis-regulation of thermal transcripts in *hvm1* compared with WT spikes at 25 °C at both W2.5 and W3.5 stages. **c**, Overlap of 9,434 DEGs (differentially expressed genes) due to temperature, genotype and developmental phase. Blue shading indicates genes co-regulated by genotype and temperature. **d**, Gene ontology (GO) analysis of DEGs between WT and *hvm1* spikes at 15 °C and 25 °C. **e**,**f**, Hierarchical clustering analysis of DEGs that are relevant to cell cycle (**e**), and plant hormone signalling (**f**). *GH3*, *Gretchen Hagen3*; *IAA*, *Aux/IAA*; *ARF*, *Auxin Response Factor*; *GA20OX*, *GA20 oxidases*; *GA2OX*, *GA2 oxidases*; *GID*, *Gibberellin-insensitive Dwarf*; *GRAS*, *GRAS-domain transcription factor*; *SHI*, *Short Internodes*; *CKX*, *Cytokinin Oxidase/dehydrogenase*; *ZOG*, *CK O-glucosides*; *ZNG*, *CK N-glucosides*; *AHK*, *Histidine-kinase receptor*; *AHP*, *Histidine Phosphotransfer protein*; *PRR*, *Pseudo-response Regulator*. Three biological repeats were performed for transcriptome.

Fig. 5 HvMADS1 binds to the CArG-box to regulate gene transcription in response to temperature. a, Artificial CArG-boxes with A-tracts (underlined, *CArG-wt*, wild-type) or non-A-tracts (red, *CArG-mu*, mutant) used to drive luciferase (*LUC*) gene expression. **b,** Normalised luciferase activity (LUC/REN) activated by artificial CArG-box promoters [from (**a**)] in the presence of HvMADS1, HvMADS3 (temperature-independent gene activation), or EV (empty vector, negative control). REN, Renilla luciferase (internal control). Data shown as mean ± s.d., n = 5. **c**, qRT-PCR analysis of reporter gene *LUC* expression from (**a**) and (**b**). Values normalised to *REN* expression. Data shown as mean ± s.d., n = 3. **d**, EMSA of HvMADS1 with DNA fragments containing *CArG-wt* and *CArG-mu* boxes [from (**a**)] at different temperatures. Homodimeric (blue arrows) and monomeric (orange arrows) HvMADS1 protein–DNA complexes are indicated. Quantification of band intensity is shown on each gel. **e**, *In vivo* co-immunoprecipitation assay showing the homodimers of HvMADS1. Tobacco leaves extracts that transiently expressed HvMADS1-Flag (tag) with HvMADS1-HA or with empty vector (EV, negative control) were immunoprecipitated by anti-HA antibody. WB, western blot. IP, immunoprecipitation. **f**, ChIP-PCR assays of regulatory regions of four selected genes from *pro::HvMADS1-eGFP* transgenic plants grown at 15 °C and 25 °C. The promoter or intron regions containing A-tract (blue text) and non A-tract (black text) CArG-boxes of *HvPIF4* (*Phytochrome-Interacting Factor*), *HvRPK4* (*Receptor-like Protein Kinase 4*), *HvTFL1L* (*TERMINAL FLOWER 1-like*), and *HvTB1L* (*TEOSINTE BRANCHED 1-like*) are indicated. Data shown as mean ± s.d., n = 6. No antibody (No Ab) served as negative control. P values indicate results from indicated pairwise comparisons of one-way ANOVA tests (**b**, **c**, **f**). All experiments were repeated independently at least three times with similar results.

Fig. 6 HvMADS1 integrates cytokinin signalling and temperature response to regulate barley inflorescence branching. a, RPKM ratio for cytokinin two-component signalling genes (8 type A *RRs* and 7 type B *RRs*) in W3.5 spikes. A ratio > 1 indicates upregulated expression in the *hvm1* mutant, n = 3. **b**, WT (GP) and *hvm1* spikes after benzylaminopurine (BAP) treatments at 15 °C. Red asterisks indicate EI. Bars = 1 mm. **c**, Phenotype of WT (WI) and *hvm1* spikes after BAP treatment at 15 °C. Arrows indicate EI. Bars = 1 mm. **d**, Phenotype of WT (GP) and *hvm1* spikes after BAP treatment at 28 °C. ES (yellow arrows) attached to the lemma are observed in WT and *hvm1* spikes, but EI (red arrows) attached to the palea are only detected in *hvm1* spikes. WT lateral spikelets under mock treatment have been removed. Bars = 2 mm. **e**, Average number of EI (*upper*) and ES (*lower*) per spike after BAP treatment. Data shown as mean ± s.d., n = 31–39 individual spikes of each set. **f**, Quantification of endogenous cytokinin content in W3.5 spikes. iP, isopentenyladenine; iPR, isopentenyladenine riboside; iP9G, isopentenyladenine 9-N-glucoside; tZ, trans-zeatin; tZR, trans-zeatin riboside; tZ9G,

trans-zeatin 9-N-glucoside; tZOG, trans-zeatin O-glucoside. Data shown as mean ± s.d., *n* = 3. *P* values indicate results from indicated pairwise comparisons of one- and two-way ANOVA tests. **g**, WT (WI) and *hvm1* spikes expressing *pTCS::YFP(n)* (cytokinin biosensor, yellow signals). Heat treatment at 28 °C was for 7 days. Green circles indicate ectopic signals. Bars = 100 μm. All experiments were repeated independently at least three times with similar results.

Fig. 7 HvMADS1 directs *HvCKX3* to regulate spike determinacy under high temperatures. a, *In vivo* binding of *HvCKX3* CArG-boxes by HvMADS1 at 15 °C and 25 °C. *Upper*, *HvCKX3* genomic region containing A-tract (blue) and non-A-tract (black text) CArG-boxes. *Lower*, seven DNA fragments tested by ChIP-PCR. Data shown as mean ± s.d., *n* = 6. No antibody (Ab), negative control. *P* values indicate results from indicated pairwise comparisons of one-way ANOVA tests. **b**, EMSA assays of HvMADS1 with *HvCKX3* promoter fragments containing A-tract (P4) and non-A-tract (P1) CArG-boxes at various temperatures. Homodimeric (blue) and monomeric (orange) protein–DNA complexes are indicated. Quantification of band intensity is shown on each gel. **c**, Normalised luciferase activity (LUC/REN) activated by the *HvCKX3* promoter in tobacco cells in the presence of HvMADS1 or empty vector (EV, negative control). Data shown as mean ± s.d., *n* = 5. *P* values indicate results from indicated pairwise comparisons of one-way ANOVA tests. **d**, *HvCKX3* transcript levels in WT (GP) and *hvm1* spikes at 15 °C and 25 °C. Data shown as mean ± s.d., *n* = 3. **e**, *In situ* hybridisation of *HvCKX3* in wild-type and *Hvmads1* spikes at 15 °C and 25 °C. Bars = 100 μm. Blue arrows indicate mRNA accumulation at the base of the CSM. **f**, Creation of the *Hvckx3* mutant using CRISPR/Cas9. *Upper*, Two targets (T1 and T2) in the first exon of *HvCKX3*. *Lower*, DNA sequences and putative encoded amino acid sequences of three independent T_0 transgenics in WT (GP). **g**, Phenotype of *Hvckx3* spikes at stages W2.5, W6 and W7 in response to high temperatures. Red circles indicate abnormal differentiation of spikelet meristems. Red arrows indicate EI. Bars = 200 μm. **h**, Proposed model of HvMADS1-mediated spike determinacy maintenance at high temperatures. *HvCKX3* expression is activated by HvMADS1 to drive cytokinin homeostasis that stabilizes meristem determinacy. In *hvm1* spikes, lack of *HvCKX3* activation causes enhanced cytokinin response and reduced meristem determinacy, triggering ectopic branching. IM, inflorescence meristem. SM, spikelet meristem. All experiments were repeated independently at least three times with similar results.

Extended Data Figures and Legends

Extended Data Fig. 1 Creation of barley *sep* mutants using CRISPR/Cas9. a, The gene structure of *HvMADS1* and positions of two sgRNA targets (T1 and T2) for CRISPR/Cas9 editing in the MADS-box domain. Blue rectangles indicate exons of *HvMADS1*. **b**, DNA sequences of independent T_0 transgenics of *Hvmads1* (*hvm1*) mutants in GP, WI, and Vla backgrounds, and *hvm1/5* and *hvm1/34* double mutants in GP, carrying putative *HvMADS1* biallelic and homozygous mutations. WT, wild-type. **c**, The putative amino acid sequences encoding HvMADS1 of *hvm1* single mutant, and *hvm1/5* and *hvm1/34* double mutants [from (**b**)]. Asterisks indicate a stop codon. **d**,**e**, Genotypes of three independent lines of two sgRNA targets of *HvMADS5* (**d**) and *HvMADS34* (**e**) in *hvm5* and *hvm34* single mutants, and *hvm1/5*, *hvm1/34* and *hvm5/34* double mutants that were used for CRISPR/Cas9 editing, respectively.

Extended Data Fig. 2 Spike phenotypes of *sep* single and double mutants under control and heat stress conditions. Images represent spike architecture of barley WT (GP), *hvm1*, *hvm5*, *hvm34* single mutants, and *hvm1/5*, *hvm1/34*, *hvm5/34* double mutants at 15 °C and 28 °C. Red arrows indicate the ectopic organs. Bars = 2 cm, bars in enlarged regions are 1 cm.

Extended Data Fig. 3 Spikelet phenotype of *Hvmads1* mutant under normal temperature. a, The awn phenotype of *hvm1* central spikelet in GP, Vla and WI backgrounds at 15 °C. Yellow asterisks indicate awn length. Bars = 1 cm. **b**, Average awn length in *hvm1* and WT plants. Data shown as mean ± s.d., n = 79–106 individual spikes of each set. P values indicate results from indicated pairwise comparisons of one-way ANOVA tests. **c**, Floret organ (lemma, palea, stamen and pistil) phenotype in the WT (GP) and *hvm1* plants at 15 °C. CS, central spikelet; LS, lateral spikelet; le, lemma; pa, palea; gl, glume; st, stamen; ca, carpel; lo, lodicule. Bars = 1 mm.

Extended Data Fig. 4 High ambient temperature induces the production of ectopic organs in *Hvmads1* inflorescences. a, WT (GP) inflorescence architecture at W9 at 28 °C. Bar = 0.5 cm. **b**, The developing *hvm1* inflorescence from W5–9 at 28 °C. Red arrows indicate ectopic organs. Bars = 0.5 cm. **c**, The *hvm1* heading spike at 28 °C. Red arrows indicate ectopic organs. Bar = 0.5 cm. **d**–**f**, The ES (ectopic spikelet) (**d**) and EI (ectopic inflorescence) (**e**,**f**) of the *hvm1* spike grown at 28 °C. CS, central spikelet. Bars = 0.2 cm. **g**, The frequency of ES and EI in *hvm1* spike sections (basal, central and apical) at different temperatures. Data shown as mean ± s.d., n = 40–47 individual spikes of each temperature set. **h**, The average ES and EI number per *hvm1* spike at five temperature conditions. Data shown as mean ± s.d., n = 46–81 individual spikes of each set. **i**, Total spikelet numbers, including spikelet from ES/EI, per WT or *hvm1* spike at W7 at different temperatures. Data shown as mean ± s.d., n = 79–85 individual spikes of each set. P values indicate results from indicated pairwise comparisons of one-way ANOVA tests. **j**,**k**, The ES and EI induced by high ambient temperatures in *hvm1* mutants of Vla (**j**) and WI (**k**) backgrounds. Bars = 0.5 cm. **l**,**m**, short (**l**) and elongated (**m**) EI branches with different spikelet morphology. s-EI, secondary EI branch; LS, lateral spikelet; le, lemma; pa, palea; st, stamen; ca, carpel; gl, glume. Bars = 0.5 cm. **n**, The frequency of short and elongated EI phenotype in *hvm1* mutants at different temperatures. **o**, Mature spike of *hvm1* (GP) mutant after treatment at high temperatures. Red arrows indicate fertile spikelets from EI or ES. Bar = 1 cm. **p**, Spikelet fertility rate of EI induced by high temperatures in *hvm1* (GP) spikes. Data shown as mean ± s.d., n = 28–32 individual EI of each temperature set. **q**, Fertility rate of ES in *hvm1* mutants of three backgrounds at 23 °C and 28 °C. Data shown as mean ± s.d., n = 39–50 individual spikes of each set. All individual biological experiments were repeated at least three times with similar results.

Extended Data Fig. 5 Loss of *Hvmads1* leads to reduced meristem determinacy and delayed inflorescence development under high temperature. a,**b**, Scanning electron microscopy of spike morphology at W2.5, W3.5 and W7 in WT (GP) (**a**) and *hvm1* (**b**) plants at 15 °C, showing the short awn in *hvm1*. **c**, Morphology of the developing WT spike at 28 °C. **d**, Reduced meristem determinacy of *hvm1* inflorescences at 28 °C. Green asterisks indicate the indeterminate inflorescence meristem likely converted from the central spikelet meristems, yellow asterisks indicate the ectopic initiated meristems or inflorescence/spikelet meristems possibly reverted from rachilla. **e**, Effects of ambient high temperatures 20 °C, 23 °C and 25 °C on morphology of *hvm1* spike. Yellow shading indicates EI, blue shading indicates ES. All bars (**a**–**e**) = 100 μm. fm, floral meristem; ls, lateral spikelet; cs, central spikelet; gl, glume; st, stamen; pi, pistil; le, lemma; esm, ectopic spikelet meristem; eim, ectopic inflorescence meristem. **f**, Rate of spike development at different temperatures, showing delay in *hvm1* (GP) at 28 °C, compared with WT, spike development. Bars = 0.5 mm. **g**, Days to reach different Waddington stages of spike development at 15 °C and 28 °C in three barley varieties and related *hvm1* mutants. Data shown as mean ± s.d., n = 31–42 individual meristems of each set. $P < 0.001$, two-way ANOVA tests of WT and *hvm1* (three background comparisons) at 28 °C. All experiments with treatment were repeated independently at least three times with similar results.

Extended Data Fig. 6 HvMADS1 represses cell division in the spike in response to high temperature. a, Indicative method of EdU (5-ethynyl-2'-deoxyuridine) tracking in barley spike. PI, propidium iodide. **b**,**c**, EdU tracking of cell division activities in WT (GP) and *hvm1* spikes (W2.5 and W3–3.5) grown at 15 °C (**b**) and 25 °C (**c**). White arrows indicate ectopic EdU clusters in non-floret meristem regions of the central spikelet, which shows high levels of cell division activity in ectopic meristems of *hvm1* plants. Numbers of ectopic clusters represent the average observed additional EdU signal clusters in non-floret meristem regions per spike (W3–3.5). Also see the Source Data. Bars = 100 μm. The pictures of EdU tracking assays represent one of three experiments performed independently with similar results.

Extended Data Fig. 7 Temperature does not alter HvMADS1 mRNA expression or protein accumulation. a, Accumulation of the HvMADS1 protein in flower organs, including lemma, palea, anther, and lodicule, in *pro::HvMADS1-eGFP* transgenic lines at 15 °C. BF, bright field. Bars = 100 μm. **b**, qRT-PCR analysis of *HvMADS1* expression in W2.5 and W3.5 WT spikes at different temperatures. A temperature-responsive gene (*HB, homeobox)* served as the positive control. Data shown as mean ± s.d., n = 3. **c**, RT-PCR analysis of *HvMADS1* expression in WT spikes (W3.5). *HvActin7* served as the control. **d**, Immunoblot analysis of HvMADS1-eGFP protein in W3.5 spikes from three independent *pro::HvMADS1-eGFP* transgenic lines in response to temperatures. Tubulin served as a loading control. All experiments were repeated independently at least three times with similar results.

Extended Data Fig. 8 HvMADS1 regulates the transcriptome of barley inflorescence in response to temperature. a, Correlation analysis of transcripts showing mis-regulation of spike developmental genes in *hvm1* plants at 15 °C (left) and, more obviously, at 25 °C (right). **b**, Venn diagram showing the number of DEGs affected by genotype and temperature at two developmental stages. **c**, Co-expression clustering of all DEGs in eight transcriptomes (W2.5 and W3.5 of WT and *hvm1* spikes at 15 °C and 25 °C). Clustering was performed on the expression-filtered data set using a Gaussian mixture model. The number of clusters was assumed to be random and was automatically learned using an empirical Bayes approach (variational Bayesian inference). **d**, DEG clusters in response to temperature, developmental phase and *HvMADS1* genotype in inflorescence meristems. Z-score represents variation in gene expression that is likely to be regulated by the interaction between phase × temperature ×

genotype, temperature × genotype, or only *HvMADS1* genotype for selected clusters. Three biological repeats were performed for transcriptome.

Extended Data Fig. 9 Effects of *Hvmads1* mutation on the expression of key regulators in response to temperature. a, Heat map showing DEGs relevant to spike development (left) and temperature response (right). **b**, qRT-PCR analysis of selected genes related to inflorescence meristem identity in W3.5 WT (GP) and *hvm1* spikes at 15 °C and 25 °C. *OSH1*, *ORYZA SATIVA HOMEOBOX1*; *AP1*, *APETALA 1*; *VRN*, *VERNALIZATION*; *TFL1*, *TERMINAL FLOWER 1*; *TB1*, *TEOSINTE BRANCHED 1*; *TAW1*, *TAWAWA1*; *RPK4*, *RECEPTOR-LIKE PROTEIN KINASE 4*; *IDS1*, *INDETERMINATE SPIKELET 1*. **c**, qRT-PCR analysis of selected genes known to regulate barley spike development, spikelet identity and row-type in W3.5 WT and *hvm1* spikes at 15 °C and 25 °C. *VRS*, *SIX-ROWED SPIKE*; *COM2*, *COMPOSITUM 2*. **d**, qRT-PCR analysis of selected genes related to temperature response in W3.5 WT and *hvm1* spikes at 15 °C and 25 °C. *PIF4*, *PHYTOCHROME-INTERACTING FACTOR 4*; *ER*, *ERECTA*; *TT1*, *THERMO-TOLERANCE 1*. *HvActin7* and *HvEF2* were used for normalisation. Data shown as mean ± s.d., $n = 3$. *P* values indicate results from indicated pairwise comparisons of one-way ANOVA tests (**b**–**d**).

Extended Data Fig. 10 HvMADS1 promotes the activity of the *HvCKX3* promoter in a temperature-dependent manner. Truncated *HvCKX3* promoter fragments containing 0, 1, 2 or 3 CArG-boxes were fused to the *LUC* reporter gene, and co-transformed with effector plasmids of EV (empty vector) and *35S::HvMADS1* into tobacco cells. Normalised LUC/REN activity is shown as mean ± s.d., $n = 5$. *P* values indicate results from indicated pairwise comparisons of one-way ANOVA tests.

Extended Data Fig. 11 Variation of *HvMADS1*. Relative positions and effect of the 4 SNPs identified in *HvMADS1* exons using morex_contig_202661 exome sequencing in 267 barley varieties[50]. Exon_pos, exon position; CDS_pos, coding DNA position; Protein_pos, protein position, Exome-seq, exome sequencing.