Cell Biology International ISSN 1065-6995
doi: 10.1002/cbin.10805

RESEARCH ARTICLE

Cold interferes with male meiotic cytokinesis in *Arabidopsis thaliana* independently of the AHK2/3-AHP2/3/5 cytokinin signaling module

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Abstract

Previously we have shown that low temperature stress in Arabidopsis causes defects in microtubule organization and cytokinesis in male meiocytes, which leads to the formation of diploid pollen. Because cytokinin (CK) mediates multiple physiological responses to cold stress, we investigated whether CK signaling is involved in cold-induced diploid pollen formation. To this end, we monitored male sporogenesis in a series of mutants defective in CK metabolism and signalling. Arabidopsis plants with altered CK homeostasis, that is, the *ahk2-2 ahk3-3* double and the *ahp2-1 ahp3 ahp5-2* triple mutant, were cold sensitive and displayed similar defective male meiotic cytokinesis as wild type plants upon cold stress. These findings demonstrate that the AHK2/3-AHP2/3/5 CK-signaling module is not required for cold-induced ploidy stability of male gamete in Arabidopsis. Cytological analysis further revealed that the cold-induced cytokinesis defects in the *ahk2-2 ahk3-3* mutant correlated with irregular organization of the radial microtubule array (RMA) in tetrad microspores at the end of male meiosis. Contrary to the *ahk* and *ahp* mutants, Arabidopsis plants defective for ARR1, a downstream target of *ahk* and *ahp* mediated CK signalling, displayed higher cold-tolerance of male meiotic cytokinesis program. We here suggest that the transcription regulator ARR1 may act independently from the CK AHK2/3-AHP2/3/5 signaling module in conveying the cold response to male meiocytes.

Keywords: cold sensitivity; cytokinin signaling; male meiotic cytokinesis; meiotic restitution; radial microtubule arrays

Introduction

The production of viable haploid gamete is important for ploidy stability and male fertility in flowering plants. Genetic alterations of meiotic regulators and/or external stresses may cause defects in male meiotic cell division and subsequently lead to meiotic restitution with associated unreduced gamete production (De Storme and Mason, 2014). The cytological mechanisms that result in male meiotic restitution can be classified into omission of meiosis cycles, fused, and/or parallel spindles and incomplete meiotic cytokinesis (Ramanna and Jacobsen, 2003). Short periods of cold stress (4–5°C for 1–40 h) induce male meiotic restitution and 2n gamete formation in Arabidopsis by specifically interfering with male meiotic cytokinesis (De Storme et al., 2012). More specifically, the low temperature stress evokes alterations in the formation and organization of meiotic cytoskeleton radial microtubule arrays (RMAs) in developing meiocytes at telophase II stage, and subsequently leads to incomplete meiotic cell wall formation at tetrad stage resulting in diploid or polyploid microspore formation(De Storme et al., 2012). Concerning the specifically targeted meiotic program (meiotic cytokinesis) and the affected cytoskeleton structure (RMA), it is considered that cold-induced meiotic restitution is mediated by cold-responsive genetic mechanisms. However, these involved molecular regulators and signaling pathways are not yet known.

Cytokinin (CK) regulates cell division, cell differentiation, and chlorophyll senescence in plants, and additionally integrates in the response of plants to abiotic stresses such as cold (Mok and Mok, 2001; Argueso et al., 2009). CKs are generally classified in four types; isopentenyladenine nucleotide (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (dZ), with iP and tZ harboring strongest ligand affinity to CK receptors (Inoue et al., 2001; Suzuki

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Abbreviations: CK, Cytokinin; WT, wild type; RMAs, radial microtubule arrays; PMCs, pollen mother cells

et al., 2001; Yamada et al., 2001; Spíchal et al., 2004; Romanov et al., 2006). De novo biosynthesis of CKs is conducted in a step-wise manner. The first step is initiated by adenosine phosphate-isopentenyltransferase (IPT), which catalyzes the conversion of dimethylallyl diphosphate (DMAPP), adenosine 5′-phosphates and/or hydroxymethylbutenyl diphosphate (HMBDP) into iP nucleotide (Sakakibara, 2005; Hirose et al., 2008). The cZ cytokinin is synthesized through tRNA degradation by tRNA-isopentenyltransferase (tRNA-IPT); and the source of dZ is considered to be a putative zeatin reductase (Hwang and Sakakibara, 2006; Frébort et al., 2011). In Arabidopsis, there are seven *IPT* genes (i.e., *IPT1* and *IPT3-8*) encoding for IPT proteins that show differential subcellular specificity (Kakimoto, 2001; Takei et al., 2001; Sun et al., 2003; Kasahara et al., 2004). *Trans*-zeatin (tZ) is synthesized by the subsequent hydroxylation of iP by the cytochrome P450 monooxygenases (P450s) CYP735A1 and CYP735A2 (Kamada-Nobusada and Sakakibara, 2009). Maintenance of proper bioactive CK levels is critical for normal plant development and requires cytokinin dehydrogenases (CKXs) that irreversibly degrade cytokinin (Frébort et al., 2011). In Arabidopsis, the CKX protein family consists of seven members (Sakakibara, 2006; Werner et al., 2006). CKX1, CKX3, and CKX5 are considered to exert redundant functions as overexpression of these genes causes similar physiological phenotypes (Werner et al., 2003). However, overproduction of CKX2 and CKX4 causes a more severe degree in CK-deficiency with the strongest enzymatic effect on iP, tZ, and the respective ribosides (Werner et al., 2003; Nishiyama et al., 2011; Köllmer et al., 2014).

In Arabidopsis, CK signalling is mainly mediated by a two-component system that is composed of Arabidopsis Histidine Kinases (AHKs) and Arabidopsis Histidine Phosphotransfers (AHPs) (To and Kieber, 2008). The AHKs are membrane-located proteins that show a conformational change and autophosphorylation upon binding with extracellular cytokinin (Heyl and Schmülling, 2003). Arabidopsis Histidine Phosphotransferases (AHPs) mediate the cytoplasmic-to-nuclear signal transfer from AHKs to nucleus-localized B-type Arabidopsis Response Regulators (B-type ARRs); B-type ARRs act as transcription regulators by binging with the promoter of A-type ARRs and consequently induce CK-dependent physiological activities (Kakimoto, 2003; Heyl and Schmülling, 2003; Ferreira and Kieber, 2005; To and Kieber, 2008; Hwang et al., 2012,). In Arabidopsis, there are three CK receptor AHK proteins; that is, AHK2, AHK3, and AHK4/CRE1, which harbor both specific and redundant functions in the transduction of CK signals (Heyl and Schmülling, 2003; Higuchi et al., 2004; Nishimura et al., 2004; Heyl et al., 2012). Arabidopsis AHP1, AHP2, AHP3, AHP4, and AHP5 are redundant regulators that act positively in CK signaling with stable transcripts upon exogenous CK treatment; AHP6, on the other hand, is a negative regulator of CK signaling and its expression decreases upon CK application (Tanaka et al., 2004; Hutchison et al., 2006; Mähönen et al., 2006). Both *AHK2*, *3*, and *AHP1-5* are expressed in Arabidopsis flowers (Nishimura et al., 2004; Hradilová and Brzobohatý, 2007). Upon phosphorylation by the AHKs, the AHPs in their turn phosphorylate ARR regulators that act downstream of CK signaling and that modulate plant development by regulating the expression of downstream target genes (Ferreira and Kieber, 2005). In Arabidopsis, there are 23 ARR proteins, and they are classified into two groups (type A and B) according to their sequence and domain structures (Brandstatter and Kieber, 1998; Imamura et al., 1999). Gene expression of A-type ARRs is inducible by exogenous CK, whereas transcript levels of B-type ARRs are relatively stable in response to exogenous CK treatment (Brandstatter and Kieber, 1998; Imamura et al., 1999; Kiba et al., 1999; Taniguchi et al., 1998).

CK is involved in the regulation of reproductive development in plants (Cucinotta et al., 2016). Genetic studies revealed that the combined loss-of-function of the CK receptors AHK2, 3, and 4 leads to delayed flowering induction, impaired female gametophyte development and decreased ovule number, indicating that CK is required for flowering initiation and ovule development (Nishimura et al., 2004; Bartrina et al., 2011; Bencivenga et al., 2012; Cheng and Kieber, 2013). Male gamete development is also dependent on proper CK signalling, since ectopic expression of *CKX1* in pollen and anthers of maize and tobacco causes male sterility, which can be restored by exogenous CK application (Huang et al., 2003). Although it is likely that CK deficiency-induced male sterility is caused by the *CKX1-OX* induced alteration of one specific anther developmental stage (Huang et al., 2003), it is yet unknown whether CK is required for male sporogenesis or pollen development, or plays a role in other male developmental processes. Studies in petunia have revealed that CK additionally mediates pollen tube growth by modulating the rearrangement of actin cytoskeleton (Kovaleva et al., 2015). Considering earlier stages in male reproductive development, CK has also been found to stimulate the initiation of meiosis in rye, suggesting a putative regulatory role for CK in male sporogenesis (Rueda and Vázquez, 1985). However, despite these preliminary findings, it is not yet known whether CK plays a regulatory role during male meiotic cell division in flowering plants.

CK signaling mediates the response of plant development to environmental stresses and in particular cold tolerance by activating the expression of downstream genes through the AHKs-AHPs two-component system (Tran et al., 2010; Ha et al., 2012; Hwang et al., 2012; Kim and Jeon, 2013). The

expression induction of cold-responsive A-type ARRs through AHKs is not through affecting *AHK* transcript or CK levels (Jeon et al., 2010). Moreover, this AHK-induced expression of cold-responsive A-type *ARRs* requires the action of AHP2/AHP3/AHP5 that function redundantly in CK signaling (Jeon and Kim, 2013). A role for cytokinin signaling in cold stress is further corroborated by the observation that the double *ahk2-2 ahk3-3* mutant shows higher tolerance to freezing temperatures (Jeon et al., 2010). Also, gene expression studies revealed the Arabidopsis *arr1* mutant exhibits reduced expression of A-type ARRs, whereas increased ARR1 activity enhances the transcript level of cold-inducible A-type ARRs, conferring enhanced freezing tolerance (Jeon and Kim, 2013).

Considering the role of CK signaling in the cold response of plant development, we hypothesized that cold-induced alterations in male meiotic cell division may be mediated by the CK signaling pathway. By monitoring the cold sensitivity of male meiotic cell division in a series of CK metabolism and signaling mutants, we here report that altered endogenous CK levels do not influence the cold response of male meiotic cytokinesis. Double *ahk2-2 ahk3-3* and triple *ahp2-1 ahp3 ahp5-2* mutant plants produce similar frequencies of meiotically restituted dyads and triads as wild type plants upon exposure to cold stress, suggesting that the AHK2/3-AHP2/3/5 CK signaling module does not confer cold-induced meiotic restitution in Arabidopsis male sporogenesis. Despite the fact that CK perception is not involved in the male meiotic cold response, *arr1* null mutant plants showed an improved tolerance of male meiotic cell division to the effects of cold, as reflected by the reduced number of cold-induced diploid (2n) pollen grains. Thus, although CK signaling through AHK2/3-AHP2/3/5 is not critical for cold-induced male meiotic restitution and 2n pollen formation in Arabidopsis, our data here suggest that the CK-related transcriptional regulator *ARR1* is somehow involved in this process.

Materials and methods

Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana* Heynh.) ecotype Columbia-0 was obtained from the Nottingham Arabidopsis Stock Centre. The double *ahk2-2 ahk3-3*, triple *ahp2-1 ahp3 ahp5-2*, and the *arr1-3* mutant were kindly shared by Jeon and Kim (2013) and Jeon et al. (2010). The *ipt* single mutants, and the *35S::CKXs* transgenic lines used in this study were kindly shared by Tatsuo Kakimoto (Miyawaki et al., 2006) and T. Schmülling (Werner et al., 2003), respectively. Primers used for plant genotyping are listed in Supplemental Table S1. The *35S::CKX2* and *35S::CKX4* transgenic lines have been described (Werner et al., 2001, 2003). Following a 3 day vernalization period (4–5°C), Arabidopsis seeds were germinated on K1 medium for 6–8 days (12 h day/12 h night, 20°C), after which seedlings were transferred to soil and cultivated in growth chambers at 12 h day/12 h night, 20°C, and less than 70% humidity. After 4 weeks of vegetative growth, the photoperiod was changed to a 16-h-day/8-h-night regime to stimulate flowering.

Measurement of cold sensitivity of *Arabidopsis thaliana* male sporogenesis

The cold treatment of flowering Arabidopsis plants and analysis of the cold sensitivity of male sporogenesis was performed according to De Storme et al. (2012) with minor modifications. Young flowering Arabidopsis plants were treated with cold (4–5°C) for 48 h, and tetrad-stage male meiocytes and/or unicellular-stage microspores were examined at 24–36 h following cold treatment.

Cytology

The analysis of male meiotic products (tetrads and microspores) using orcein staining was performed according to the methodology described previously (Liu et al., 2016). Flower buds producing significant numbers of mature meiotic products or young microspores were used for quantification and monitoring assays.

Tubulin immunolocalization

The α-tubulin immunolocalization was performed according to the method of De Storme et al. (2012) with minor modifications. After m-maleimidobenzoyl N-hydrosuccinimide ester treatment (100 μM in 50 mM potassium phosphate buffer and 0.05% [v/v] Triton X-100, pH 8; 30 min under vacuum) and fixation for 1 h in 4% (w/v) paraformaldehyde, inflorescences were washed in 50 mM potassium phosphate buffer (pH 8) and digested in an enzyme mixture consisting of 0.3% (w/v) pectolyase (Sigma), 0.3% (w/v) cytohelicase (Sigma), and 0.3% (w/v) cellulase (Sigma) in a humid chamber at 37°C for 3 h. Following a washing step in 50 mM potassium phosphate buffer (PPB), enzyme-digested anthers were dissected, squashed, and fixed on a slide by freezing in liquid nitrogen. Released cells were then digested again at 37°C for 1.5 h using the same enzyme mix. After rinsing with PPB, immobilized cells were incubated overnight at room temperature with rat α-tubulin primary antibody (0.3% [v/v]; clone B-5-1-2; Sigma–Aldrich) in 50 mM PPB containing 0.1% (v/v) Triton X-100, and 4.5 g L^{-1} bovine serum albumin. Next, cells were rinsed three times with phosphate-buffered saline and then treated for 3–5 h with 0.5% (v/v) secondary antibody (labeled goat anti-rat) at 37°C in the dark. Finally, after

five rinses with phosphate-buffered saline, a small droplet of 4′,6-diamidino-2-phenylindole (DAPI; 2 mg mL^{-1}) in Vectashield mounting medium (Vector Laboratories) was added to visualize the cell's chromosomes. DAPI was used for staining and visualization of DNA.

Microscopy

Both bright-field and fluorescence microscopy were performed using an Olympus IX81 inverted fluorescence microscope equipped with an X-Cite Series 120Q UV lamp and an Olympus XM10 camera. Bright field images were taken with a 60× objective and fluorescence images of immunostained microtubule structures were imaged with a 100× objective (Olympus). Bi-fluorescent images and Z-stacks were processed using ImageJ. Brightness and contrast settings were adjusted using Photoshop CS6.

Results

Reduced endogenous CK levels do not influence the cold sensitivity of male sporogenesis in arabidopsis

To determine the effect of a reduced endogenous CK level on the cold sensitivity of male meiotic cell division in Arabidopsis, we quantified the cold-induced male meiotic response (i.e., meiotic non-reduction) of a series of mutant lines with reduced CK biosynthesis or promoted CK catabolism (Figure 1). Previous studies have shown that the combined loss-of-function of *IPT1*, *IPT3*, *IPT5*, and *IPT7* significantly reduces the level of bioactive CK in developing *Arabidopsis thaliana* seedlings, and thereby concomitantly confers an enhanced tolerance to salt and drought stress (Nishiyama et al., 2011). *AtIPT1*, *3*, *5*, and *7* are expressed at low levels in Arabidopsis flowers with *IPT1* displaying highest transcripts compared with other *IPTs* (Miyawaki et al., 2004; Takei et al., 2004). In our growth assays, single *ipt1/3/5/7* and quadruple *ipt1357* mutant plants displayed different severity of vegetative growth phenotypes, with the *ipt1357* mutant showing defects in leaf development, and the *ipt5* mutant exhibiting the strongest phenotype among the single mutant plants (Supplement Figure S1A). In line with previous reports, loss-of-function of all four CK biosynthesis genes causes defects in vegetative growth and development, and leaf development in Arabidopsis (Miyawaki et al., 2006; Nishiyama et al., 2011). Since the quadruple *ipt1357* and the single *ipt5* mutant plants do not produce sufficient flower buds to detect cold-induced diploid pollen formation, quantification analysis was not possible. We therefore analyzed the single *ipt1*, *ipt3*, and *ipt7* mutants.

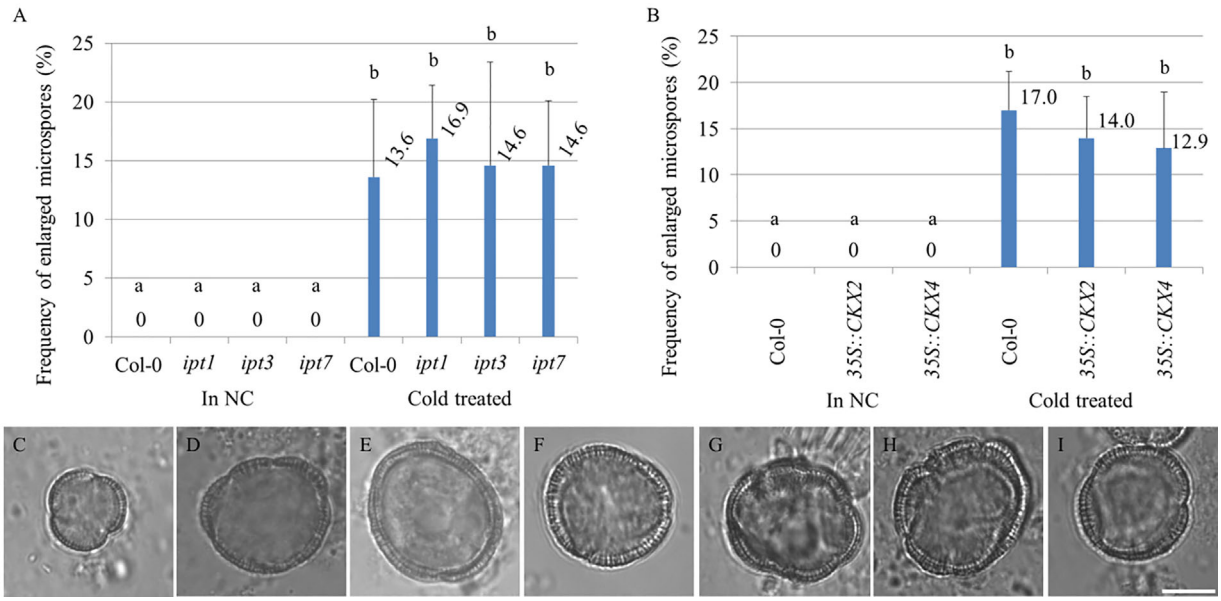

Figure 1 Cold sensitivity of male meiotic cell division in cytokinin metabolism mutants. A, Histogram showing the frequency of enlarged 2n microspores produced by cold-stressed *ipt1*, *ipt3*, and *ipt7* single mutants. B, Histogram showing the frequency of enlarged 2n microspores produced by cold-stressed *35S::CKX2* and *35S::CKX4* transgenic plants. Student's *t*-test and/or Wilcoxon rank test was performed for significance comparison analysis ($P > 0.05$). C, Haploid unicellular stage microspore produced by wild type Col-0 plants under control temperature conditions. D–I, Cold-induced meiotically restituted 2n unicellular stage microspores in wild type Col-0 (D) and *ipt1* (E), *ipt3* (F), *ipt7* (G), *35S::CKX2* (H), and *35S::CKX4* (I) plants. Scale bar = 10 μm.

Under normal temperature conditions, *ipt1*, *ipt3*, and *ipt7* mutant lines produce normal haploid microspores (Figures 1A and 1C), similar as in wild type Col-0 plants, indicating that IPT1, IPT3, and IPT7 are not essentially required for male meiotic cell division. Moreover, at 24–36 h after cold treatment, the frequency of enlarged, meiotically restituted microspores produced by these three mutant lines was similar to that in cold-stressed wild type Col-0 plants ($P > 0.05$) (Figures 1A and 1D, wild type plant; 1E, *ipt1*; 1F, *ipt3*; 1G, *ipt7*). These data demonstrate that reduced CK levels by single *ipt1*, *3*, and/or *7* mutations do not influence the cold sensitivity of Arabidopsis male sporogenesis.

CKX proteins catabolize CKs and the overexpression of *CKXs* (*35S::CKXs*) leads to a reduction of endogenous CK levels (Werner et al., 2001, 2003). Transgenic plants (*35S::CKXs*) that overexpress *CKX1*, *CKX2*, *CKX3*, or *CKX4*, were selected for the cold sensitivity analysis of male sporogenesis. All four *CKX*-overexpression lines contain a reduced endogenous CK level and show altered vegetative development (Nishiyama et al., 2011) (Supplement Figure S1A); however, overexpression of *CKX1* and *CKX3* causes greater reduction of endogenous CKs (Werner et al., 2003; Nishiyama et al., 2011) and a more severe inhibition of lateral leaf development than in the *35S:CKX2* and *35S:CKX4* plants (Supplement Figure S1A). Since both *35S:CKX1* and *35S:CKX3* plants failed to produce enough flower buds for meiotic outcome assessment, we only analyzed the cold response of male meiotic cell division in *35S:CKX2* and *35S:CKX4* plants. *AtCKX2* and *4* has been shown to expressed in shoot, shoot apex and in the apical stem of Arabidopsis (Werner et al., 2003). Similar to wild type and to the *ipt1*, *ipt3*, and *ipt7* mutant plants, both the *35S:CKX2* and *35S:CKX4* transgenic plants produce normally sized, haploid microspores under control temperature conditions (Figures 1B and 1C) and yield meiotically restituted 2n microspores at a similar frequency as wild type Arabidopsis Col-0 plants after 24–36 h of cold treatment ($P > 0.05$) (Figure 1B; 1H, *35S:CKX2* plant; 1I, *35S:CKX4* plant), indicating similar cold sensitivity of male sporogenesis as wild type plants.

Collectively, these findings demonstrate that an endogenous reduction of CKs caused by either single *IPT1/3/7* mutation or by overexpression of *CKX2/4* does not inhibit or alter the frequency of cold-induced male meiotic restitution in *Arabidopsis thaliana*.

Altered CK-signaling does not affect cold-induced male meiotic restitution in arabidopsis

Since the combined loss-of-function of *AHK2* and *AHK3*, and/or *AHK3* and *AHK4* leads to enhanced freezing tolerance, and the triple *ahp2-1 ahp3 ahp5-2* mutant exhibits reduced cold-inducible gene expression (Jeon et al., 2010; Jeon and Kim, 2013), we further tested the possibility whether AHK-mediated signaling is involved in cold-induced male meiotic restitution in Arabidopsis (Figure 2), independently of CK homeostasis. To test the roles of AHK2/3 cytokinin receptors as well as the downstream signal mediators AHP2/3/5 herein, corresponding mutants were analyzed for their response of male meiotic cell division to cold.

Under control temperature conditions, the double *ahk2-2 ahk3-3* and triple *ahp2-1 ahp3 ahp5-2* mutant display regular male meiotic cytokinesis, as exemplified by the production of normal tetrad figures, together with the formation of normally sized, haploid microspores (Figure 2). Upon exposure to cold stress, however, meiotically restituted figures, such as dyads and triads, together with enlarged 2n microspores were observed in both types of CK signalling mutants, at a similar rate as those formed in cold-stressed wild type Arabidopsis plants (Figure 2). Our data, therefore, suggest that the CK signaling AHK2/3-AHP2/3/5 regulatory module is not required for causing male meiotic restitution in response to cold spells.

Cold-induced alterations in male meiotic radial microtubule array (RMA) and cell wall formation occur independently from cytokinin signaling

Cold stress interferes with male meiotic cytokinesis in wild type Arabidopsis plants by disrupting the organization of the radial microtubule arrays (RMAs) at telophase II. To determine whether cold-induced disorganization of male meiotic RMA depends on CK-signaling, we performed tubulin immunolocalization on cold-stressed male meiocytes in the double *ahk2-2 ahk3-3* mutant plants (Figure 3). Under normal temperature conditions, *ahk2-2 ahk3-3* mutant meiocytes (Figures 3I–3L) exhibit a regular organization of all microtubular cytoskeletal structures (i.e., spindles, phragmoplast, and RMAs) throughout meiotic cell division (Figure 3), supporting our previous notion that *ahk2-2 ahk3-3* plants undergo normal male meiosis and meiotic cytokinesis. Following 48 h cold treatment, *ahk2-2 ahk3-3* mutant male meiocytes were found to display a subset of disorganized RMAs at telophase II stage, at a similar rate as those observed in cold-stressed wild type plants (Figure 3). Triad-like RMA figures were observed in both Col-0 (Figure 3H) and *ahk2 ahk3* mutant meiocytes (Figures 3P–3T), showing lack of microtubule structures between two adjacent haploid nuclei (Figure 3 red arrows). At other meiosis stages, that is, from prophase I to anaphase II, no alterations in the microtubular cytoskeletal structures were observed in both Col-0 and *ahk2 ahk3* mutant meiocytes (Figure 3). Because CK signaling mutants produce regular microspores under normal temperatures and show cold-induced disorganization of the RMA, at a similar rate as in cold-stressed wild type Arabidopsis plants, we conclude

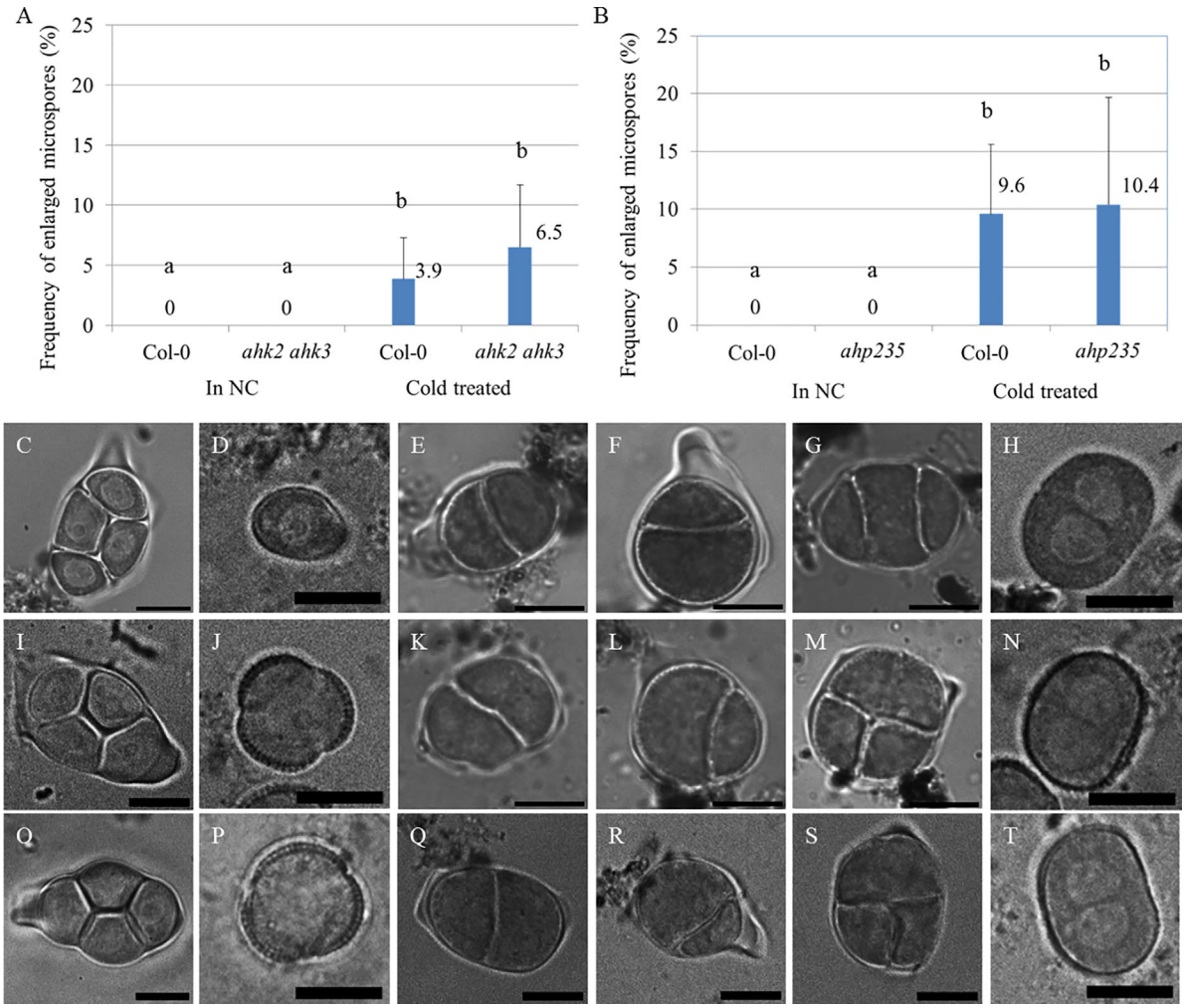

Figure 2 Cold sensitivity of male meiotic cell division in cytokinin signaling mutants. A and B, Histograms showing the frequency of enlarged 2n microspores produced by cold-stressed *ahk2-2 ahk3-3* (A) and *ahp2-1 ahp3 ahp5-2* mutant plants (B). Transmission images of orcein-stained tetrad stage male meiocytes and microspores (C–T). C–H, Tetrad (C), haploid unicellular microspore (D), balanced dyad (E), unbalanced dyad (F), triad (G) and 2n microspore (H) produced by wild type Col-0 plants under normal temperature conditions (C and D) or after cold stress (E–H). I–N, Tetrad (I), haploid unicellular microspore (J), balanced dyad (K), unbalanced dyad L), triad (M) and 2n microspore (N) produced by *ahk2-2 ahk3-3* mutant plants under normal temperature conditions (I and J) or after cold stress (K–N). O–T, Tetrad (O), haploid unicellular microspore (P), balanced dyad (Q), unbalanced dyad (R), triad (S) and 2n microspore (T) produced by *ahp2-1 ahp3 ahp5-2* mutant plants under normal temperature conditions (O and P) or after cold stress (Q–T). Scale bars = 10 μm.

that the AHK2/3-AHP2/3/5 CK signaling module is not involved in male sporogenesis, and is not required for the cold sensitivity of male meiotic cytokinesis in Arabidopsis.

Male meiotic cell division in the *arr1* arabidopsis mutant shows reduced sensitivity to cold stress

The lack of involvement of the AHK/AHP two-component signaling module in the male meiotic cold response implies that downstream response regulators are also not critical for cold-induced meiotic restitution. An important downstream regulator of cold signaling is ARR1 as the corresponding loss-of-function *arr1* mutation causes a severely reduced cold-inducible expression of type A ARRs (Jeon and Kim, 2013). To test the putative functional requirement of ARR1 in cold-induced male meiotic non-reduction, we exposed *arr1-3* mutant plants to cold and subsequently analyzed the male meiotic outcome. Under control temperature conditions, *arr1* male meiocytes exhibit regular male meiotic cytokinesis together with the consistent production of haploid microspores, similar as in wild type Col-0 (Figure 4). Interestingly, upon exposure to cold, *arr1-3* male meiocytes exhibited a significant reduction in cold sensitivity compared to wild type Col-0 male meiocytes, as reflected by the

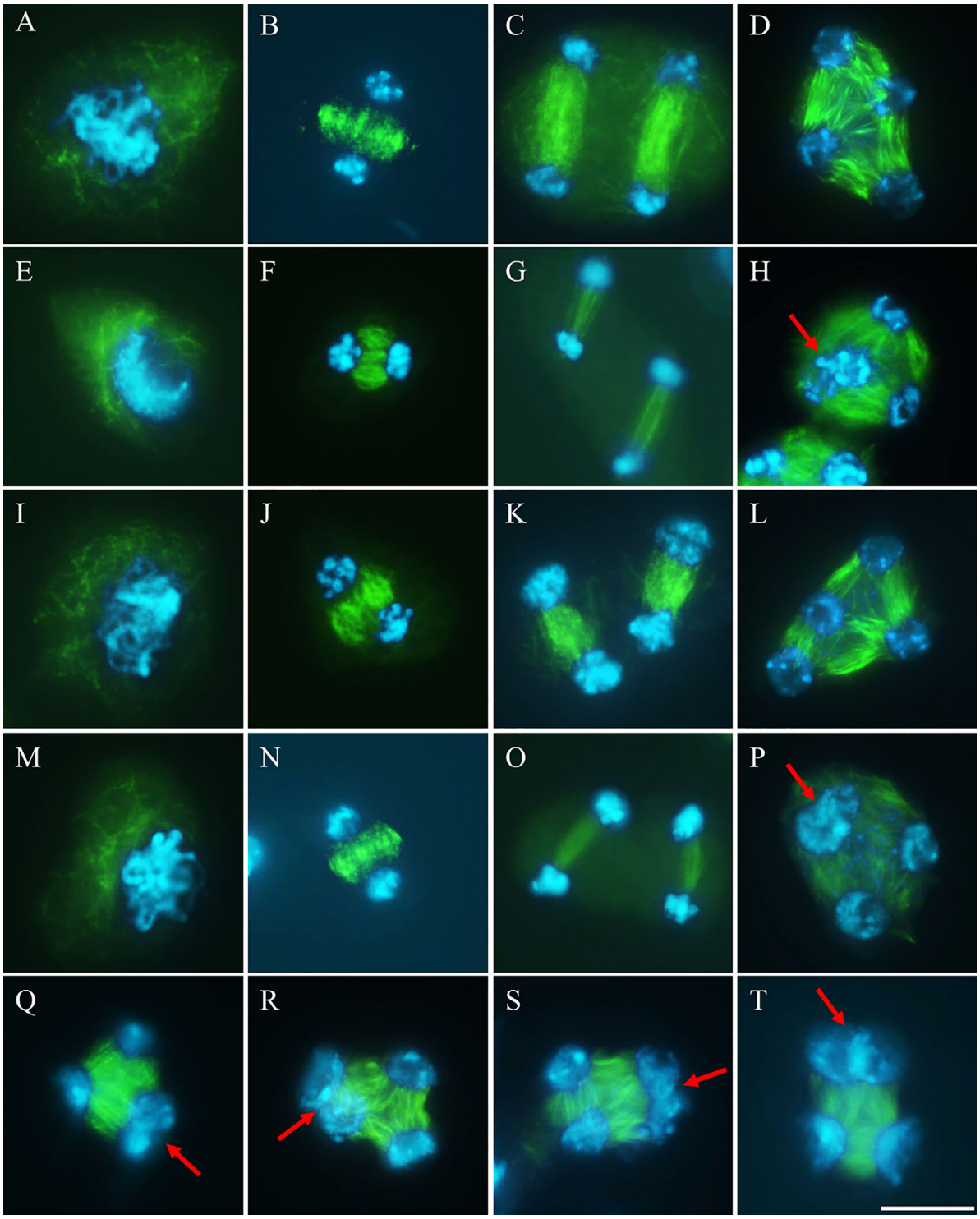

Figure 3 α-Tubulin immunostaining in wild type (A-H) and *ahk2-2 ahk3-3* male meiocytes (I-T) to visualize microtubular structures. Male meiocytes isolated from plants grown under normal temperature conditions (A–D and I–L) or following a 48 h cold treatment (E–H and M–T). Illustrated stages are prophase I (A, E, I, M), interkinesis (B, F, J, N), anaphase II (C, G, K, O), and telophase II (D, H, L, P–T). Green: α-tubulin, cyan: DAPI-stained chromosomes. Scale bar = 10 μm.

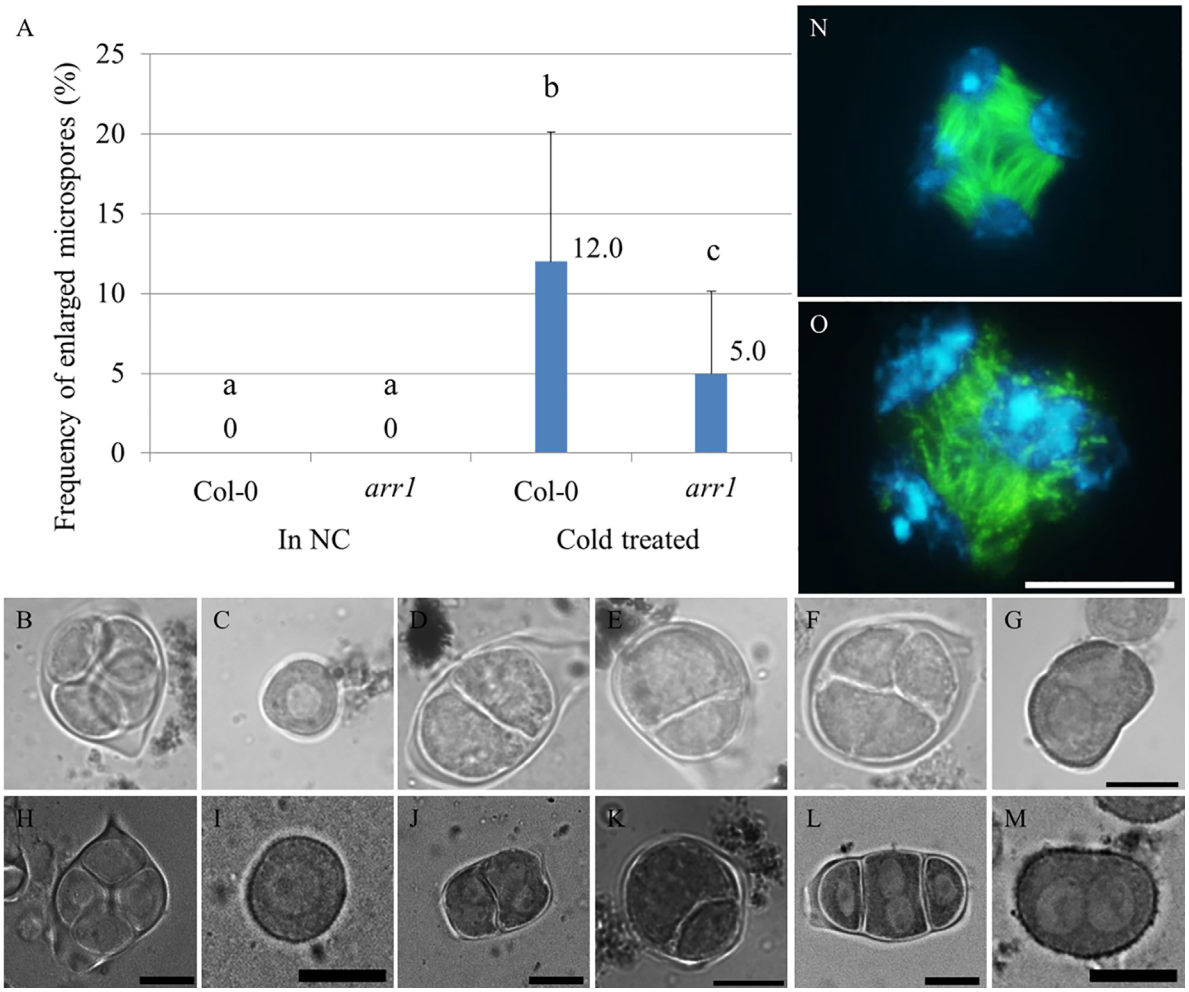

Figure 4 Cold sensitivity of male meiotic cell division in the Arabidopsis *arr1-3* mutant. A, Histogram showing the frequency of enlarged 2n microspores produced by cold-stressed wild type Col-0 and *arr1-3* mutant plants (A). Wilcoxon rank test was performed for significance comparison analysis. B–G, Tetrad (B), haploid unicellular stage microspore (C), balanced dyad (D), unbalanced dyad (E), triad (F), and enlarged 2n unicellular stage microspore (G) produced by wild type Col-0 plants under normal temperature conditions (B and C) or upon cold stress (D–G). H–M, Tetrad (H), haploid unicellular stage microspore (I), balanced dyad (J), unbalanced dyad (K), triad (L) and enlarged 2n unicellular stage microspore (M) produced by *arr1-3* mutant plants under normal temperature conditions (H and I) or upon cold stress (J–M). N and O, RMAs of *arr1-3* male meiocytes under normal temperature conditions (N) and upon 48 h cold shock (O). Green: α-tubulin, cyan: DAPI-stained chromosomes. Scale bars = 10 μm.

significant reduction in meiotically restituted gametes upon cold stress ($P < 0.01$) (Figure 4). This observation suggests that male meiotic cytokinesis of *arr1* mutant plants is more tolerant to cold stress, and that the cold response of male meiotic cell division in Arabidopsis is partially dependent on ARR1 function.

Discussion

Cytokinin regulates multiple abiotic stress responses of plants (Zwack and Rashotte, 2015) and the downstream AHK-AHP two-component CK signalling pathway is specifically involved in the modulation of gene expression upon exposure to cold (Jeon et al., 2010; Jeon and Kim, 2013). In support of this, it has been shown that the double *ahk2-2 ahk3-3* mutant displays enhanced freezing tolerance, and that the CK signalling factors AHP2, AHP3, and AHP5 act positively in cold signaling to induce the expression of cold-responsive A-type ARRs (Jeon et al., 2010; Jeon and Kim, 2013). Here we test the hypothesis whether cold-induced alterations in male meiotic cytokinesis are mediated by CK homeostasis and signaling. Quantitative analysis of the male meiotic outcome of double *ahk2-2 ahk3-3* and triple *ahp2-1 ahp3 ahp5-2* mutant plants revealed that cold sensitivity of male meiotic cytokinesis and the associated production of meiotic restituted 2n gamete is similar as in wild type plants. Hence, the results therefore indicate that the AHK2/3-AHP2/3/5 module is not essentially required

for conferring cold-induced meiotic restitution through alterations in male meiotic cytokinesis in Arabidopsis.

Besides AHK2 and AHK3, Arabidopsis has another cytokinin receptor, which is CRE1/AHK4 that also functions in cytokinin reception and signal transduction (Yamada et al., 2001; Hwang et al., 2012). Since the *ahk3 ahk4* Arabidopsis mutant is also more tolerant to freezing temperatures than wild type plants (Jeon et al., 2010), our findings cannot exclude the possibility that AHK4 mediates the cold response of Arabidopsis male meiotic cell division, and that AHK2, AHK3, and AHK4 function redundantly in mediating cold-induced male meiotic restitution. To this end, the cold sensitivity of male meiosis in the *ahk3 ahk4* mutant and/or a higher combination of *AHK* mutations should be checked. In Arabidopsis, the AHP family consists of six histidine phosphotransferase members (AHP1-6), with AHP1-5 redundantly promoting CK signal transduction (Tanaka et al., 2004; Hutchison et al., 2006; Mähönen et al., 2006). Since this study only focuses on the putative role of AHP2, 3, and 5 in conferring cold-induced male meiotic restitution, the putative involvement of the other two AHPs, that is, AHP1 and AHP4, remains to be investigated.

Although the CK signaling components AHK2/3 and AHP2/3/5 are not involved in the cold sensitivity of male meiotic cell division, we found that the *arr1-3* mutation dampens the response, suggesting that the transcriptional regulator ARR1 may play a role in cold-induced alterations in meiotic cytokinesis and associated meiotic non-reduction. ARR1 has recently been shown to mediate the drought tolerance of vegetative development in Arabidopsis (Nguyen et al., 2016). It has been reported that many genes that are preferentially expressed in Arabidopsis male meiocytes share conserved motifs in their putative 1000 bp promotor regions, and that these conserved sequences are the binding sites of transcription regulators (Li et al., 2014). In among fifty promotors of male meiocyte-expressed genes, a conserved binding site of ARR1 protein was identified (Li et al., 2014), suggesting that ARR1 is an important regulator of gene expression during male meiocyte development. These downstream target genes, however, are clearly not critical for male meiotic cell division, as *arr1-3* male meiosis produces normal tetrads and regular numbers of haploid spores, eventually leading to fully fertile mutant plants. The functional requirement for ARR1 in the cold responsiveness of Arabidopsis male meiotic cell division, together with the lack of requirement for the CK signalling proteins AHK2/3 and AHP2/3/5, implies that cold stress mediates ARR1 activity independently from cytokinin signaling to modulate factors involved in male meiotic cytokinesis. Actually, ARR1 also acts in other hormone signaling pathways. For example, ARR1 plays a role in ethylene-mediated root development in Arabidopsis, with null arr1 mutant displays reduced ethylene sensitivity of root apical meristem (Street et al., 2015). Ethylene suppresses freezing tolerance in Arabidopsis by inhibiting the expression of A-type ARRs including ARR7, which is one of the cold-inducible targets of ARR1 (Shi et al., 2012; Jeon and Kim, 2013). In addition, ARR1 is regulated by endogenous GA and GA suppresses ARR1 activity by inhibiting DELLAs, which physically interact with ARR1 and positively co-regulate gene expression (Marín-de la Rosa et al., 2015; Fonouni-Farde et al., 2016). Since both ethylene and GA are stress-responsive hormones, and both are involved in the cold-response of plant development (Zinn et al., 2010; Colebrook et al., 2014; Kazan, 2015; Wani et al., 2016), the function of ARR1 in mediating the cold sensitivity of male meiotic cell division in Arabidopsis may be controlled by a complex hormone cross-talk mechanism involving GA and ethylene. Previously we have shown that functional GA-DELLA is critical for regular male meiotic cytokinesis and gametophytic ploidy consistency in Arabidopsis (Liu et al., 2016). Further research is thus needed to clarify the putative role of ARR1 together with the plant stress hormones GA and ethylene in the control of cold-induced male meiotic restitution and associated 2n pollen formation in *Arabidopsis thaliana*.

Acknowledgments and funding

We thank Jungmook Kim and Jin Jeon for kindly sharing the seeds of the double *ahk2-2 ahk3-3* and triple *ahp2-1 ahp3 ahp5-2*, as well as the *arr1-3* mutants. We thank Tatsuo Kakimoto and T. Schmülling for sharing the *ipt* single mutants and the *35S::CKXs* transgenic lines, respectively. The funding is supported by China Scholarship Council (CSC) No. 201306760005 and FWO PostDoc grant 1293014N.

References

Argueso CT, Ferreira FJ, Kieber JJ (2009) Environmental perception avenues: the interaction of cytokinin and environmental response pathways. Plant Cell Environ 32: 1147–60.

Bartrina I, Otto E, Strnad M, Werner T, Schmülling T (2011) Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in arabidopsis thaliana. Plant Cell 23: 69–80.

Bencivenga S, Simonini S, Benkova E, Colombo L (2012) The transcription factors BEL1 and SPL are required for cytokinin and auxin signaling during ovule development in Arabidopsis. Plant Cell 24: 2886–97.

Brandstatter I, Kieber JJ (1998) Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in arabidopsis. Plant Cell 10: 1009–19.

Cheng C-Y, Kieber JJ (2013) The role of cytokinin in ovule development in Arabidopsis. Plant Signal Behav 8: e23393.

Colebrook EH, Thomas SG, Phillips AL, Hedden P (2014) The role of gibberellin signalling in plant responses to abiotic stress. J Exp Biol 217: 67–75.

Cucinotta M, Manrique S, Guazzotti A, Quadrelli NE, Mendes MA, Benkova E, Colombo L (2016) Cytokinin response factors integrate auxin and cytokinin pathways for female reproductive organ development. Development 143: 4419–24.

De Storme N, Copenhaver GP, Geelen D (2012) Production of diploid male gametes in arabidopsis by cold-induced destabilization of postmeiotic radial microtubule arrays. Plant Physiol 160: 1808–26.

De Storme N, Mason A (2014) Plant speciation through chromosome instability and ploidy change: cellular mechanisms, molecular factors and evolutionary relevance. Curr Plant Biol 1: 10–33.

Ferreira FJ, Kieber JJ (2005) Cytokinin signaling. Curr Opin Plant Biol 8: 518–25.

Fonouni-Farde C, Diet A, Frugier F (2016) Root development and endosymbioses: DELLAs lead the orchestra. Trends Plant Sci 21: 898–900.

Frébort I, Kowalska M, Hluska T, Frébortová J, Galuszka P (2011) Evolution of cytokinin biosynthesis and degradation. J Exp Bot 62: 2431–52.

Ha S, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Tran L-SP (2012) Cytokinins: metabolism and function in plant adaptation to environmental stresses. Trends Plant Sci 17: 172–9.

Heyl A, Riefler M, Romanov GA, Schmülling T (2012) Properties, functions and evolution of cytokinin receptors. Eur J Cell Biol 91: 246–56.

Heyl A, Schmülling T (2003) Cytokinin signal perception and transduction. Curr Opin Plant Biol 6: 480–8.

Higuchi M, Pischke MS, Mähönen AP, Miyawaki K, Hashimoto Y, Seki M, Kobayashi M, Shinozaki K, Kato T, Tabata S, Helariutta Y, Sussman MR, Kakimoto T (2004) In planta functions of the Arabidopsis cytokinin receptor family. Proc Natl Acad Sci USA 101: 8821–6.

Hirose N, Takei K, Kuroha T, Kamada-Nobusada T, Hayashi H, Sakakibara H (2008) Regulation of cytokinin biosynthesis, compartmentalization and translocation. J Exp Bot 59: 75–83.

Hradilová J, Brzobohatý B (2007) Expression pattern of the AHP gene family from Arabidopsis thaliana and organ specific alternative splicing in the AHP5 gene. Biologia Plantarum 51: 257–67.

Huang S, Cerny RE, Qi Y, Bhat D, Aydt CM, Hanson DD, Malloy KP, Ness LA (2003) Transgenic studies on the involvement of cytokinin and gibberellin in male development. Plant Physiol 131: 1270–82.

Hutchison CE, Li J, Argueso C, Gonzalez M, Lee E, Lewis MW, Maxwell BB, Perdue TD, Schaller GE, Alonso JM (2006) The arabidopsis histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. Plant Cell 18: 3073–87.

Hwang I, Sakakibara H (2006) Cytokinin biosynthesis and perception. Physiol Plant 126: 528–38.

Hwang I, Sheen J, Müller B (2012) Cytokinin signaling networks. Ann Rev Plant Bio 63: 353–80.

Imamura A, Hanaki N, Nakamura A, Suzuki T, Taniguchi M, Kiba T, Ueguchi C, Sugiyama T, Mizuno T (1999) Compilation and characterization of Arabidopsis thaliana response regulators implicated in His-Asp phosphorelay signal transduction. Plant Cell Physiol 40: 733–42.

Inoue T, Higuchi M, Hashimoto Y, Seki M, Kobayashi M, Kato T, Tabata S, Shinozaki K, Kakimoto T (2001) Identification of CRE1 as a cytokinin receptor from Arabidopsis. Nature 409: 1060–3.

Jeon J, Kim J (2013) Arabidopsis response Regulator1 and Arabidopsis histidine phosphotransfer Protein2 (AHP2), AHP3, and AHP5 function in cold signaling. Plant Physiol 161: 408–24.

Jeon J, Kim NY, Kim S, Kang NY, Novak O, Ku SJ, Cho C, Lee DJ, Lee EJ, Strnad M, Kim J (2010) A subset of cytokinin two-component signaling system plays a role in cold temperature stress response in Arabidopsis. J Biol Chem 285: 23371–86.

Köllmer I, Novák O, Strnad M, Schmülling T, Werner T (2014) Overexpression of the cytosolic cytokinin oxidase/dehydrogenase (CKX7) from Arabidopsis causes specific changes in root growth and xylem differentiation. Plant J 78: 359–71.

Kakimoto T (2001) Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate:ATP/ADP isopentenyltransferases. Plant Cell Physiol 42: 677–85.

Kakimoto T (2003) Perception and signal transduction of cytokinins. Ann Rev Plant Bio 54: 605–27.

Kamada-Nobusada T, Sakakibara H (2009) Molecular basis for cytokinin biosynthesis. Phytochemistry 70: 444–9.

Kasahara H, Takei K, Ueda N, Hishiyama S, Yamaya T, Kamiya Y, Yamaguchi S, Sakakibara H (2004) Distinct isoprenoid origins of cis- and trans-zeatin biosyntheses in Arabidopsis. J Biol Chem 279: 14049–54.

Kazan K (2015) Diverse roles of jasmonates and ethylene in abiotic stress tolerance. Trends Plant Sci 20: 219–29.

Kiba T, Taniguchi M, Imamura A, Ueguchi C, Mizuno T, Sugiyama T (1999) Differential expression of genes for response regulators in response to cytokinins and nitrate in Arabidopsis thaliana. Plant Cell Physiol 40: 767–71.

Kim J, Jeon J (2013) Cold signaling via the two-component signaling system. Mol Plant 6: 15–7.

Kovaleva LV, Voronkov AS, Zakharova EV (2015) Role of auxin and cytokinin in the regulation of the actin cytoskeleton in the in vitro germinating male gametophyte of petunia. Russ J Plant Physiol 62: 179–86.

Li J, Yuan J, Li M (2014) Characterization of putative cis-regulatory elements in genes preferentially expressed in arabidopsis male meiocytes. BioMed Res Int 2014: 708364.

Liu B, De Storme N, Geelen DN (2016) Gibberellin induces diploid pollen formation by interfering with meiotic cytokinesis. Plant Physiol 173: 338–53.

Mähönen AP, Bishopp A, Higuchi M, Nieminen KM, Kinoshita K, Törmäkangas K, Ikeda Y, Oka A, Kakimoto T, Helariutta Y (2006) Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. Science 311: 94–8.

Marín-de la Rosa N, Pfeiffer A, Hill K, Locascio A, Bhalerao RP, Miskolczi P, Grønlund AL, Wanchoo-Kohli A, Thomas SG, Bennett MJ (2015) Genome wide binding site analysis reveals transcriptional coactivation of cytokinin-responsive genes by DELLA proteins. PLoS Genet 11: e1005337.

Miyawaki K, Matsumoto-Kitano M, Kakimoto T (2004) Expression of cytokinin biosynthetic isopentenyltransferase genes in

Arabidopsis: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant J 37: 128–38.

Miyawaki K, Tarkowski P, Matsumoto-Kitano M, Kato T, Sato S, Tarkowska D, Tabata S, Sandberg G, Kakimoto T (2006) Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. Proc Natl Acad Sci USA 103: 16598–603.

Mok DW, Mok MC (2001) Cytokinin metabolism and action. Annu Rev Plant Physiol Plant Mol Biol 52: 89–118.

Nguyen KH, Ha CV, Nishiyama R, Watanabe Y, Leyva-González MA, Fujita Y, Tran UT, Li W, Tanaka M, Seki M, Schaller GE, Herrera-Estrella L, Tran LS (2016) Arabidopsis type B cytokinin response regulators ARR1, ARR10, and ARR12 negatively regulate plant responses to drought. Proc Natl Acad Sci USA 113: 3090–5.

Nishimura C, Ohashi Y, Sato S, Kato T, Tabata S, Ueguchi C (2004) Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in arabidopsis. Plant Cell 16: 1365–77.

Nishiyama R, Watanabe Y, Fujita Y, Kojima M, Werner T, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Kakimoto T, Sakakibara H, Schmulling T, Tran LS (2011) Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic acid responses, and abscisic acid biosynthesis. Plant Cell 23: 2169–83.

Ramanna M, Jacobsen E (2003) Relevance of sexual polyploidization for crop improvement-A review. Euphytica 133: 3–8.

Romanov GA, Lomin SN, Schmülling T (2006) Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. J Exp Bot 57: 4051–8.

Rueda J, Vázquez AM (1985) Effect of auxins and cytokinins upon the start of meiosis in cultured anthers of rye (Secale cereale L.). Can J Genet Cytol 27: 759–65.

Sakakibara H (2005) Cytokinin biosynthesis and regulation. Vitam Horm 72: 271–87.

Sakakibara H (2006) Cytokinins: activity, biosynthesis, and translocation. Annu Rev Plant Biol 57: 431–49.

Shi Y, Tian S, Hou L, Huang X, Zhang X, Guo H, Yang S (2012) Ethylene signaling negatively regulates freezing tolerance by repressing expression of CBF and type-A ARR genes in arabidopsis. Plant Cell 24: 2578–95.

Spíchal L, Rakova NY, Riefler M, Mizuno T, Romanov GA, Strnad M, Schmülling T (2004) Two cytokinin receptors of Arabidopsis thaliana, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. Plant Cell Physiol 45: 1299–305.

Street IH, Aman S, Zubo Y, Ramzan A, Wang X, Shakeel SN, Kieber JJ, Schaller GE (2015) Ethylene inhibits cell proliferation of the arabidopsis root meristem. Plant Physiol 169: 338–50.

Sun J, Niu QW, Tarkowski P, Zheng B, Tarkowska D, Sandberg G, Chua NH, Zuo J (2003) The Arabidopsis AtIPT8/PGA22 gene encodes an isopentenyl transferase that is involved in de novo cytokinin biosynthesis. Plant Physiol 131: 167–76.

Suzuki T, Miwa K, Ishikawa K, Yamada H, Aiba H, Mizuno T (2001) The Arabidopsis sensor His-kinase, AHK4, can respond to cytokinins. Plant Cell Physiol 42: 107–13.

Takei K, Sakakibara H, Sugiyama T (2001) Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in Arabidopsis thaliana. J Biol Chem 276: 26405–10.

Takei K, Ueda N, Aoki K, Kuromori T, Hirayama T, Shinozaki K, Yamaya T, Sakakibara H (2004) AtIPT3 is a key determinant of nitrate-dependent cytokinin biosynthesis in Arabidopsis. Plant Cell Physiol 45: 1053–62.

Tanaka Y, Suzuki T, Yamashino T, Mizuno T (2004) Comparative studies of the AHP histidine-containing phosphotransmitters implicated in His-to-Asp phosphorelay in Arabidopsis thaliana. Biosci Biotechnol Biochem 68: 462–5.

Taniguchi M, Kiba T, Sakakibara H, Ueguchi C, Mizuno T, Sugiyama T (1998) Expression of Arabidopsis response regulator homologs is induced by cytokinins and nitrate. FEBS Lett 429: 259–62.

To JPC, Kieber JJ (2008) Cytokinin signaling: two-components and more. Trends Plant Sci 13: 85–92.

Tran L-SP, Shinozaki K, Yamaguchi-Shinozaki K (2010) Role of cytokinin responsive two-component system in ABA and osmotic stress signalings. Plant Signal Behav 5: 148–50.

Wani SH, Kumar V, Shriram V, Sah SK (2016) Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. Crop J 4: 162–76.

Werner T, Köllmer I, Bartrina I, Holst K, Schmülling T (2006) New insights into the biology of cytokinin degradation. Plant Biol 8: 371–81.

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T (2003) Cytokinin-Deficient transgenic arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15: 2532–50.

Werner T, Motyka V, Strnad M, Schmülling T (2001) Regulation of plant growth by cytokinin. Proc Natl Acad Sci 98: 10487–92.

Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Yamashino T, Mizuno T (2001) The arabidopsis AHK4 histidine kinase is a cytokinin-Binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol 42: 1017–23.

Zinn KE, Tunc-Ozdemir M, Harper JF (2010) Temperature stress and plant sexual reproduction: uncovering the weakest links. J Exp Bot 61: 1959–68.

Zwack PJ, Rashotte AM (2015) Interactions between cytokinin signalling and abiotic stress responses. J Exp Bot 66: 4863–71.

Received 21 April 2017; accepted 11 June 2017.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Vegetative growth of cytokinin-metabolism and −signaling mutant plants.

Table S1. Primers used for mutant genotyping.