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

Running title: Role of Cytokinin Receptors in Seed Development

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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 seed development are difficult to assess and maybe mascaraed by their major impact on plant development.

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 pattern 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 metabolite's abundance differently, with a remarkably high concentration of tannins in *ahk2* 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

Graphical abstract

Graphical abstract of the findings on expression, functional, and metabolomic analyses in the seeds of CK single-receptor mutants presented in this study.

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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. CKs were first described by their role in the activating of cell division, cytokinesis (Miller et al. 1955, 1956; Amasino 2005) and then by their influential role in organ primordia initiation (Skoog 1957). 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: (Kakimoto 2003; Bishopp et al. 2006)).

In *Arabidopsis thaliana* (Arabidopsis) there are three paralogs that encode the CKs 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) which are formed by an extracytosolic cyclase. The extracellular sensory domain, CHASE, associated with the Histidine kinase domain in the cytosolic region, 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 then is transferred via ARABIDOPSIS HISTIDINE-CONTAINING PHOSPHOTRANSFER PROTEINS (AHPs) that 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

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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, CKs regulate many plant traits such as in 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); CKs 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, CKs levels are highly (Rock and Quatrano 1995) reviewed by (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).

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 et al. 2016; Xu and Zhang 2023). In *Arabidopsis*, at pre-globular embryo stage, in unpollinated ovules, 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 CKs in activating morphogenetic events during reproductive development, understanding the phenotype and expression patterns of each cytokinin receptor is important to better apprehend their specific roles, similarities and differences. This can allow to exploit this biological knowledge for breeding purposes aimed at improve seed quality for critical aspects like grain production and seed germination. 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-

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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 CKs receptors differ in their expression patterns, biochemical properties, and biological 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 (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). Accordingly, AHK2 behaves as a positive regulator of leaf senescence and AHK3 as a negative regulator (Kim et al. 2006; Danilova et al. 2017); longer plant longevity regulated by these CK receptors results in higher fruit and seed yield (Bartrina et al. 2011, 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 CKs 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. 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 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 in 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*. 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 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 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 to those previously reported for other functions of CK receptors in the plant such as the regulation of transition to reproductive development and leaf senescence.

This study provides evidence on the relationship between cytokinin receptors and secondary metabolite accumulation, particularly in seed morphogenesis, an area that remains largely unexplored. The research sheds light on how CKs signaling pathways could influence the biosynthesis of essential metabolites during seed formation, presenting potential targets for improving seed quality. By uncovering the molecular and signalling mechanisms underlying these pathways, this study not only advances our understanding of plant morphogenesis, but also provides practical tools and breeding strategies to optimize seed traits. For instance, this approach could have significant applications in crop improvement, aiming to increase seed nutritional value, enhancing plant resistance and yield by manipulating CK-associated pathways or by controlling synchrony in germination and its impact on overall plant fitness.

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 (Figure 1 and Supplementary Figure 2). 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 CKs receptors. For instance, we detected expression in seeds for AHK3 and AHK4 but no apparent signal of *AHK2* was detected in wild-type seeds which may suggest very low or null expression of this receptor in seeds Supplementary Figure 2). The expression of *AHK4* in wild-type seeds was restricted to a few cells of the embryo (Supplementary Figure 2). As for the expression of *AHK3,* it was detected in the embryo

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throughout seed development (Figure 1A-F) and also was localized in the peripheral endosperm (Figure 1A-C) and then throughout endosperm development (Figure 1D-F).

We performed also expression analysis of the AHK3 in the other receptor's 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 (Figure 1G-H). Expression that was not detected onwards later *ahk2* seed development (Figure 1I-J). In *ahk4* mutant seeds, the expression of *AHK3* is detected in the embryo, endosperm, and also was extended to the seed coat (Figure 1K-L).

Overall this data suggested a potential role of AHKs during control of seed development and also the potential compensatory mechanisms among them to exert a role in the different tissues.

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

Cytokinin receptor mutants are known to have small plant growth (Supplemental Figure 1) as well as larger seeds (Riefler et al. 2005), which we have corroborated by measuring the dry seeds surface area of (Figure 2A and 2B). In all genotypes, the increase in seed size is significant compared to the wild-type seeds. Overall *ahk2* shows larger seeds, and between *ahk3* and *ahk4*, there are no significative differences in seed size (Figure 2B). The development in the CKs receptor mutants appears to be delayed, at 3 Days After Pollination (DAP) there are still many unfertilized ovules than seeds, at this developmental stage, and was not possible to determine whether these are unfertilized ovules or delayed seeds (Supplemental Figure 3). 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; (Figure 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 (Figure 2D). At 6DAP, the CKs 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 (Figure 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 - (Figure 2E). At 4 DAP, a direct correlation between seed size and endosperm size was observed, however, at 6DAP no significant difference was detected

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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 (Figure 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 (Figure 2F). This together with previous observations on the very specific expression pattern of AHK3 in embryo developmental stages suggest 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. 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. This phenomenon can be exploited to test and validate the potential roles of effectors, transcription factors, signaling mechanisms, and signaling cascades involved in the complex mechanisms of seed growth. 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). These functional studies, which integrate developmental research with genetics, epigenetics, and

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population analysis, suggest that the various seed tissues play key roles in activating the postzygotic barrier. This is consistent with the need to coordinate tissue growth for proper seed formation. In essence, each seed tissue—despite its distinct genetic origin—harbors control mechanisms that regulate the activation of this barrier, preventing the formation of viable progeny in cases of genomic dosage imbalance. Given that AHK3 receptor were specifically expressed in embryo and caused significant developmental 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 paternal impact on rescuing seed development by crossing with a paternal excess (using 4X pollen). 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) 89%; while type (2x *ahk4* X 4x Col) presented 95% of abortions (Supplemental Figure 4). Similarly, abortions were observed in seeds from crosses of the 2X mutants with 2X pollen, suggesting that receptors for cytokinins have a maternal effect in proper seed development due to their "early" effect during the development of the ovule, 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. Future research could explore the specific contribution of each receptor—whether in the integument, fertilization, endosperm, or embryo.

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 Figure 5). 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 Figure 6). To understand which molecules were responsible for such a sample distribution, we performed a supervised multivariate statistical

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analysis, O2PLS-DA (orthogonal bidirectional partial least square - discriminant analysis) dividing the samples into 4 different classes, one for each genotype (Supplemental Figure 7). The loading plot showed that specific metabolites characterized one class better than the others. In detail, the wild-type and *ahk4* seeds contain glucosinolates (Figure 3), a characteristic compound of the Brassicaceae family (Wittstock and Burow 2010; Nguyen et al. 2020), *ahk3* seeds contain sinapoylated glucosinolates and some hydroxycinnamic acids, in particular glycosylated sinapic acid (HCA glycosylated) while *ahk2* seeds contain more condensed tannins, procyanidins, respect to the other seeds analyzed (Figure 3).

In fact, the relative abundance of single tannins classes appears to be higher in *ahk2* mutant seeds than in the other seeds (Figure 4A) and *ahk3* has less, while *ahk4* appears with content similar to that of wild-type (Figure 4A). All the seeds 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 (Figure 4B). *ahk2* seeds present the same amount of soluble procyanidins as the wild-type seeds and a significant increase in the amount of soluble procyanidins (Figure 4B). In contrast, *ahk3* has a significantly lower amount of both insoluble and soluble procyanidins (Figure 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 (Figures 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 glucosinolates and glycosylated sinapic acid (Figure 4). Indeed, showing a higher relative abundance of individual sinapoylated glucosinolates in *ahk3* compared to all other samples (Figure 5A). While in *ahk2* and *ahk4* seeds, the amount of these compounds is slightly higher than in the wild-type (Figure 5A), the most abundant being 4-Benzoyloxy-n-butyl-glucosinolate sinapoylated, glucoconringiin sinapoylated and glucoerucin sinapoylated (Figure 5A).

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In order 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* biological 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 (Figure 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 (Figure 5B).

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

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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 relative impact on reproductive structures, and more in concrete in seed size and number (Riefler et al. 2005; Heyl et al. 2012; Cerbantez-Bueno et al. 2020) has been very little studied. To fill this gap of knowledge here we focused on the developmental and functional characterization of the single mutants during seed development with two main objectives: 1) to increase the understanding of the individual roles of these receptors; 2) to elucidate the singularity of their specific functions because even if there is a large redundancy in the roles of these receptors here we show, differences in their individual expression patterns in reproductive structures which may suggest specific roles of their own. 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 (Figure 1) and independently controls seed size, all the cytokinin receptor single mutants exhibit bigger seeds (Figure 2A, B). Seed growth is coupled to endosperm growth, the larger endosperm the larger the seed (Sundaresan 2005). Since cytokinin homeostasis is directly linked to endosperm development in several species (Yang 2002; Mizutani et al. 2010; Basunia and Nonhebel 2019), we also performed the assessment of this phenotype.

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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). In fact, 4 days after pollination, cytokinin receptor mutants have smaller seeds and endosperm with respect to the wild type (Figure 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 be also 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 (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 (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 (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 (Figure 2D, E). Compared to wild type seeds, changes in endosperm size of cytokinin receptors throughout seed development, also appear to be linked to delayed embryo development (Figure 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

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major impact is observed in the single mutant *ahk3*, which is consistent with the high expression in the endosperm and embryo (Figure 2). In fact, *ahk3* shows more severe effects in endosperm size, besides the fact that embryo development is notoriously more delayed (Figure 2).

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 its 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 seed, the metabolic profile changes in the absence of different cytokinin receptors (Figure 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 seed, some flavonoids polymerize to form condensed tannins also known as proanthocyanidins (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 proanthocyanidins and *ahk3* seeds have less (Figure 4).

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 glucosinolates accumulation in the case of *ahk2* seeds, where the level of accumulation is lower while it is higher in *ahk3* seeds (Figure 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 glucosinolates exert a fine control role in maintaining inter-hormone homeostasis properly for optimal developmental processes.

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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 (*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. The 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 would be necessary, to further elucidate, which parts of the flavonoid and glucosinolate pathway interfere with the cytokinin pathway and what their roles 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

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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 manufacturer's instructions. 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 manufacturer's instructions. DNA templates for RNA synthesis probe were obtained by PCR amplification of 319bp - 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 manufacturer's protocol.

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 hours. 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).

Morphological analyses and microscopy

To detect the presence of proanthocyanidins 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

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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 (This site will be publicly available when accepted).

Sample preparation for metabolomic analyses of seeds

Mature dry seeds (at least 200mg) were collected in liquid nitrogen, ground and stored at -80°C until use. Approximately 30mg 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 analyed 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 x 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 minutes, 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 by using Argon gas in the collision cell and applying a voltage of 35V. Scanning was between 50 and 2000 m/z and the scan time set to 0.3s.

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

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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. 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. (Gao et al. 2010), from 400uL (of the supernatant, *see sample preparation section*) with 1ml of Butanol:HCl (95.5) at 98°C for 70min. 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 400uL of methanol:water 70:30, 1ml of butanol:HCl 95:5 and 100ul of NH4Fe(SO4)2*12H2O solution in 2M HCl and put in 98°C for 70min. 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, 400ul were added to 1ml of 95:5 (v/v) butanol:HCl solution and 100 ul of NH4Fe(SO4)2*12H2O solution in 2M HCl to allow the conversion reaction of procyanidin B2 to cyanidin take place. This analysis was conducted twice.

Author contributions

DL, CZ, MC performed the experiments, IE conceptualized the research, DL, CZ and IE wrote the manuscript and contributed to performing the literature search. LC, FG, BA have read the final version of the manuscript, CZ and IE revised and finalized the manuscript.

Data availability

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

Conflict of interest

The authors declare that there are no conflicts of interest.

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Author contribution—

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

Figure legends—

Figure 1: *In situ* hybridization expression patterns of *AHK3*. A-F Expression in wild type seeds. 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:* 50µm.

Figure 2: Seed phenotypes in the three cytokinin receptor single mutants. A Dry seed pictures for each of the genotypes analysed. 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. 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). p-value annotation legend: ns: 5.00e-02 < p <= 1.00e+00; *: 1.00e-02 < p <= 5.00e-02; **: 1.00e-03 < p <= 1.00e-02; ***: 1.00e-04 < p <= 1.00e-03;m****: p <= 1.00e-04. Scale bars:1cm (A); 50µm (C).

Figure 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

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semitransparent 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.

Figure 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 3 biological 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 3 biological replicates.

Figure 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.

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Supplemental material—

Supplemental Table 1: list of primers used for this study

Supplemental Figure 1: Morphology of the A. thaliana plants used in this study. All cytokinin receptor mutant plants are smaller respect to the wild type. ahk3 plants have a very severe phenotype as it has bene previously described, very small plants, few siliques and with low germination rates. Morphologically, ahk4 is more similar in phenotype to the wild type plants.

Supplemental Figure 2: In situ hybridization of AHK2 and AHK4 in wild type background seeds. A. no expression was detected for AHK2. B-E expression of AHK4 restricted to the embryo throughout its development. *Scale bars* 100μm.

Supplemental Figure 3: Number of ovules and seeds counted at 3DAP in a silique of the different genotypes. The cytokinin receptor mutants have a high number of ovules.

Supplemental figure 4: Percentage of aborted ovules observed in the different genotypes after crossing with 2x and 4x pollen. On the left, silique at 3DAP with *asterisks* showing what was considered to be aborted, and *black arrowheads* pointing to a normally developing seed. The x-axis of the graph shows the maternal line, blue is for pollen 2x and orange for pollen 4x as the top right convention shows.

Supplemental Figure 5: Negative-mode LC-MS chromatograms and main identified molecules of hydroalcoholic extracts of *A. thaliana* seeds from 2x Col, *ahk2*, *ahk3* and *ahk4*.

Supplemental Figure 6: PCA shows that the different samples are well separated from each other and that the three biological replicates cluster together.

Supplemental Figure 7: O2PLS-DA score scatter plot. Highlighting the 4 different classes corresponding to each genotype.

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