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ORIGINAL ARTICLE

The role of cytokinin receptors in *Arabidopsis thaliana* seed development and how they affect the metabolomic profile

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Abstract

Main conclusion **Based on expression, functional, and metabolomic analyses in the seeds of the single-receptor mutants, each receptor has a specific function during seed development. Their redundant roles during this process are difficult to assess; moreover, the impact they have on plant development must also be taken into account.**

Abstract In this study, we investigated the role of cytokinin receptors in *Arabidopsis thaliana* seed development and their impact on the metabolomic profile. Our findings reveal distinct expression patterns among them in the seed: *AHK2* expression is not detected in seed tissues, *AHK3* is expressed in embryo, endosperm, and peripheral endosperm, while *AHK4/CRE1* expression is restricted to a few embryo cells. These patterns are consistent with the observed phenotypes where *ahk3* exhibits more severe seed phenotypes such as delayed embryo development and increased seed and endosperm size. Metabolomic analyses showed that the receptors impact the abundance of metabolites, with a remarkably high concentration of tannins in *ahk2* with respect to wild type seeds, while *ahk3* mutant seeds have a very low amount of tannins but elevated levels of other compounds such as sinapoylated glucosinolates (GSLs), important for plant defense. The metabolic profile performed further supports a link between cytokinin and the regulation of secondary metabolites such as flavonoids and glucosinolates. Our results suggest that each cytokinin receptor independently contributes to this regulation, reflected in the distinct metabolic profiles of each mutant.

Keywords Seed development · Cytokinin receptors · Metabolome · In situ *hybridization* · *Arabidopsis thaliana* · Flavonoids

Introduction

Plant hormones are signaling molecules that play a crucial role in many plant developmental processes, regulating seed germination, plant reproduction and growth and plant response to biotic and abiotic stress (Bari and Jones 2009; Pieterse et al. 2012; Checker et al. 2018). Cytokinin (CK) is an adenine-derived hormone (Letham 1973; Romanov and Schmülling 2022) that is involved in the regulation of various processes through the plant life cycle. CK were first described by their role in the activation of cell division, cytokinesis (Miller et al. 1955, 1956; Amasino 2005) and then by their influential role in organ primordia initiation (Skoog 1957). Cytokinin receptors are primarily in the membrane of the endoplasmic reticulum (ER), but have also been reported in the plasma membrane (Romanov et al. 2018). In the cell, the cytokinin signal is perceived by receptors and translated by a two-component signaling system (TCS) consisting of a membrane-localized sensor kinase that perceives the stimuli and a response regulator that propagates the signal, often by directly regulating the transcription of target genes (Reviewed by: Hu and Shani 2023; Wulfetange et al. 2011; Bishopp et al. 2006; Kakimoto 2003).

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In *Arabidopsis thaliana* (Arabidopsis) there are three paralogs that encode the CK receptors (His kinase receptors) ARABIDOPSIS HISTIDINE KINASE2 (*AHK2*), *AHK3*, WOODEN LEG/CRE1/AHK4 (*WOL/AHK4* (Mähönen et al. 2000; Ueguchi et al. 2001; Bishopp et al. 2006), *AHK4* hereafter). The extracellular sensory domain called the Cyclase/Histidine kinase-Associated Sensing Extracellular (CHASE) domain, associated with the Histidine kinase domain in the cytosolic region, which is recognized by the cytokinin, flanked by two transmembrane domains: a canonical receiver domain, and a diverged receiver domain (Hwang and Sheen 2001; Inoue et al. 2001; Suzuki et al. 2001; Yamada et al. 2001; Nishimura et al. 2004; Higuchi et al. 2004). The signal is then transferred via ARABIDOPSIS HISTIDINE-CONTAINING PHOSPHOTRANSFER PROTEINS (AHPs) which are small cytoplasmic proteins (Hutchison et al. 2006) to ARABIDOPSIS RESPONSE REGULATORS (ARRs) (Suzuki et al. 1998; Tanaka et al. 2004). Among these ARRs, there are two types: A and B. Type-B ARRs (ARR-B) are transcription factors that belong to the GARP family controlling the expression of cytokinin regulated genes, including ARRs type A (Sakai et al. 2001; Mason et al. 2004).

During plant vegetative development, cytokinin regulates cell division, apical dominance, shoot initiation and growth, phyllotaxis, leaf senescence as well as root development (Werner and Schmülling 2009; Hwang et al. 2012; Schaller et al. 2014; Kieber and Schaller 2018; Wybouw and De Rybel 2019; Cortleven et al. 2019). Furthermore, by quantifying anthocyanins, it was reported that the hormone CK can increase the anthocyanin accumulation in the shoot after the application of cytokinin (Deikman and Hammer 1995).

During the reproductive stage, CK regulate many plant traits such as organ size and maintaining the meristematic activity of the carpel marginal meristem, from which tissues such as the placenta and ovules are derived (Reyes-Olalde et al. 2017; Zuñiga-Mayo et al. 2018); CK are also involved in the regulation of ovule number (Cucinotta et al. 2016, 2018) and therefore in seed yield (Bartrina et al. 2011). They also play a role in female gametophyte development specifying the functional megaspore, during ovule development (Bencivenga et al. 2012; Cheng and Kieber 2013; Cheng et al. 2013). In seed development, CK levels are deeply (Rock and Quatrano 1995) reviewed to (Tomaz and Marina 2010) being involved in seed size, seed development and germination, as well as in procambial cell division and vascular differentiation during embryo development (Riefler et al. 2005; Bartrina et al. 2011; Heyl et al. 2012). The developing seed is a site of cytokinin biosynthesis and have been reported that endogenous cytokinin changes are major regulators controlling the phase of nuclear and cell divisions and establishment of sink size, this fine control involves CK homeostasis controlled by isopentenyltransferases and cytokinin oxidase/dehydrogenase gene family members for both cytokinin biosynthesis and degradation respectively (Jameson 2023; Jameson and Song 2016).

In most angiosperms, upon double fertilization, the ovule undergoes a series of ontogenetic transformations during which each of the three tissues: seed coat, endosperm and embryo, that form the seed of an angiosperm begins to grow and differentiate in a synchronous manner, ensuring the proper development of the entire structure (Ingram 2010; Orozco-Arroyo et al. 2015; Figueiredo et al. 2016). This complex process is tightly controlled by several regulators such as genes specific to each tissue of the seed, genes with maternal or paternal imprinting and hormones (Garcia et al. 2005; Aw et al. 2010; Zhang et al. 2011; Roszak and Köhler 2011; Batista et al. 2019a). Specifically, within the seed, after fertilization during the rapid nuclear division phase, cytokinin levels increase in the endosperm (Bennett et al. 1973; Day et al. 2008; Jameson and Song 2016; Xu and Zhang 2023). In Arabidopsis, at the pre-globular embryo stage, cytokinin activity appears to be low in the seed coat, and in the endosperm, decreasing to be restricted to the chalazal endosperm (Li et al. 2013).

Due to the crucial role of CK in activating morphogenetic events during reproductive development, understanding the phenotype and expression patterns of each cytokinin receptor is important to unveil their specific roles, similarities and differences. The influence of CK receptor sites of action can be observed in the triple mutant *ahk2 ahk3 ahk4* that exhibits severe developmental defects and is almost sterile (Nishimura et al. 2004; Higuchi et al. 2004; Riefler et al. 2005; Kinoshita-Tsujimura and Kakimoto 2011), while single mutants and all combinations of double mutants exhibit less severe defects (Wu et al. 2021), suggesting a high degree of redundancy. The three CK receptors differ in their expression patterns, biochemical properties, and biologic functions in plant development (Bishopp et al. 2006; Heyl et al. 2012). The role of each cytokinin receptor, AHK2, AHK3 and AHK4, during plant development can be observed through the single mutant phenotype (Supplemental Fig. 1). While AHK2 and AHK3 control transition to reproductive development in opposite ways, the *ahk2* mutant shows an earlier reproductive transition whereas *ahk3* mutant shows a delay (Igarashi et al. 2009; Danilova et al. 2017). AHK4, best known for its role in root elongation and response to exogenous cytokinin, has little effect on reproductive tissues (Riefler et al. 2005), *ahk4* mutant treated with exogenous cytokinin 6-Benzylamino purine (BAP) developed two integuments just as the wild type, whereas the AHK2 and AHK3 appear to play a major role in the response to CK in the chalazal region, as single mutants show only one integument (Bencivenga et al. 2012). The triple mutant *ahk2 ahk3 ahk4* shows a dramatic reduction in cytokinin response with defects in female gametophyte formation (Higuchi et al.

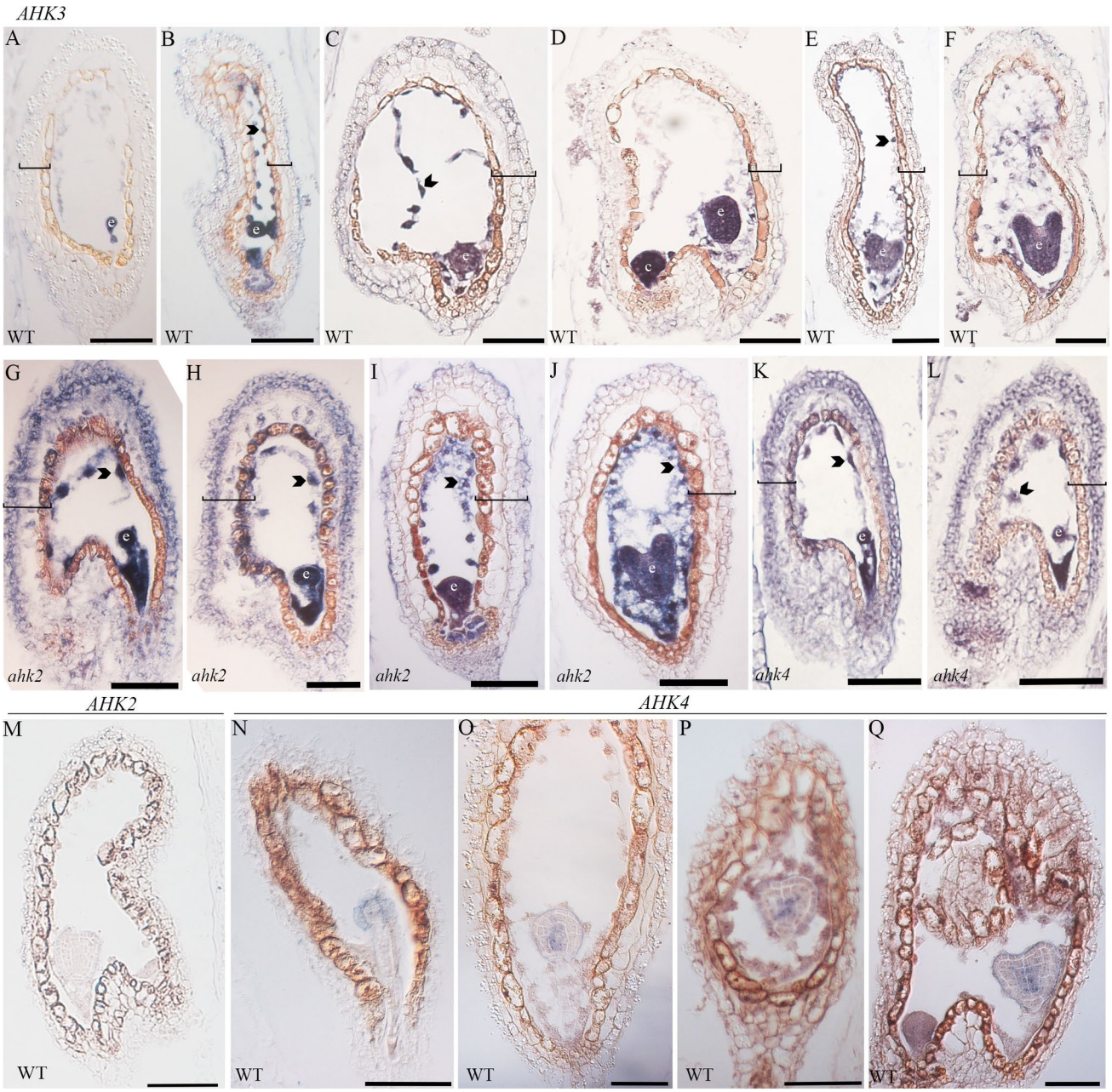


Fig. 1 In situ *hybridization* expression patterns of *AHK3*, *AHK2* and *AHK4*. **A-F** Expression in wild type seeds of *AHK3*. **A** *AHK3* is present in the embryo at 4-cell stage. **B** At 16-cell embryo stage the expression is also detected in the peripheral endosperm. **C** At globular stage *AHK3* is expressed in the embryo and peripheral endosperm. **D** At triangular stage, the expression is detected in the endosperm, embryo and chalaza. **E** Expression in the embryo at early heart stage and the endosperm. **F** Expression in the embryo at heart stage and in the endosperm. **G–J** Expression of *AHK3* in *ahk2* mutant background. **K–L** Expression of *AHK3* in *ahk4* mutant. *Black arrowhead* pointing to the peripheral endosperm; *squared bracket* to show the seed coat; *c*, chalaza; *e*, embryo; *WT*, wild type. *Scale bars:* 50 µm. **M–Q** Expression of *AHK2* and *AHK4* in wild type background seeds. **M** No expression was detected for *AHK2*. **N–Q** expression of *AHK4* restricted to the embryo throughout its development. *Scale bars* 100 µm

2004; Kinoshita-Tsujimura and Kakimoto 2011; Bencivenga et al. 2012). Although the number of seeds produced by the triple mutant is reduced, these seeds are larger than those of the wild type, a direct consequence of the role of these receptors in growth control (Riefler et al. 2005). Moreover, under the dark conditions, cytokinin receptors contribute differently to the regulation of seed germination. In this regard, AHK4 has been shown to promote germination by significantly enhancing it with respect to the wild type as well as the other CK receptors (Riefler et al. 2005).

In this study, we focused on the functions of different cytokinin receptors during seed development in *Arabidopsis thaliana*. Even though other factors involved in cytokinin pathway may have also functions in seed development, such as isopentenyltransferases (IPTs) that synthesize cytokinin, appear to be more involved in response to stress (Nguyen et al. 2021), and CYTOKININ OXIDASE/DEHYDROGENASES (CKXs), which degrade cytokinin, control ovule number due to the regulation of the meristematic activity of the placenta (Bartrina et al. 2011; Galbiati et al. 2013), however, their direct role in seed development has been little studied. We characterized the spatiotemporal expression patterns of each CKs receptor, *AHK2*, *AHK3* and *AHK4*, during seed development to determine their site of activity as well as to predict the localization of cytokinin accumulation sites. We describe, with emphasis on tannins, the seed phenotype of each single mutant, *ahk2*, *ahk3*, and *ahk4* and provide relative metabolite quantification, present in each mutant with respect to the wild type seeds. Although *AHK2* expression was not detected by in situ *hybridization*, we show that the cytokinin receptors have different expression patterns in the seed, that *AHK3* is expressed in the endosperm and embryo and *AHK4* is restricted to a few cells of the embryo. Furthermore, with the metabolic profile in the seeds we show that *AHK2* and *AHK3* appear to have an opposite effect in seed flavonoid accumulation. Thus, *ahk2* mutant seeds present more accumulation of tannins while *ahk3* mutant seeds have less tannin accumulation. In contrast, *ahk4* seeds have similar tannin accumulation compared to the wild type seeds, these patterns are consistent with those previously reported for other functions of cytokinin receptors in the plant such as the regulation of transition to reproductive development and leaf senescence. This research sheds light on how the cytokinin signaling pathway could influence the biosynthesis of essential metabolites during seed formation, presenting potential targets for improving seed quality.

Results

Expression patterns of the cytokinin receptors in the seed by in situ *hybridization*

To first assess where each of these receptors plays a specific role in the seed, we performed in situ *hybridization* for each receptor (Fig. 1). Although the site of expression is not necessarily correlated with the site where translation occurs, this procedure seemed relevant to address the differences in the phenotypes. This approach allowed us to detect very different expression patterns between the different CK receptors. For instance, expression of *AHK3* (Fig. 1A–F) and *AHK4* (Fig. 1N–Q) was detected in wild type seeds, whereas no detectable signal of *AHK2* was observed in wild type seeds (Fig. 1M). This suggests a very low or absent signal of *AHK2* receptor in seeds. The low *AHK2* signal may reflect low expression or expression below detection limits. We cannot exclude that the post-translational localization of the receptor may differ from its transcription domain, and thus the transcription pattern may not directly correlate with the receptor site of accumulation.

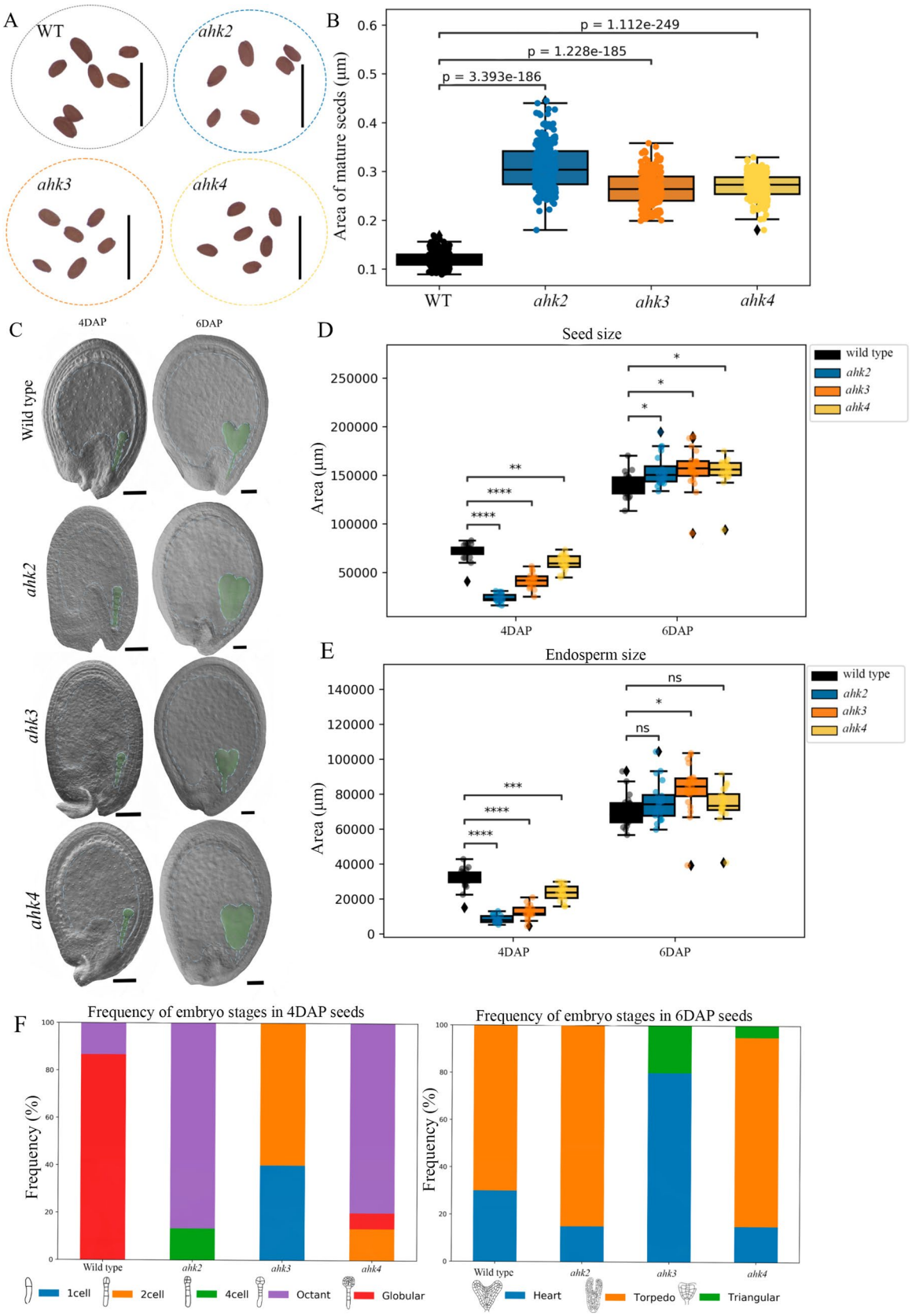
The expression of *AHK4* in wild type seeds was restricted to a few cells of the embryo (Fig. 1N–Q). As for the expression of *AHK3,* it was detected in the embryo throughout seed development (Fig. 1A–F) and was also localized in the peripheral endosperm (Fig. 1B, C), and then throughout endosperm development (Fig. 1D–F).

We also performed expression analysis of the *AHK3* in the other receptor mutants to uncover potential genetic interaction among them. The expression of *AHK3* in *ahk2* mutant seeds at early stages of seed development was detected not only in the embryo and peripheral endosperm but also extended to the seed coat (Fig. 1G–H). Expression that was not detected onwards later in *ahk2* developed seed (Fig. 1I, J). In *ahk4* mutant seeds, the expression of *AHK3* is detected in the embryo, endosperm, and also extended to the seed coat (Fig. 1K, L).

Overall, these data suggest a potential regulatory role of AHKs during seed development. It also highlights the potential compensatory mechanisms to which the three receptors are subjected, allowing them to play a role in different tissues.

Cytokinin receptor mutant seeds are bigger at the end of their development

Cytokinin receptor mutants are known to exhibit reduced plant growth (Supplemental Fig. 1) and larger seed size, which we confirmed by examining the dry seed surface area (Fig. 2A, B). In all genotypes, the increase in seed size is significant compared to the wild type seeds. Despite *ahk2* having larger seeds between the mutant lines, there are no significant differences to each other's (Fig. 2B). The seed development in the CK receptor mutants appears to be delayed; at 3 Days After Pollination (DAP) there are still many unfertilized ovules than seeds, and it was not possible to determine whether these are unfertilized ovules or delayed seeds (Supplemental Fig. 2). However, as we found more seeds at 4DAP and the subsequent developmental stages, it may suggest a delay in development. Thus, to detect the major differences in seed phenotypes, we measured the seed size at two times in the development process, 4 and 6 DAP


A
WT
ahk2
ahk3
ahk4
B
p = 1.112e-249
p = 1.228e-185
p = 3.393e-186
Area of mature seeds (μm)
0.6
0.5
0.4
0.3
0.2
0.1
WT
ahk2
ahk3
ahk4
C
4DAP
6DAP
Wild type
ahk2
ahk3
ahk4
D
Seed size
Area (μm)
250000
200000
150000
100000
50000
4DAP
6DAP
wild type
ahk2
ahk3
ahk4
E
Endosperm size
Area (μm)
140000
120000
100000
80000
60000
40000
20000
0
4DAP
6DAP
wild type
ahk2
ahk3
ahk4
F
Frequency of embryo stages in 4DAP seeds
Frequency (%)
Wild type
ahk2
ahk3
ahk4
1cell
2cell
4cell
Octant
Globular
Frequency of embryo stages in 6DAP seeds
Frequency (%)
Wild type
ahk2
ahk3
ahk4
Heart
Torpedo
Triangular

◂ **Fig. 2** Seed phenotypes in the three cytokinin receptor single mutants. **A** Dry seed pictures for each of the genotypes analyzed. **B** Box-plot showing measurements of the dry seeds. With the respective *p* value shows statistical significance between the wild type and all the mutant seeds that are bigger, 100 seeds were measured by genotype. There is no significative difference between *ahk3* and *ahk4* seeds. **C** Selected seed clearing for each genotype showing in green the embryo and a dotted line in light blue showing the cavity of the endosperm. **D** Box-plot showing the seed size at 4 and 6 days after pollination, all mutants are different compared to the wild type. **E** Box-plot showing the difference between the endosperm cavity size (as shown with the dotted line in the seed clearings). **F** Percentage frequency of embryo stages (shown in different colors) detected at 4 and 6 DAP. At least 25 seeds per genotype were measured for **D**–**F**. *p* value annotation legend: ns (not significative) p value > 0.05; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ Scale bars:1 cm (**A**); 50 µm (**C**)

(Fig. 2C). At 4DAP, cytokinin receptors mutant seeds are smaller than those of the wild type, suggesting that there might be a considerable delay in seed development (Fig. 2D). At 6DAP, the CK receptors mutants are larger than those of the wild type seeds, indicating a considerable accelerated increase in seed size during the later stages of development. These phenotypical differences will remain as *ahk2, 3* and *4* have a larger final seed size than the wild type (Fig. 2B, D). To detect whether there is a correlation between the endosperm size and final seed size, we measured the internal cavity of the endosperm—known as a major driver of seed expansion—(Fig. 2E). At 4 DAP, a direct correlation between seed size and endosperm size was observed, however, at 6DAP no significant difference was detected between the endosperm of both *ahk2* and *ahk4* seeds compared to the wild type seeds, and only *ahk3* shows a correlation between endosperm size and seed size, meaning that as the *ahk3* seed grows, the endosperm cavity also grows. This could suggest that (1) *ahk2* and *ahk4* final increase in seed size does not depend on the endosperm size but rather on the seed coat; or (2) that when there is a delay in endosperm development, the growth dynamics increase will only be visible later during seed development (Fig. 2E). The embryo stages were also documented at 4 and 6 DAP, and the frequency of occurrence of each stage was calculated as a final percentage. This allowed us to observe that the delayed seed development for *ahk3* (seen in Fig. 2C) is accompanied by a considerable delay in embryo development in the *ahk3* seeds, which are still at the "heart" stage at 6 DAP. In contrast, at 6DAP the wild type, *ahk2*, and *ahk4* seeds mostly had embryos at the "torpedo" stage (Fig. 2F). This, together with previous observations on the very specific expression pattern of *AHK3* in embryo developmental stages, suggests a major role of AHK3 in embryo development growth.

Cytokinin receptors do not play a major role in genome dosage response mechanisms control

In recent years, interploid crosses have emerged as a valuable tool in Arabidopsis research for testing molecular models related to the control mechanisms of seed development and growth (Zumajo-Cardona et al. 2023). By crossing diploid Arabidopsis with 2x (diploid) and 4x (tetraploid) plants in the Columbia ecotype, researchers can create seeds with different genome dosages, impacting seed formation. For example, a 2x Col maternal plant crossed with a 4x Col paternal plant results in seeds with a higher paternal genome dosage and a high percentage of abortions. Given that the *AHK3* receptor was specifically expressed in the embryo and the observations that *AHK3* mutation causes significant defects in this tissue, we hypothesized that it might be involved in the genome dosage response to interploidy crosses. To test this, we assessed the potential maternal impact on rescuing seed development by crossing with a paternal excess 4x pollen donor. The results showed a similar percentage of seed abortions compared to the wild type. A cross of unbalanced type (2x Col X 4x Col) resulted in 80% of abortions (as reported in different papers Dilkes et al. 2008; Zumajo-Cardona et al. 2023); the type (2x *ahk2* X 4x Col) showed 85% of abortions; type (2x *ahk3* X 4x Col) resulted 89%; while type (2x *ahk4* X 4x Col) presented 95% of abortions (Supplemental Fig. 3). Similarly, abortions were observed in seeds from crosses of the 2x mutants with 2x wild type pollen, suggesting that receptors for cytokinins have a maternal effect in proper seed development due to their effect during ovule development, on integument and female gametophyte formation (Bencivenga et al. 2012). This also indicates that they play little or no role in the genome-dosage response mechanism.

Different metabolic profiles in cytokinin receptor mutant seeds in relation to wild type seeds

Considering that the cytokinin hormone regulates flavonoid biosynthesis (Ng et al. 2015; Abdelrahman et al. 2021; Kurepa et al. 2023), we hypothesized that the absence of cytokinin receptors may have an impact on the accumulation of metabolites such as flavonoids. In this respect, we performed an untargeted metabolomics investigation on dry seeds in order to have a relative quantification and to identify the differences between the metabolomic profiles of wild type seeds and *ahk2*, *ahk3* and *ahk4* seeds. The chromatograms from the liquid chromatography–mass spectrometry (LC–MS) show differences in the main molecules of hydroalcoholic extracts identified for each genotype (Supplemental Fig. 4). The data resulting from the LC–MS were subjected to multivariate statistical analysis, generating a PCA-X model, showing that all samples are different from

each other (Supplemental Fig. 5). To understand which molecules were responsible for such a sample distribution, we performed a supervised multivariate statistical analysis, O2PLS-DA (orthogonal bidirectional partial least square—discriminant analysis) dividing the samples into four different classes, one for each genotype (Supplemental Fig. 6). The loading plot showed that specific metabolites characterized one class better than the others. In detail, the wild type and *ahk4* seeds contain glucosinolates (GSLs) (Fig. 3), a characteristic compound of the Brassicaceae family (Wittstock and Burow 2010; Nguyen et al. 2020), *ahk3* seeds contain sinapoylated GSLs and some hydroxycinnamic acids, in particular glycosylated sinapic acid (HCA glycosylated) while *ahk2* seeds contain more condensed tannins, procyanidins, with respect to the other seeds analyzed (Fig. 3).

In fact, the relative abundance of single tannin classes appears to be higher in *ahk2* mutant seeds than in the other seeds (Fig. 4A) and *ahk3* has less, while *ahk4* appears with content similar to that of wild type (Fig. 4A). All the seeds

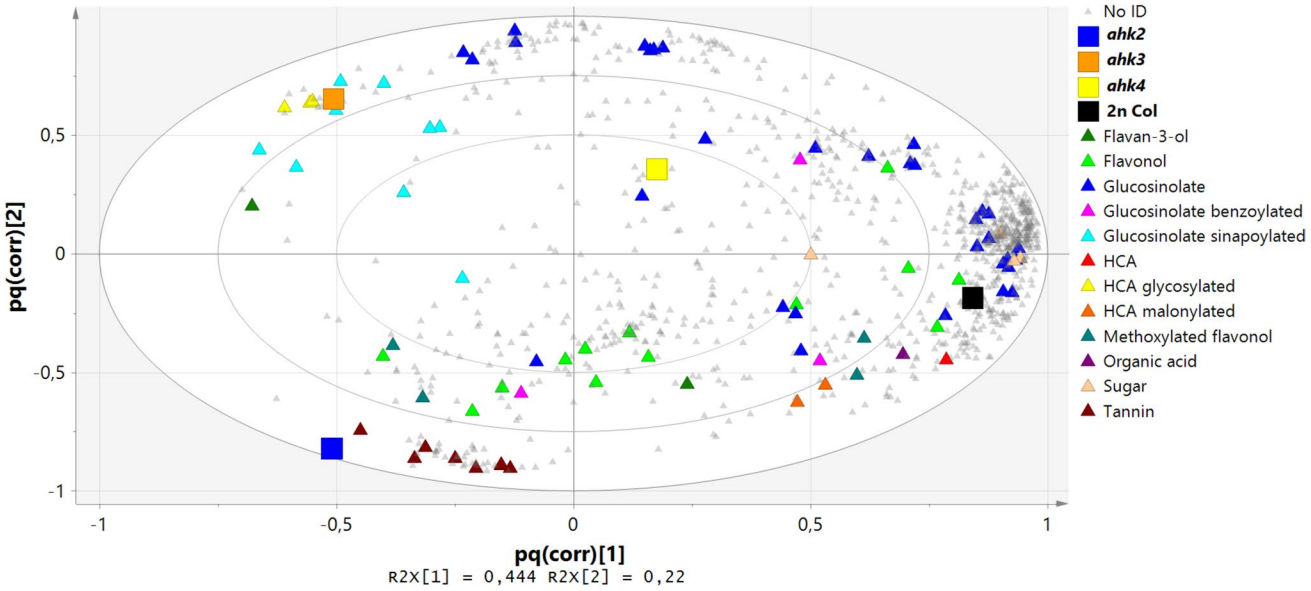


Fig. 3 Orthogonal bidirectional partial least square—discriminant analysis (O2PLS-DA) loading plot. Metabolites were colored based on the genotype they belong to. Identified metabolites are indicated by colored triangles, unidentified metabolites by shrunken and semi-transparent triangles. The squares represent the 4 classes: in black the 2n Col, in yellow the *ahk4*, orange the *ahk3* and in blue the *ahk2*. Triangles with colored borders (putatively identified metabolites) enclose to a specific class (square) positively correlate with that specific group of samples

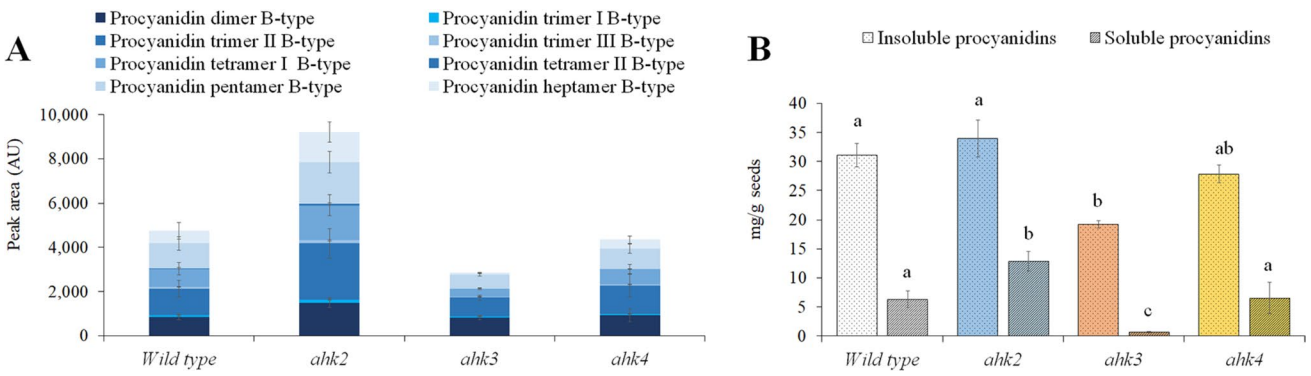


Fig. 4 Abundance of tannins in the seeds of *A. thaliana* samples. **A** Relative abundance of individual tannins in the different seed samples was obtained through UPLC-HRMS untargeted metabolomics analysis. *AU* arbitrary unit. The error bar indicates the standard deviation based on the three biologic replicates investigated. **B** Absolute values (mg/g seed) of procyanidins in the various genotypes of *A. thaliana* seeds. Levels of insoluble procyanidins are shown in dotted bars and those of soluble procyanidins in stripped bars. Error bars indicate the standard deviation from three biologic replicates

analyzed contain more insoluble procyanidins than soluble, with a relative amount that varies with respect to the wild type, except for *ahk4* seeds which have a similar amount of insoluble and soluble procyanidins compared to the wild type (Fig. 4B). *ahk2* seeds present the same amount of insoluble procyanidins as the wild type seeds and a significant increase in the amount of soluble procyanidins (Fig. 4B). In contrast, *ahk3* has a significantly lower amount of both insoluble and soluble procyanidins (Fig. 4B). In summary, AHK2 and AHK3 receptors may have opposite roles in tannin accumulation, as *ahk2* seeds contain more procyanidins, especially soluble procyanidins, and *ahk3* seeds contain less insoluble and soluble procyanidins. The *ahk4* seeds show no significant differences compared with wild type seeds, suggesting that the AHK4 receptor does not play a role in tannin accumulation in the seed (Figs. 3 and 4).

Glucosinolates, particularly high in *ahk3* cytokinin receptor mutant seeds compared to wild type seeds

Cytokinin receptor *ahk3* mutant seed samples are characterized by higher values of sinapoylated GSLs and glycosylated sinapic acid (Fig. 3), showing a higher relative abundance of individual sinapoylated GSLs in *ahk3* compared to all other samples (Fig. 5A). While in *ahk2* and *ahk4* seeds, the amount of these compounds is slightly higher than in the wild type (Fig. 5A), the most abundant being 4-Benzoyloxy-*n*-butyl-glucosinolate sinapoylated, glucoconringiin sinapoylated and glucoerucin sinapoylated (Fig. 5A).

To observe the trends of all sinapic acid derivatives in a single graph, the values of individual metabolites were converted from absolute to relative by setting the highest accumulation value of a specific metabolite observed among the samples as 100%. For example, the highest level of sinapic acid dihexose was observed in sample *ahk3* biologic replicate 2: this value was placed as 100% and was used as the basis for calculating the relative abundances of sinapic acid dihexose in the other samples. In detail, the relative abundances of individual sinapic acid derivatives show that *ahk3* has higher values of the glycosylated forms of sinapic acid and low levels for those conjugates with malic acid and choline (Fig. 5B). We could also establish that the overall level of sinapic acid derivatives accumulation in the *ahk3* mutant is still comparable to the other genotypes (Fig. 5B).

In terms of the different aliphatic GSLs, wild type seeds show a positive correlation with short-chain aliphatic GSLs such as 4-Methylsulphinylbutyl—(4MSOB) and 4-Methylthiobutyl—(4MTB) for which we observe a higher relative abundance (Fig. 5C). On the other hand, *ahk4* seeds seem to be positively correlated with some GSLs (Fig. 3) and, more specifically, these are long-chain aliphatic GSLS such as 8-Methylsulphinyloctyl—(8MSOO) and 8-Methylthiooctyl-(8MTO) (Fig. 5C).

Discussion

Due to their diverse roles in plant development, cytokinin receptors have been extensively studied. Double and triple mutants result in a general reduction of plant size (Riefler et al. 2005). The three receptors have been reported to be involved in the apical–basal patterning of the gynoecium with, probably, not completely redundant roles (Zúñiga-Mayo et al. 2014). The triple mutant, *ahk2 ahk3 ahk4*, shows defects in the formation of the female gametophyte (Bencivenga et al. 2012; Cheng et al. 2013). Although the single receptors present also unique and specific functions (Riefler et al. 2005; Igarashi et al. 2009; Danilova et al. 2017; Cerbantez-Bueno et al. 2020) their overall influence on reproductive structures, particularly regarding seed development and yield, has been little studied (Riefler et al. 2005; Heyl et al. 2012; Cerbantez-Bueno et al. 2020). To fill this gap of knowledge, here we focused on the developmental and functional characterization of the single mutants during seed development. Our main objectives were: (1) to increase the understanding of the individual roles of these receptors; (2) to elucidate the singularity of their specific functions, as despite a significant overlap in the functions of these receptors. We demonstrate variations in their individual expression patterns within reproductive structures that may imply unique functions for each. Addressing the study of seed development from double mutants represents a challenge in itself, considering that double mutants such as *ahk2 ahk3* have fewer ovules and fewer seeds (Cerbantez-Bueno et al. 2020). Additionally, since the triple mutant *ahk2 ahk3 ahk4* produces very few flowers and those that develop are sterile, without seeds (Nishimura et al. 2004; Higuchi et al. 2004; Kinoshita-Tsujimura and Kakimoto 2011), the study of seed development cannot be carried out with the triple mutant.

AHK3 expression consistent with its severe effects on seed development

The function of cytokinin and the cytokinin receptors in seed development has been previously described to be linked to seed size (Riefler et al. 2005; Hutchison et al. 2006; Argyros et al. 2008). Here we corroborate that each receptor has its own expression pattern (Fig. 1) and independently controls seed size, all the cytokinin receptor single mutants exhibit bigger seeds (Fig. 2A, B). Seed growth is coupled to endosperm growth, the larger the endosperm the larger the seed (Sundaresan 2005). Since cytokinin homeostasis is directly linked to endosperm development in several species (Mizutani et al. 2010; Basunia and Nonhebel 2019), we also performed the assessment of this phenotype.

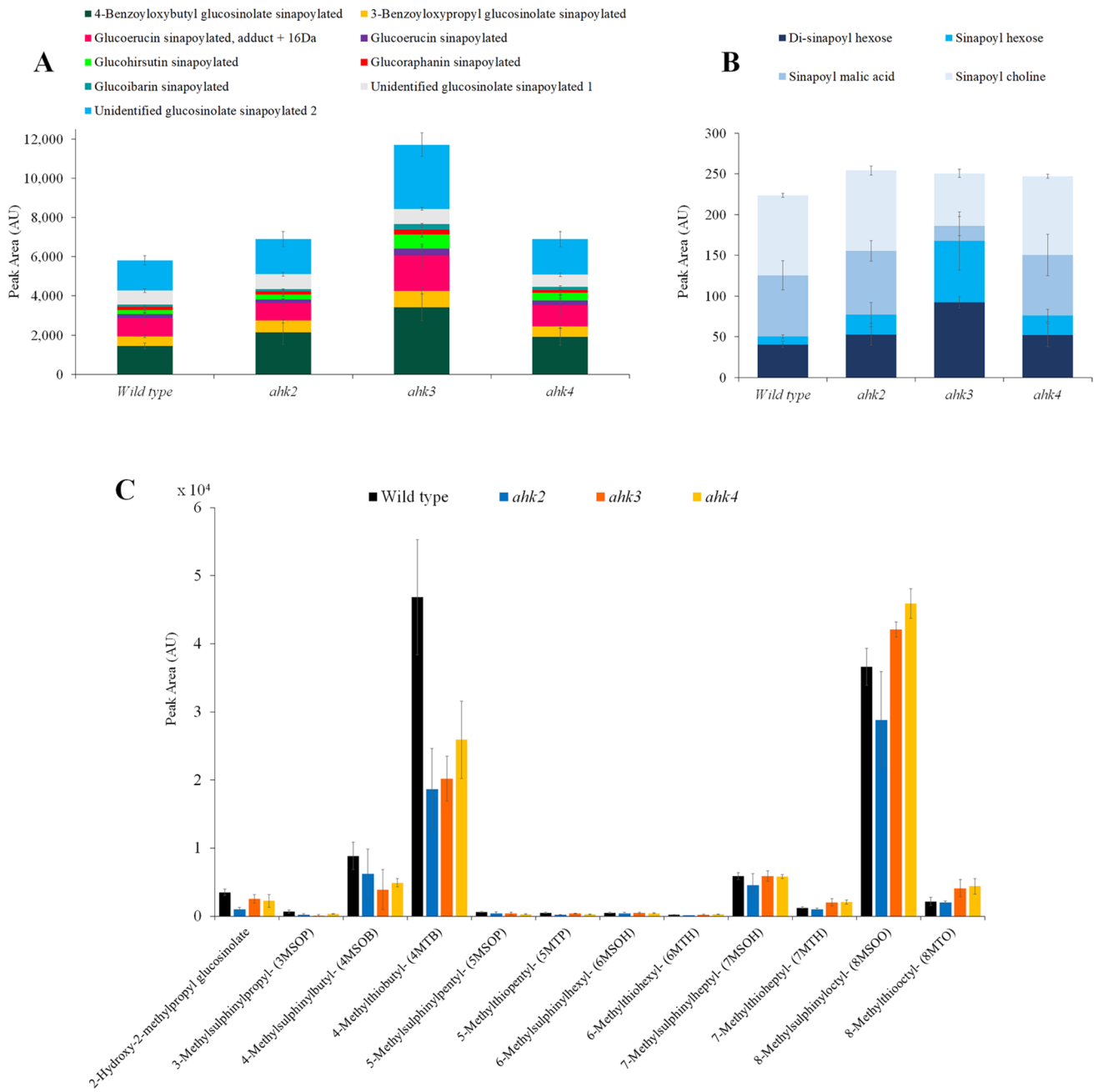


Fig. 5 Quantification of glucosinolates and sinapic acid derivatives. **A** Relative amount of sinapolated glucosinolates. **B** Relative amount of sinapic acid derivatives. **C** Relative abundances of the major aliphatic chain glucosinolates. The aliphatic chain length increases as one moves to the right along the *x* axis. All graphs are in terms of *AU* arbitrary unit

In the wild type, at the early stages of development, the levels of the hormone cytokinin are high in the endosperm during the syncytial stage, as the nuclei divide, controlling the growth of the different seed tissues (Mok and Mok 2001; Day et al. 2008; Jameson and Song 2016). In fact, 4 days after pollination, cytokinin receptor mutants have smaller seeds and endosperm with respect to the wild type (Fig. 2D, E). Earlier stages of seed development were difficult to characterize because fertilized seeds were difficult to identify, which could be a result of the fertilization defects previously reported for these mutants, in which the anthers do not dehisce, the pollen grains do not mature, the stigma does not induce the pollen to germinate, and there is a failure to form the female gametophyte (Kinoshita-Tsujimura and Kakimoto 2011). Alternatively, it could also be due to a delay in seed development. Later, in wild

type seeds, during cell wall formation in endosperm cells, high expression of gene families involved in cytokinin catabolism, is observed (Werner et al. 2003; Cheng and Kieber 2013; Li et al. 2013) suggesting differences in cytokinin levels throughout seed development.

However, control of endosperm and seed growth is a tightly regulated characteristic with environmental, and epigenetic factors as well as different gene pathways (Garcia et al. 2005; Jofuku et al. 2005; Ohto et al. 2005, 2009; Köhler and Makarevich 2006). One of the best characterized mechanisms regulating endosperm and seed growth is the IKU pathway (Werner et al. 2003; Cheng and Kieber 2013; Li et al. 2013), the *iku1* and *iku2* mutants produce smaller seeds, which may be linked to the fact that the IKU pathway is upstream of cytokinin activity, a circumstance that appears to impact cytokinin activity, repressing it. The double mutant of the IKU genes with each of the cytokinin receptors shows larger seeds (Werner et al. 2003; Cheng and Kieber 2013; Li et al. 2013). Thus, the IKU cytokinin pathway may explain the observed phenotype exhibiting larger seeds, a trait that becomes evident 6 days after pollination, with an increase in seed and endosperm size (Fig. 2D, E). Compared to wild type seeds, changes in endosperm size of cytokinin receptors mutants throughout seed development, also appear to be linked to delayed embryo development (Fig. 2F). Correct seed development relies on the synchronized growth, differentiation, and functional integration of all tissues, embryo, endosperm, and seed coat. This synchronized development occurs due to an exchange of nutrients and hormones (Figueiredo and Köhler 2014).

The detailed phenotypes described here in endosperm and embryo development, show that the role of cytokinin is crucial for the proper development of endosperm mainly, which is linked to the widely previously reported phenotype in seed size (Riefler et al. 2005; Hutchison et al. 2006; Argyros et al. 2008). Moreover, even if all cytokinin receptor single mutants show defects, the major impact is observed in the single mutant *ahk3,* which is consistent with the high expression in the endosperm and embryo (Fig. 1). Interestingly, it has been previously reported expression of *AHK4* in the endosperm (Day et al. 2008), expression that we have not been able to detect with our methods, which may due to low levels of expression of *AHK4* in this particular tissue, compared to *AHK3* (Fig. 1). In fact, *ahk3* shows more severe effects in endosperm size, besides the fact that embryo development is notably more delayed (Fig. 2).

In interploidy crosses, an excess in paternal genomic dosage is associated with endosperm over-proliferation and cellularization failure, leading to larger F1 seeds, including those that may collapse or abort. Conversely, precocious endosperm cellularization and reduced F1 seed size are correlated with F1 seeds having maternal-excess genome dosage. During the last years, interploidy tests screenings has been exploited to test and validate the potential roles of effectors, transcription factors, signaling mechanisms and molecular cascades involved in seed growth mechanisms. Substantial research in the field has reported during the last decades the important role of the different tissues involved (embryo, endosperm and seed coat) in the control response to the proper genome dosage balance (Dilkes et al. 2008; Huang et al. 2017; Jiang et al. 2017; Batista et al. 2019b; Zumajo-Cardona et al. 2023). To test the maternal effect on seed rescue, here we performed crosses using 4x pollen. Specifically, 2x Col X 4x Col crosses showed 80% abortions, consistent with previous reports (Dilkes et al. 2008; Zumajo-Cardona et al. 2023). Crosses with cytokinin receptor mutants showed similar high abortion rates in 2x *ahk2* X 4x Col, 2x *ahk3* X 4x Col, and 2x *ahk4* X 4x Col (Supplemental Fig. 3). Interestingly, similar abortion rates were already observed in isogenic progenies crosses (when 2x mutants were crossed with 2x wild type pollen), suggesting the existence of a maternal role of cytokinin receptors in the control of early ovule and female gametophyte development.

ahk2 and *ahk3* cytokinin receptors have opposite effects on metabolite accumulation in the seed

Interactions between the cytokinin and the flavonoid pathways seem to have a significant effect on nodule formation; in particular, cytokinin seems to induce several flavonoid-related genes (Ariel et al. 2012; Ng et al. 2015). However, regarding the link between cytokinin and flavonoids, there is still a long way to go to better understand their interactions. In the roots of some species, the induction of flavonoids by cytokinin alters auxin transport; flavonoids affect the expression and localization of auxin transporter proteins, PIN proteins (Peer et al. 2004; Santelia et al. 2008; Ng et al. 2015).

In this study, we found that in the seed, the metabolic profile changes in the absence of different cytokinin receptors (Fig. 3), based on previous results which highlight a link between cytokinin and flavonoids in other plant tissues (Ariel et al. 2012; Ng et al. 2015), we focused on the metabolic profile of flavonoids. In the seed, some flavonoids polymerize to form condensed tannins also known as procyanidins (De La Iglesia et al. 2010). Interestingly, *ahk4,* which has been shown to affect flavonoid accumulation in roots (Ng et al. 2015), has almost no effect on the seed, whereas *ahk2* shows more procyanidins and *ahk3* seeds have less (Fig. 4). This metabolic impact of *ahk2* is particularly interesting given that we did not detect *AHK2* transcript signal in the seed tissues analyzed (4 and 6 days after pollination). Considering that tannin accumulation occurs at late developmental stages of Arabidopsis seeds and the flavonoid profiling was conducted on dry mature seeds, we can hypothesize that AHK2 protein may be

mobilized from a different transcriptional domain into the seeds (mentioned before in results section), or that *AHK2* expression could have been activated at later developmental stages not included in this analysis.

Hormone–anthocyanin interactions have already been reported to be determinant of seed traits such as germination. In recent studies with viviparous Rhizophoraceae mangroves, it has been reported that the loss of the gene DELAY OF GERMINATION 1 (*DOG1*) function disrupts hormonal balance, reducing ABA sensitivity and proanthocyanidin accumulation, while promoting brassinosteroid-mediated seed coat weakening and vivipary (Qiao et al. 2024). There, authors report that the seed coat of non-viviparous *Ca. brachiata* is darker and higher in PA content than that of viviparous K. obovata at the comparable developmental stages before the hypocotyl breaks the seed coat (Fig S2 of Qiao et al. 2024). Additionally, the control of procyanidins in reinforcing the strength of the seed coat has been reported, showing that increased pigmentation leads to a tougher seed coat that is more difficult to penetrate, with reduced permeability (Debeaujon and Koornneef 2000; MacGregor et al. 2015). Given this, future studies should also examine the role of cytokinins in this network and controlling this aspect of the seed coat, considering the evolutionary patterns observed in the cytokinin gene pool described here.

Moreover, it has been shown that, tight phytohormonal regulation involving CKs, ABA, JAs are required for proper endosperm development, including the effect of crosstalks between them and other plant growth regulators in terms of highly coordinated signaling pathways An extended detail of specific genes involved can be seen in the paper of Day et al 2008. In consequence, it would be interesting to extend our results regarding tannin - cytokinin, with other hormones involved in seed development.

Glucosinolates, another type of secondary metabolite containing sulfur and nitrogen, are abundant in the order Brassicales to which Arabidopsis belongs (Radojčić Redovniković et al. 2008). As for the flavonoid accumulation and glucosinolates, we detected similarities between *ahk4* seeds and the wild type seeds, suggesting that the AHK4 receptor does not play a role in tannin accumulation in the seed. On the other hand, we found differences in the levels of glucosinolate accumulation in the case of *ahk2* seeds, where the level of accumulation is lower while it is higher in *ahk3* seeds (Fig. 5). Glucosinolates have also been reported to interact with plant hormones such as auxins, cytokinins, and ethylene, influencing processes such as cell elongation, differentiation, and tissue patterning (Malka and Cheng 2017). Together with the metabolic profile we present here, further efforts are still required to uncover whether GSLs exert a fine control role in maintaining inter-hormone homeostasis properly for optimal developmental processes.

Conclusions

Our study focused on having insight into independent roles of cytokinin receptors in seed development which sets up a foundation for further studies, aimed at better understanding the complex role of a hormone such as cytokinin in seed development. From this study, performed using homozygous plants, it appears that each receptor has a different expression profile, *AHK3* is expressed throughout the embryo and the endosperm, and *AHK4* is only detected in the embryo, while *AHK2* expression was not detected. These expression profiles correspond to the phenotypes we have described for each of these mutants in which *ahk3* has stronger effects on embryo and endosperm development compared to the other mutants. Understanding whether these phenotypes are solely maternally determined will require crosses using heterozygote maternal plants.

On the other hand, the metabolic profile performed provides evidence supporting the link between cytokinin and the regulation of secondary metabolites, such as flavonoids and glucosinolates. These results also suggest that each cytokinin receptor plays an independent role in this regulation, as each mutant has a different metabolic profile. In fact, it is observed that *ahk2* has a high concentration of tannins, while in *ahk3* the concentration is low. To understand its impact on seed development, it is essential to clarify which components of the flavonoid and glucosinolate pathways interact with the cytokinin pathway and what their functions are in seed development.

Materials and methods

Plant material and growing conditions

For this study, the following *Arabidopsis thaliana* lines were used: wild type (ecotype Columbia 2x and 4x, Col-0) and T-DNA insertion mutant lines of *ahk2-2* (SALK_052531), *ahk3-3* (SALK_069269.54.25.x) and *cre1-12* (SALK_048970.48.60.x*; CRE1* = *AHK4*). Plants were grown under long-day conditions (16 h light–8 h dark) at 22 °C in growth chambers at the University of Milan and its botanical garden and genotyped using primers listed in Supplemental Table 1. The seeds analyzed here are all the result of self-pollination.

Expression analyses by in situ *hybridization*

For the probe synthesis, flowers in anthesis and fruits were collected in liquid nitrogen, total RNA was extracted using plant RNeasy miniKit (Qiagen), following the instruction

of the manufacturer. First-strand cDNA was synthesized from total RNA to obtain a final concentration of 700 ng/µl using the iScript Kit (Bio-Rad, Hercules, CA, USA), following the instructions of the manufacturer. DNA templates for RNA synthesis probe were obtained by PCR amplification of 319–500-bp fragments with primers specific to each cytokinin receptor (Supplemental Table 1). Fragments were purified using the PCR purification kit (Macherey–Nagel by Fisher Scientific, Duren, Germany). Digoxigenin-labeled RNA probes were prepared using T7 RNA polymerase (Promega, Fitchburg, USA), murine RNAse inhibitor (Promega, Fitchburg, WI, USA) and RNA labeling mix according to each protocol of the manufacturer.

Self-pollinated carpels and fruits of all different genotypes at different stages of development were immediately fixed in 3.7% formaldehyde, 5% glacial acetic acid and 50% ethanol for 3 h. Samples were then dehydrated in a standard ethanol series and embedded in paraffin (Thermo Fisher Scientific, Waltham, MA, USA). RNA in situ *hybridization* was performed according to (Ferrandiz et al. 2000; Ambrose et al. 2000), with overnight hybridization at 55 °C. Probes were diluted at 1:25 for all the experiments. Slides were permanently mounted in Permount. Sections were viewed on a Zeiss Axiophot D1 optical microscope and digitally photographed with an Axiocam MRc5 digital camera and Axiovision software (v.4.1).

Morphologic analyses and microscopy

To detect the presence of procyanidins in the seed coat, seeds from the different ecotypes were collected 4 days after pollination. These seeds were stained with 1% (w/v) vanillin (4-hydroxy-3-methoxybenzaldehyde) in 6-*n* HCl (Pellegrini 1980) and photographed with a light microscope as described for in situ *hybridization* results.

For seed clearing, to analyze the embryo stage and endosperm area, 4 and 6 days after pollination carpels were fixed over-night in ethanol:acetic acid [9:1] and hydrated in an ethanol series. The seeds of the fruit were dissected, mounted in chloral hydrate:glycerol:water [8:1:3, w/v/v], and immediately observed under a Zeiss Axiophot D1 microscope equipped with DIC optics and an Axiocam MRc5 camera (Zeiss) with Axiovision software (version 4.1). The endosperm size was measured in ImageJ (https://imagej.nih.gov/ij/) using the 'analyze particles' function and the embryonic stage was observed and documented.

All graphs shown here were obtained with Jupyter Lab Notebook (Python v3), using Pandas, Matplotlib, and Seaborn libraries. All data and code sources used for the graphs are available on the GitHub page (https://github.com/cezumajo).

Sample preparation for metabolomic analyses of seeds

Mature dry seeds (at least 200 mg) were collected in liquid nitrogen, ground and stored at − 80 °C until use. Approximately 30 mg of samples were placed in 20 volumes of methanol:water (70:30) at 4 °C overnight. Secondary metabolites were extracted with an ultrasound ice bath for 15 min, the extracts were collected, and the pellet was reprocessed in the same manner to extract all the metabolites (Gao et al. 2010). Here, in a single extraction, we combined both LC–MS-based analysis and absolute quantification of soluble and insoluble procyanidins.

LC–MS untargeted metabolomic analysis

The generated extracts (*see sample preparation for metabolomic analysis* section) were diluted with 1:500 water LC–MS grade (Honeywell), filtered and analyzed with LC–MS. Reversed-phase chromatography was performed with an Ultra Performance Liquid Chromatography (UPLC) Acquity I-class (Waters) equipped with a BEH C-18 column (100 × 2.1 mm; 2.7 µm) at 30 °C and an FTN autosampler (Waters) refrigerated at 7 °C. The chromatographic method used features of gradient elution, lasting a total of 25 min, using two solvents: water with the addition of 0.1 percent formic acid (solvent A) and acetonitrile (solvent B). Molecules were detected using a Xevo G3 mass spectrometer (Waters) equipped with an Electrospray Ionization (ESI) type source and a qTOF (Quadrupole-Time-of-Flight) analyzer (0.8-kV capillary voltage, 30-V cone voltage, 120 °C source temperature). Exact mass calculation and calibration maintenance were carried out by direct infusion, at a flow rate of 10 µl/min of Leucine Enkephaline 100 pg/µl. Fragmentation was conducted using Argon gas in the collision cell and applying a voltage of 35 V. Scanning was between 50 and 2000 m/z and the scan time was set to 0.3 s.

The data matrix, in negative ionization mode, and extrapolation of the fragmentation spectra (ms/ms) for the various metabolites were generated using ProgenesisQI software (http://www.nonlinear.com/). The identification of the metabolites was conducted by comparing the retention time, exact mass and fragmentation pattern with those of a proprietary library of authentic commercial standards, and by comparison of exact mass and the fragmentation pattern with those of databases available online. Metabolite identification raw data based on peak detection profiles are presented in Supplemental Table 2. In particular, the most widely used database was Metlin (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage).

Quantification of soluble and insoluble procyanidins

Soluble procyanidins were quantified as previously described by Gao et al. (2010), from 400 ul (of the supernatant, *see sample preparation section*) with 1 ml of Butanol:HCl (95.5) at 98 °C for 70 min. Samples were diluted 1:10 with methanol acidified with 1% HCl before reading in the spectrophotometer.

For quantification of insoluble tannins, the pellet (*see sample preparation section*) was resuspended in a solution containing 400 µl of methanol:water 70:30, 1 ml of butanol:HCl 95:5 and 100 µl of $NH_4Fe(SO_4)_2$*$12H_2O$ solution in 2 M HCl and put in 98 °C for 70 min. The sample was diluted 1:20 with methanol acidified with 1% HCl before reading in the spectrophotometer.

For absolute quantification, a calibration line was prepared with Procyanidine B2 at different concentrations (mg/ml), 0.25, 0.1, 0.075, 0.05, 0.025, 0.01, 0.005, 0.001. From each solution, 400 µl were added to 1 ml of 95:5 (v/v) butanol:HCl solution and 100 µl of $NH_4Fe(SO_4)_2$*$12H_2O$ solution in 2 M HCl to allow the conversion reaction of procyanidin B2 to cyanidin to take place. This analysis was conducted twice.

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Author contributions IE, LC and CZ-C conceptualized the study; DL & CZ-C performed the morphologic analyses; MC and FG performed the metabolomic analyses. All authors wrote, revised, and approved the final version of this manuscript.

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Data availability The data presented in this study are available in the manuscript.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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