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Cytokinin Regulates the Activity of Reproductive Meristems, Flower Organ Size, Ovule Formation, and Thus Seed Yield in *Arabidopsis thaliana* [C][W][OA]

Isabel Bartrina,[a] Elisabeth Otto,[a] Miroslav Strnad,[b] Tomáš Werner,[a] and Thomas Schmülling[a,1]

[a] Institute of Biology/Applied Genetics, Dahlem Centre of Plant Sciences, Freie Universität Berlin, D-14195 Berlin, Germany
[b] Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany Academy of Sciences of the Czech Republic, CZ-78371 Olomouc, Czech Republic

The size and activity of the shoot apical meristem is regulated by transcription factors and low molecular mass signals, including the plant hormone cytokinin. The cytokinin status of the meristem depends on different factors, including metabolic degradation of the hormone, which is catalyzed by cytokinin oxidase/dehydrogenase (CKX) enzymes. Here, we show that *CKX3* and *CKX5* regulate the activity of the reproductive meristems of *Arabidopsis thaliana*. *CKX3* is expressed in the central WUSCHEL (WUS) domain, while *CKX5* shows a broader meristematic expression. *ckx3 ckx5* double mutants form larger inflorescence and floral meristems. An increased size of the WUS domain and enhanced primordia formation indicate a dual function for cytokinin in defining the stem cell niche and delaying cellular differentiation. Consistent with this, mutation of a negative regulator gene of cytokinin signaling, *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6*, which is expressed at the meristem flanks, caused a further delay of differentiation. Terminal cellular differentiation was also retarded in *ckx3 ckx5* flowers, which formed more cells and became larger, corroborating the role of cytokinin in regulating flower organ size. Furthermore, higher activity of the *ckx3 ckx5* placenta tissue established supernumerary ovules leading to an increased seed set per silique. Together, the results underpin the important role of cytokinin in reproductive development. The increased cytokinin content caused an ~55% increase in seed yield, highlighting the relevance of sink strength as a yield factor.

INTRODUCTION

Plants grow and form new organs throughout their life cycle. Shoot organs are derived from the growing tip, the shoot apical meristem (SAM) (Steeves and Sussex, 1989). The vegetative SAM forms new leaves, whereas the reproductive SAM, called the inflorescence meristem, produces flowers that form seeds after fertilization. The activity of the SAM is regulated by many factors, including transcriptional regulators, receptor kinases, and plant hormones (Tucker and Laux, 2007; Veit, 2009). Among the hormones, cytokinin has a central function. Metabolism and signal transduction of cytokinin has been elucidated during recent years (Sakakibara, 2006; Werner and Schmülling, 2009). Experimental reduction of the cytokinin status, which has been achieved either by lowering the cytokinin content or by reducing cytokinin signaling, abbreviates the activity of the SAM, demonstrating that cytokinin is a positive regulator of SAM activity (Werner et al., 2001, 2003; Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006; Heyl et al., 2008). In rice (*Oryza sativa*), disruption of an enzymatic activation step of cytokinin by the *log* mutation caused the arrest of SAM activity (Kurakawa et al., 2007). By contrast, increased cytokinin production is associated with the formation of larger vegetative meristems (Chaudhury et al., 1993; Rupp et al., 1999). Similarly, mutation of a cytokinin signaling gene in the *aberrant phyllotaxy 1* (*abph1*) maize (*Zea mays*) mutant increased meristem size (Giulini et al., 2004). *ABPH1* encodes an A-type response regulator and thus is expected to play a role in negative regulation of cytokinin responses. The cytokinin pathway is linked to transcriptional factors regulating SAM activity through reciprocal interactions involving suppression and enhancement of gene expression (Jasinski et al., 2005; Leibfried et al., 2005; Yanai et al., 2005).

The activity of the reproductive SAM is one parameter determining seed yield. Yield is the most important trait in plant breeding, and yield enhancement is required to meet increasing food demand. In addition, there is an increasing demand for plant-derived products for non-food purposes, such as energy production. Thus, yield enhancement is a central issue on the global agricultural agenda. Yield is a complex trait that is governed by many genes (quantitative trait loci), each contributing only a small portion to the total yield. Consequently, it is difficult to achieve large increases in seed yield by altering single or only a few genes. On average, the yearly increases achieved by classical breeding approaches in crop plants are in the range of 1 to 2% per year (Aizen et al., 2008).

[1] Address correspondence to tschmue@zedat.fu-berlin.de.
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The breakdown of cytokinin is catalyzed by cytokinin oxidases/dehydrogenases (CKXs). Recently it was shown that the *Gn1a* locus of rice, which makes a major contribution to the regulation of grain yield, carries a mutation in the promoter region of the Os-*CKX2* gene (Ashikari et al., 2005). This mutation caused a decreased expression of Os-*CKX2* in the inflorescence meristem, leading to increased cytokinin content and the formation of more reproductive organs. The dicotyledonous model plant *Arabidopsis thaliana* codes for seven CKX enzymes, which differ in their expression domains, subcellular localization, and biochemical characteristics (Werner et al., 2003; Galuszka et al., 2007). Here, it is shown that in *Arabidopsis*, the simultaneous mutation of two *CKX* genes delays the differentiation of cells in the reproductive meristems, thereby causing the formation of more and larger flowers forming more seeds. Moreover, a previously undiscovered function of cytokinin in regulating the activity of the ovule-forming placenta tissue was revealed. Together, these findings lend support for a central function of cytokinin in regulating the growth of reproductive meristems and organs and pinpoint the relevance of sink strength as a factor determining yield.

RESULTS

Insertional Mutants of *CKX* Genes

To study the functions of individual *CKX* genes, T-DNA insertion alleles of five of the seven *CKX* genes were identified by screening different insertional populations, and homozygote lines were established (Figure 1). *Arabidopsis* plants harboring T-DNA insertions in single *CKX* genes showed no gross morphological changes. However, various double mutants, which all included the *ckx3-1* allele, formed more flowers, indicating a more active inflorescence meristem. The activity was strongest when a *ckx5* insertion allele was combined with a mutated *CKX3* gene. Therefore, further work was focused on this mutant combination. All reported results were obtained with the allele combination *ckx3-1 ckx5-1* (named *ckx3 ckx5*) and were confirmed in the *ckx3-1 ckx5-2* double mutant. RT-PCR analysis showed that the T-DNA insertions in the *ckx3-1*, *ckx5-1*, and *ckx5-2* mutants completely abolished the expression of the respective gene (Figure 1). Because only a single *ckx3* mutant allele was available, we further tested the role of *CKX3* loss of function by transformation of the *ckx3 ckx5* double mutant with the wild-type *CKX3* gene. Supplemental Figure 1 online shows that the inflorescences of independent transformants resembled wild-type inflorescences. Together, this proves that the mutant phenotype is caused by mutation of *CKX3* and *CKX5*.

CKX Genes Regulate Inflorescence Meristem Activity

Flowers are continuously formed by the indeterminate inflorescence meristem. The *ckx3 ckx5* mutant produced significantly more flowers than the wild type and each of the parental lines (Figures 2A to 2C and 3A). Wild-type inflorescences carried, on the average, eight flowers of stage 13-16 (Smyth et al., 1990), whereas *ckx3 ckx5* mutants carried 11 flowers of that stage, with

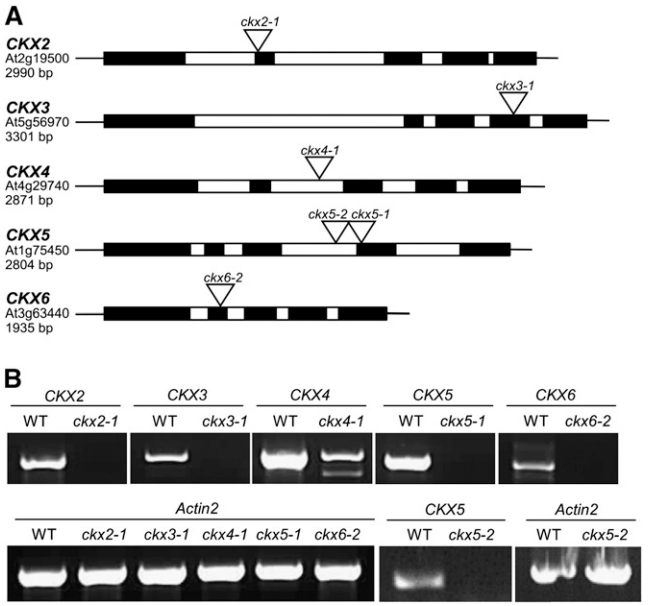

Figure 1. Characterization of *ckx* T-DNA Insertion Alleles.

(A) Positions of T-DNA insertions in the *ckx* mutants. The length of the genomic *CKX* gene sequences are given in base pairs. The insertional mutants were identified by PCR screening and the site of insertion determined by DNA sequencing of the border fragments. Black boxes represent exons, white boxes represent introns, and triangles indicate T-DNA insertion sites.

(B) *CKX* gene expression in the wild type (WT) and insertional mutants. RNA from 10-d-old seedlings was used as template for the RT-PCR. *Actin2* was included as a control.

longer pedicels (Figure 2C). Scanning electron microscopy analysis revealed the formation of a larger inflorescence meristem in the *ckx3 ckx5* mutant compared with the wild type (Figures 2D to 2G). The outer cell layer of mutant meristems had smaller cells (48.3 ± 3.2 cells per 500 μm^2 compared with 30.0 ± 7.1 per 500 μm^2 in the wild type; $n = 3$; $P < 0.05$, Student's t test) (Figures 2I and 2J), indicating that an increased number of meristematic cells, and not cell size, was the cause of the increase in meristem size. The mean number of floral primordia of stage 2-6 (Smyth et al., 1990) produced by wild-type and *ckx5* inflorescence meristems was 9, whereas a *ckx3* inflorescence meristem produced, on average, 12, and a *ckx3 ckx5* inflorescence meristem produced 14 stage 2-6 flower primordia (Figures 2D to 2G). By contrast, plants overexpressing a *CKX* gene showed a drastically diminished inflorescence meristem and the production of only two stage 2-6 flowers (Figure 2H). These data show that the *CKX3* and *CKX5* genes are negative regulators of inflorescence meristem size and activity.

ckx3 ckx5 Mutants Form More Siliques

The larger number of flowers formed by the inflorescence meristem would eventually lead to a larger number of siliques. The number of siliques of the main stem after formation of the last flower was compared. The single mutants *ckx3* and *ckx5* produced about as many siliques as wild-type plants. Mutants

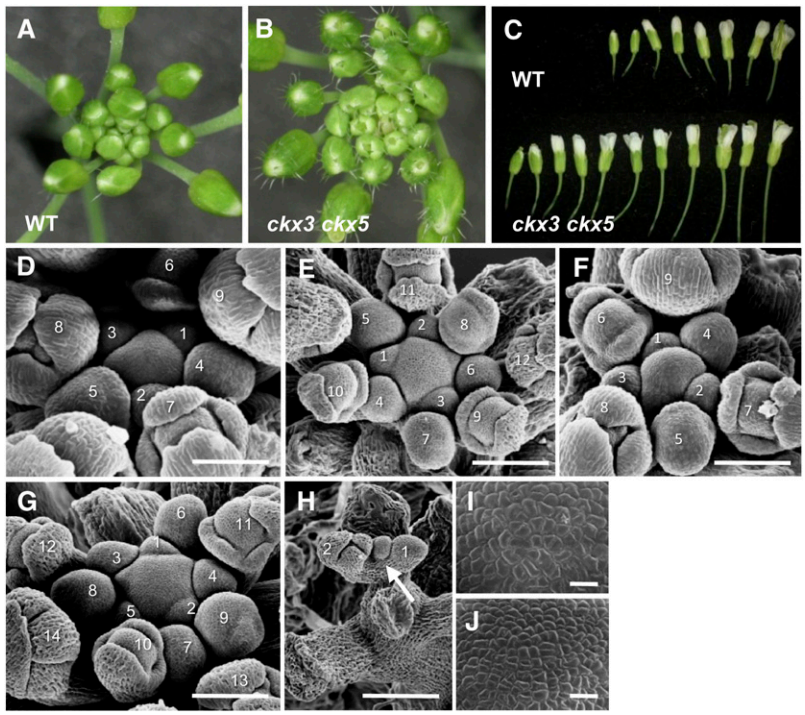

Figure 2. The Formation of Flower Primordia Is Increased in the *ckx3 ckx5* Mutant.

(A) and **(B)** Inflorescences of the wild type (WT) **(A)** and *ckx3 ckx5* **(B)**.
(C) Alignment of flowers from wild-type and *ckx3 ckx5* inflorescences, approximately stage 13-16 (Smyth et al., 1990).
(D) to **(H)** Scanning electron micrographs of the main flowering apex of the wild type **(D)**, *ckx3* **(E)**, *ckx5* **(F)**, *ckx3 ckx5* **(G)**, and *35S:CKX1* **(H)**. The arrow in **(H)** indicates the inflorescence meristem. Stage 2-6 floral buds are numbered from the youngest to oldest.
(I) and **(J)** Close-up pictures of wild-type **(I)** and *ckx3 ckx5* **(J)** inflorescence meristems.
Bars = 100 μm in **(D)** to **(H)** and 10 μm in **(I)** and **(J)**.

harboring insertions in the *CKX2*, *CKX4*, *CKX5*, or *CKX6* genes, as well as various combinations thereof, did not show enhanced numbers of siliques (Figure 3A). However, combination of a *ckx3* mutant allele with *ckx2*, *ckx4*, *ckx5*, or *ckx6* significantly increased the formation of siliques. The *ckx3 ckx5* double mutant showed the largest increase, generating ~40% more siliques than did the wild type. More flowers were formed per time unit (Figure 3B), indicating a shorter floral plastochron. It is noteworthy that the length of the reproductive phase in *ckx3 ckx5* plants was not altered. In the later stages of development, mutant plants often showed an irregular pattern of siliques along the stem, indicating that the larger meristem size interfered with positional signals regulating the spacing and/or timing of primordium initiation (Figure 3C). *ckx3 ckx5* mutants formed a stronger stem, which had about a 15% larger diameter compared with the wild type (Figure 4). This difference is most likely due to increased cambial activity, which is known to be affected by cytokinin (Matsumoto-Kitano et al., 2008; Nieminen et al., 2008).

Cytokinin Content in *ckx3 ckx5* Mutants

The cytokinin profile of *ckx3 ckx5* mutant inflorescences was compared with that of wild-type inflorescences. Table 1 shows that the levels of the biologically active *trans*-zeatin and *trans*-zeatin riboside were approximately fourfold higher in inflorescences of *ckx3 ckx5* mutants compared with the wild type. The levels of biologically inactive *trans*-zeatin-type cytokinins (i.e., glucosides and nucleotides) also increased up to eightfold. The levels of the iP-type cytokinins were low, and among them, only the level of isopentenyladenosine 5′-monophosphate increased about threefold. These results indicate that *CKX3* and *CKX5* regulate inflorescence meristem activity by modulating the cytokinin concentration and that *trans*-zeatin-type cytokinins are the predominant form of the hormone in the inflorescence.

Flower Development of *ckx3 ckx5* Mutants

Development of *ckx3 ckx5* mutant flowers also differed from the wild type. Figure 5A shows that flowers of *ckx3 ckx5* mutants were significantly larger compared with wild-type flowers and flowers of single mutants. The petal surface increased by ~40% (Figure 5B), which was due to a larger cell number (Figure 5C). Growth of *ckx3 ckx5* gynoecia, in particular, was enhanced and their final size was strongly increased (Figure 5D). The increase in stamen size of *ckx3 ckx5* mutants was less pronounced, causing reduced self-pollination of mutant flowers. With low frequency (~10 to 15%), flowers showed other morphological abnormalities to a varying degree (e.g., an altered number of floral organs)

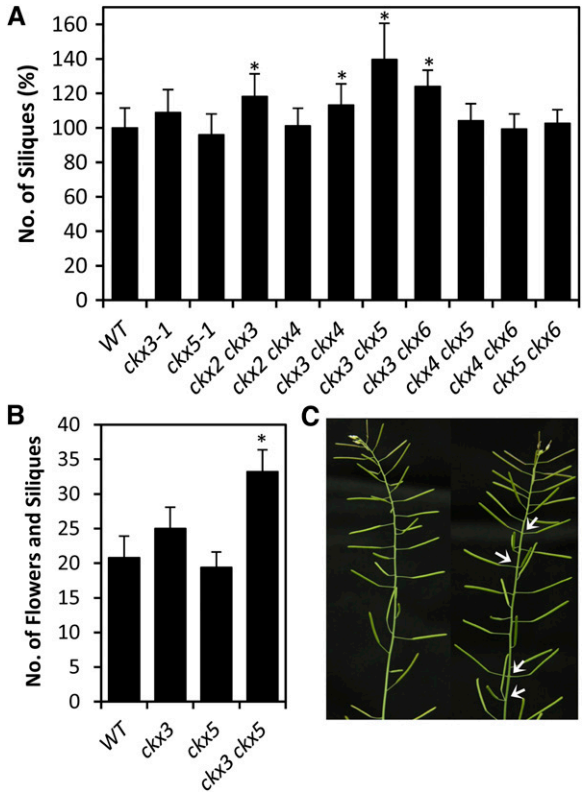

Figure 3. Lateral Organ Initiation and Phyllotaxis of *ckx* Mutants.

(A) The number of siliques on the main stem during one life cycle is shown (n = 15). Wild-type (WT) plants formed 54.7 siliques (100%).
(B) Number of flowers and siliques (stage 13-18) of 5-week-old plants. The plants had started to flower at the same time. Staging of floral meristems was according to Smyth et al. (1990) (n = 5).
(C) Irregular pattern of siliques on the main stem of the *ckx3 ckx5* mutant (right) indicated by arrows compared with the wild type (left).
Data represents mean value ± SD. *P < 0.01, calculated by Student's t test.
[See online article for color version of this figure.]

(Figure 5E; see Supplemental Figure 2 online). The increased size and cell number of floral organs suggest that cytokinin prolongs the duration of the cell division phase during organ growth.

ckx3 ckx5 Mutants Form More Seeds

Inspection of *ckx3 ckx5* mutant gynoecia revealed that they contained almost twice as many ovules as wild-type gynoecia did (Figure 5G), indicating an enhanced activity of the mutant placenta tissue (Figure 5F). The mutant gynoecia were densely filled, and some ovules were differently shaped, probably due to spatial constraints (see Supplemental Figure 2 online). The *ckx3* and *ckx5* parents also showed an increased number of ovules per gynoecium, with a stronger effect in *ckx3* (Figure 5G). To test whether the increased ovule number together with the larger flower number would lead to the formation of more seeds, *ckx3 ckx5* mutants were grown in a growth chamber, which facilitated self-pollination. Siliques of *ckx3 ckx5* mutants were longer than in the wild type (20 mm compared with 17 mm) and contained up to 110 seeds, compared with an average of 65 seeds in wild-type siliques, due to more densely packed seeds. The total seed yield of *ckx3 ckx5* mutants increased by 55% compared with the wild type (Figure 5H).

CKX3 and *CKX5* Expression Pattern during Inflorescence Development

CKX3 transcripts were detected in the center of the inflorescence meristem and in the floral meristems of stage 2 to stage 5 flowers (Figures 6A to 6D). This coincides with the expression of *WUSCHEL* (*WUS*) (Figures 6E and 6F). However, *CKX3* expression appeared later in flower development than that of *WUS*, which is seen already in stage 1 flowers (Mayer et al., 1998), and its expression domain became less defined, appearing in stage 5 mainly in the region between long stamen primordia and gynoecia primordia (Figure 6D) and disappearing thereafter. *CKX5* expression was strong in the procambium of the inflorescence stems and flowers (Figures 6G to 6I). Weaker *CKX5* expression was also detected in the inflorescence and floral meristems. In developing flowers, *CKX5* transcripts were detected in stamen and gynoecia primordia during different developmental stages (Figures 6J and 6K). During gynoecia development, *CKX5* RNA accumulated in the medial cells of the cylinder where the two main vascular bundles begin to differentiate at stage 7 (Figure 6L) (Ferrándiz et al., 1999). At stage 8, the transcripts accumulated specifically at the flanks of the medial ridges marking the developing placentas, and they were detected in the initiated ovule primordia during stage 9 (Figures 6M and 6N). During ovule differentiation at stage 10-12, *CKX5* was expressed in the cells of the chalazal region directly adjacent to the nucellus but not in the differentiating integuments (Figure 6O).

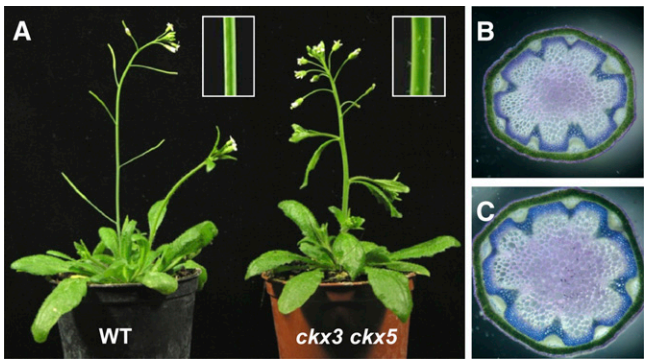

Figure 4. Phenotype of the Inflorescence Stem of *ckx3 ckx5* Mutants Compared with the Wild Type.

(A) The inflorescence stem of *ckx3 ckx5* mutants (right) is thicker than in the wild type (WT). The insets show a close-up of the inflorescence stems.
(B) and **(C)** Hand-cut transverse sections of the inflorescence stem of the wild type **(B)** and the *ckx3 ckx5* mutant **(C)** stained with toluidine blue. Sections were made at the base of the stem of 5-week-old plants.

Table 1. Cytokinin Content of Inflorescences

Genotype	Cytokinin Metabolite				
	tZ	tZR	tZRMP	tZ9G	tZOG
Wild type	4.1 ± 1.1	10.7 ± 5.9	174.6 ± 79.1	2.3 ± 0.3	3.4 ± 0.8
ckx3 ckx5	15.5 ± 3.7	46.0 ± 5.4	1129.0 ± 295.9	18.0 ± 2.0	17.3 ± 1.7
	tZROG	iP	iPR	iPRMP	iP9G
Wild type	3.3 ± 1.1	0.23 ± 0.09	0.16 ± 0.13	30.0 ± 10.2	0.20 ± 0.24
ckx3 ckx5	24.6 ± 2.8	0.29 ± 0.10	0.35 ± 0.10	100.5 ± 25.0	0.20 ± 0.03

Approximately 0.5 g of *Arabidopsis* inflorescences per sample was harvested and pooled 30 d after germination. Five independent biological samples were harvested for each genotype. Data shown are pmol/g fresh weight ± SD; $n = 5$. tZ, *trans*-zeatin; tZR; *trans*-zeatin riboside; tZRMP, *trans*-zeatin riboside 5′-monophosphate; tZ9G, *trans*-zeatin 9-glucoside; tZOG, *trans*-zeatin *O*-glucoside; tZROG, *trans*-zeatin riboside *O*-glucoside; iP, N^6-(Δ^2isopentenyl)adenine; iPR, N^6-(Δ^2isopentenyl)adenosine; iPRMP, N^6-(Δ^2isopentenyl)adenosine 5′-monophosphate; iP9G, N^6-(Δ^2isopentenyl)adenine 9-glucoside.

CKX Genes Regulate the Expression Domain of *WUS*

The WUS/CLAVATA (CLV) pathway regulates SAM size, and the domain of *WUS* expression becomes larger in mutants with a larger meristem (Carles et al., 2004). To study whether this central regulatory module was affected by the *ckx3 ckx5* mutations or whether meristem enlargement occurred independently, the *WUS* expression domain was analyzed by in situ hybridization. Figure 7 shows that the WUS domain marking the organizing center of the SAM was enlarged in *ckx3 ckx5* mutants, suggesting that the cytokinin status regulates the size of the WUS expression domain and, eventually, the *WUS* expression level.

Mutation of *AHP6* Causes a Higher Activity of the Inflorescence Meristem

Next, we investigated whether other components of the cytokinin system exert an additive negative regulatory activity in the inflorescence meristem. To this end, the *ahp6* mutation was introgressed into the *ckx3 ckx5* mutant background. *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*) encodes a pseudo-phosphotransfer protein that has been shown to act as an inhibitor of cytokinin signaling during formation of root vascular tissue (Mähönen et al., 2006). However, its regulatory activity in other developmental programs is unknown. In situ hybridization revealed expression of *AHP6* in the inflorescence meristem at the position where the next flower primordium will arise (stage P0) and in early stage 1 primordium, in floral organ primordia of stage 3 flowers, and in the distal part of developing gynoecia in stage 6 flowers (Figures 8A to 8C). Analysis of mutants harboring the *ahp6-1* or *ahp6-3* null alleles (Mähönen et al., 2006) showed that the size of the inflorescence meristem was only marginally increased (similar results were obtained for both *ahp6* mutant alleles). However, the combination of *ahp6* with *ckx3* and, in particular, *ckx3 ckx5* increased further the size of the inflorescence meristems and formation of flower primordia (Figures 8D to 8I). At the end of their life cycle, *ckx3 ckx5 ahp6* triple mutants had 23% more siliques on the main stem compared with *ckx3 ckx5* and 47% more compared with the wild type (Figure 8J). These results clearly show that *AHP6* negatively regulates the activity of the inflorescence meristem depending on the cytokinin status. By contrast, flower size and the number of ovules per gynoecium was similar in the *ckx3 ckx5* and *ckx3 ckx5 ahp6* mutants (Figure 8K), indicating that AHP6 may not be involved in regulating flower size and the activity of the placenta.

DISCUSSION

Several activities of cytokinin and in particular of *CKX3* and *CKX5* in regulating organ size were revealed in this study. First, the size of the reproductive meristems of *Arabidopsis* was promoted by cytokinin. An increase of endogenous cytokinins in inflorescences caused the formation of larger inflorescence meristems capable of initiating more flower primordia. The larger number of meristematic cells indicates that cytokinin delays the developmental switch that causes cells to exit from their meristematic cell cycle activity. Second, the enhanced cytokinin content increased the cell number and, thus, the size of floral organs and particularly of the ovule-bearing gynoecia. Third, the capacity of the placenta tissue to initiate ovule primordia was enhanced, resulting in a higher density of ovules and seeds within the carpels. Together, these changes led to an increase in seed yield of ~55%. An even higher increase could potentially be achieved but was probably restricted by a heterostylous phenotype that reduced self-fertilization.

Cytokinin Regulates the Activity of the Reproductive SAM by Distinct Mechanisms

It was found that *CKX3* and other *CKX* genes regulate the size of the reproductive SAM in a partially redundant fashion. The *CKX3* expression domain was similar to that of *WUS* in inflorescence and floral meristems, although in the latter its expression appeared later and ceased earlier than that of *WUS*. Therefore, CKX3 may be part of the circuitry regulating the cytokinin status and, thus, the size of the WUS domain. Indeed, it was shown recently that cytokinin acts as a positional cue for patterning *WUS* expression in both a CLV1-dependent and CLV1-independent fashion (Gordon et al., 2009). WUS, in turn, directly represses the expression of several A-type response regulator (*ARR*) genes that code for feedback repressors of the cytokinin signaling pathway and, thus, increases the cytokinin status in the organizing center (Leibfried et al., 2005; Zhao et al., 2010).

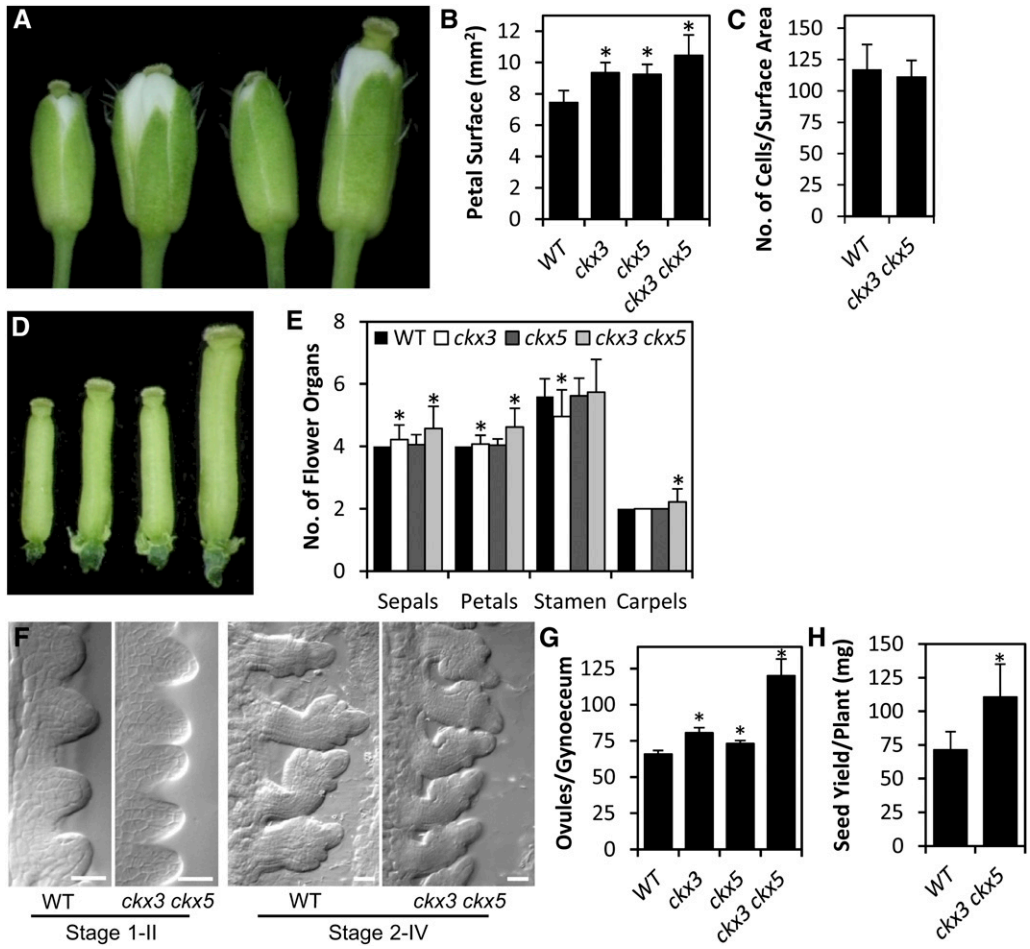

Figure 5. Flower Phenotype and Seed Yield of *ckx* Mutants.

(A) Stage 13 flowers. From left to right: the wild type, *ckx3*, *ckx5*, and *ckx3 ckx5*.
(B) Petal surface of *ckx* mutants, stage 14 flowers, 39 DAG (n = 30). *WT*, wild type.
(C) Cell number per surface area in wild-type and *ckx3 ckx5* mutant petals (n = 11).
(D) The corresponding gynoecia of flowers shown in **(A)**. From left to right: the wild type, *ckx3*, *ckx5*, and *ckx3 ckx5*.
(E) Floral organ number per flower in the wild type, *ckx3*, *ckx5*, and *ckx3 ckx5* (n = 50).
(F) Young ovules of the wild type and *ckx3 ckx5*. Staging of ovules is according to Schneitz et al. (1995). Bars = 10 μm.
(G) Number of ovules per gynoeceum (n = 12).
(H) Seed yield of the wild type and *ckx3 ckx5* under growth chamber conditions (n = 30).
Data represents mean value ± SD. *P < 0.01, calculated by Student's t test.

Furthermore, cytokinin downregulates the expression of the *CLV1* gene, which functions to restrict the WUS domain (Brand et al., 2000; Schoof et al., 2000; Lindsay et al., 2006; Gordon et al., 2009). Together, this indicates that the cytokinin status of the organizing center is relevant for determining the size of the stem cell niche and of the meristem. However, mutation of *CKX3* alone was not sufficient to increase strongly meristem size, and *CKX5* was expressed more broadly in the meristem. Thus, the cytokinin status outside of the central domain might be relevant as well, and another aspect of cytokinin activity in the SAM may operate independently of the WUS pathway. An essential transcription factor for SAM function, STM, which is required for cell division in the peripheral zone and to maintain meristem cells in the undifferentiated state (Endrizzi et al., 1996; Long et al., 1996), induces cytokinin-synthesizing *IPT* genes (Jasinski et al., 2005; Yanai et al., 2005) and is itself subject to regulation by cytokinin (Rupp et al., 1999). An enhanced cytokinin status of the SAM as a consequence of *CKX* gene mutation may enhance *STM* expression and thus increase meristem activity. The links between cytokinin and the network of transcription factors and receptor kinases regulating SAM identity and size are summarized in a model shown in Figure 9.

Enlargement of the SAM may be the result of different causes. It may be due to a disturbed transition of cells from the central zone to the periphery or due to an accumulation of cells at the periphery (Laufs et al., 1998). Thus, it could be that cytokinin

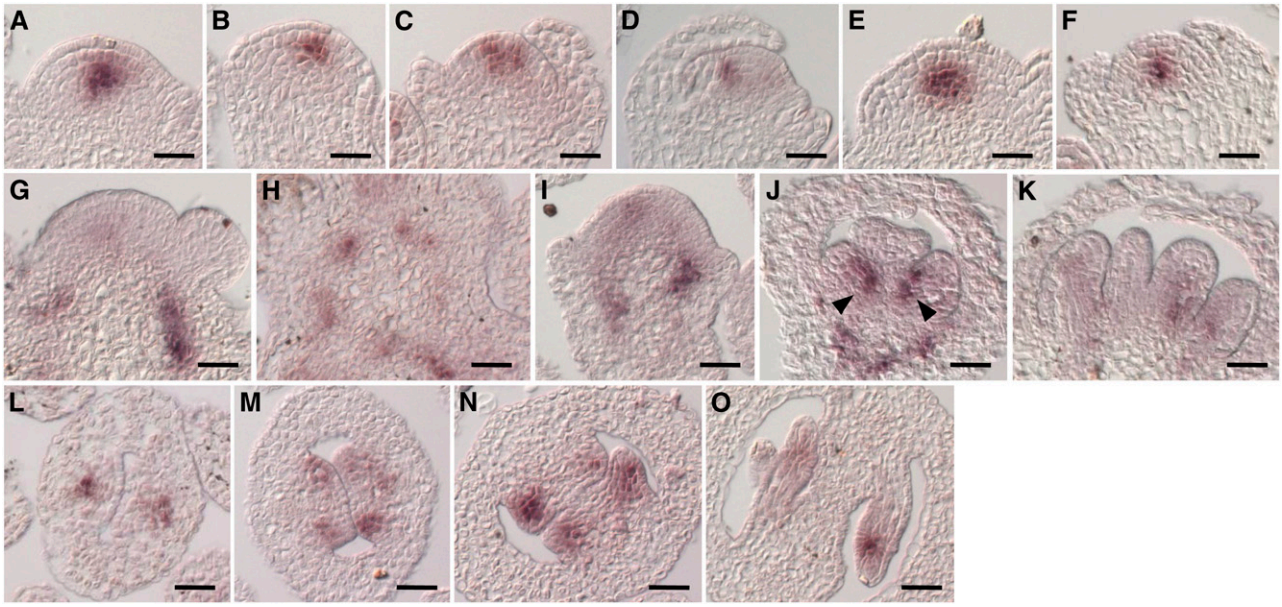

Figure 6. *CKX3* and *CKX5* mRNA Expression Pattern in Inflorescence and Flower Tissues.

RNA localization by in situ hybridization with *CKX3* (**[A]** to **[D]**), *WUS* (**[E]** and **[F]**), and *CKX5* (**[G]** to **[O]**) antisense probes hybridized to wild-type tissues. **(A)** to **(G)** and **(I)** to **(K)** show longitudinal sections through inflorescence meristems, floral meristems, and flowers. Staging of floral meristems is according to Smyth et al. (1990). Bars = 25 μm.
(A) and **(B)** *CKX3* is expressed in the center of the inflorescence meristem **(A)** and in the center of the floral meristem at stage 2 **(B)**.
(C) In stage 4 flowers, *CKX3* is expressed in a broader central area of the floral meristem.
(D) In stage 5 flowers, the *CKX3* signal appears between long stamen primordia and gynoecia primordia.
(E) and **(F)** *WUS* is expressed in the organizing center of the inflorescence **(E)** and floral meristems **(F)** during different developmental stages (a stage 4 flower is shown here).
(G) to **(I)** *CKX5* is expressed in the procambium of inflorescence stems (**[G]** and **[H]**) and flowers **(I)**. **(H)** shows a transverse section through an inflorescence stem below the meristem.
(J) *CKX5* is expressed in stage 6 flowers between long stamen primordia and gynoecia primordia (arrowheads).
(K) *CKX5* is expressed in stage 7 flowers.
(L) to **(O)** Transverse section through ovaries. *CKX5* transcripts are detected in the developing placentas of stage 7 **(L)**, stage 8 **(M)**, stage 9 **(N)**, and stage 11 **(O)** flowers.

operates through different pathways to regulate SAM size. It is tempting to speculate that *ckx3* loss-of-function causes an enhanced cytokinin concentration in the central zone and alters a morphogenic gradient that decreases toward the flanks of the meristem and regulates organ initiation and/or the establishment of organ boundary domains. Interestingly, the highest expression of *CKX5* was found in the procambial cells, suggesting that it also has, beside its possible role in regulating vascular differentiation (Mähönen et al., 2000), the function of restricting cytokinin flow derived from the vasculature. Together, cytokinin gradients in the SAM could provide positional information regulating cell differentiation.

Consistent with a role for cytokinin at the meristem borders is the expression of *AHP6* in differentiating cells at the meristem flanks and the increased inflorescence meristem size caused by *ahp6* mutation in the *ckx3* mutant and even more so in the *ckx3 ckx5* double mutant. The slight increase of meristem size caused by the simultaneous mutation of the *CKX3* and *AHP6* genes, which are expressed in distinct meristematic domains, indicates the existence of a non-cell-autonomous regulation of meristem size by these genes. The consequences of *ahp6* mutation were not strong and consistent in the wild type under our growth conditions, indicating that the cytokinin system is normally well buffered. Interestingly, another negative regulator of cytokinin signaling, the maize A-type response regulator-encoding *ABPH1* gene, is expressed also at the site of the incipient leaf primordium at the meristem border, and its mutation causes the formation of

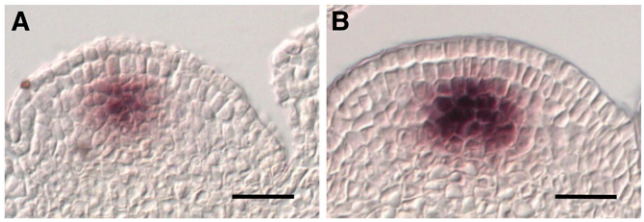

Figure 7. Detection of *WUS* mRNA Expression in the Inflorescence Meristem.

(A) The wild type.
(B) The *ckx3 ckx5* mutant.
Transcripts were identified by in situ hybridization. Bars = 25 μm.

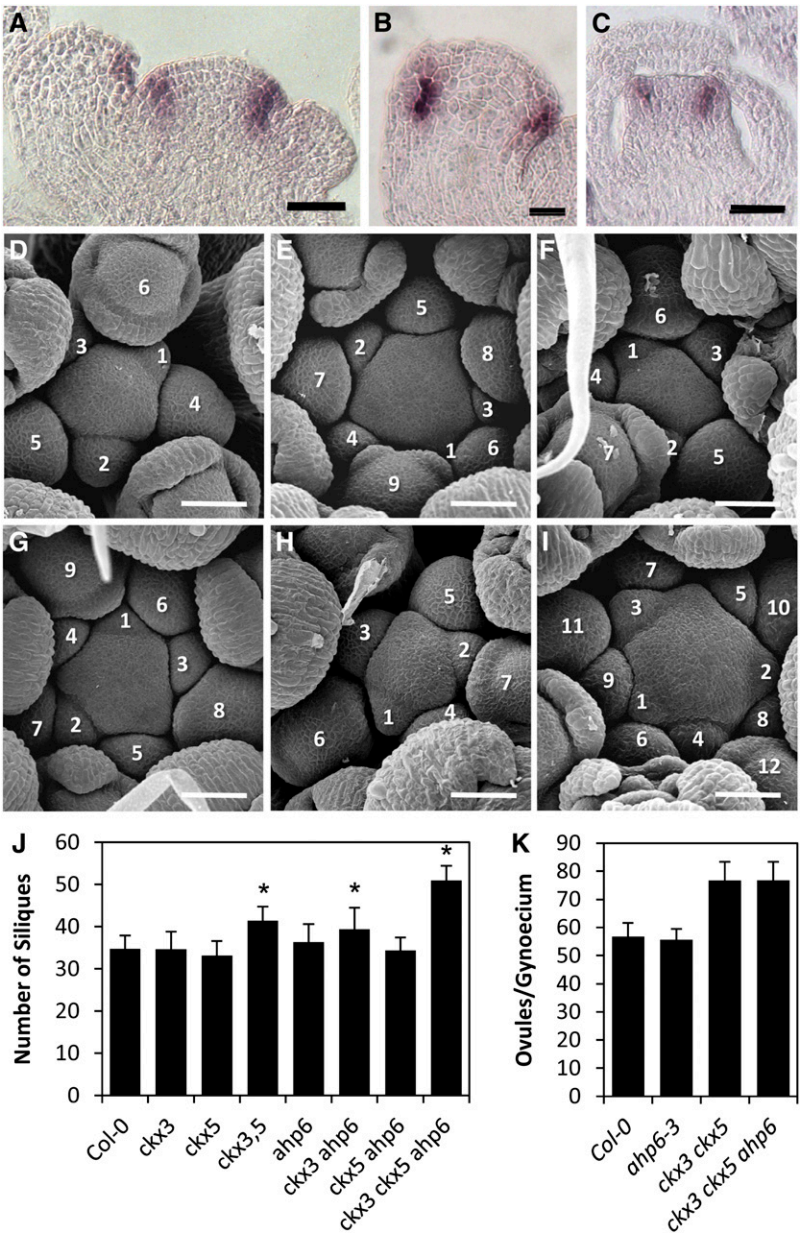

Figure 8. Enhanced Cytokinin Signaling Leads to Even More Active Inflorescence Meristems.

(A) to **(C)** mRNA localization by in situ hybridization with *AHP6* antisense probes hybridized to wild-type tissues. *AHP6* is expressed in the inflorescence meristem at the position where the next flower primordia will arise and the youngest primordia **(A)**. *AHP6* transcripts can also be detected in floral organ primordia of stage 3 flowers **(B)** and in stage 6 flowers at the distal end of the developing gynoecium **(C)**.

(D) to **(F)** Scanning electron micrographs of inflorescence meristems of 4-week-old wild-type **(D)**, *ckx3 ckx5* **(E)**, *ahp6* **(F)**, *ckx3 ahp6* **(G)**, *ckx5 ahp6* **(H)**, and *ckx3 ckx5 ahp6* **(I)** plants. Floral buds are numbered from the first primordium of stage 2 to the first primordium stage 3.

(J) *ckx3 ckx5 ahp6* mutants form more siliques on the main stem than do *ckx3 ckx5* mutants ($n = 20$).

(K) The gynoecia of *ckx3 ckx5 ahp6* triple mutants do not form more ovules than *ckx3 ckx5* mutants do ($n = 18$).

Bars = 20 μm (**[A]** to **[C]**) and 50 μm (**[D]** to **[F]**). Data represents mean value ± SD. Values of mutant lines were compared with the wild type. *$P < 0.01$, calculated by Student's *t* test.

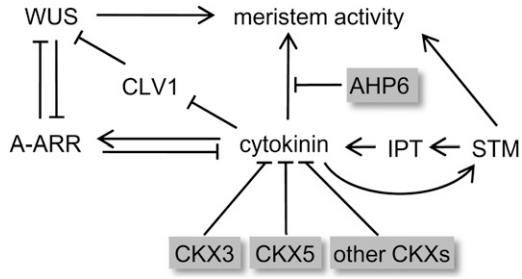

Figure 9. Model of *CKX* and *AHP6* Gene Function in Regulating Inflorescence Meristem Size and Activity.

The model shows the action of *CKX* and *AHP6* genes and cytokinin in concert with other known factors regulating shoot meristem size in *Arabidopsis*. The model integrates results of this article and other work described in the text. The genes studied in this work are shown in gray boxes.

larger meristems (Giulini et al., 2004). In *Arabidopsis*, mutation of several A-type response regulator genes was necessary to alter SAM activity (To et al., 2004; Leibfried et al., 2005). Thus, negative regulation of cytokinin-induced expansion of the shoot meristem at the meristem borders is relevant in both monocots and dicots. The regulatory modules might have been at least partly conserved since the monocot-dicot divergence.

Cytokinin Regulates Gynoecium Size and Placenta Activity

This analysis revealed an as yet unanticipated role for cytokinin in regulating gynoecium size and the activity of the placenta. The molecular events that govern development of the placenta, which is the internal surface of the carpels giving rise to ovule primordia, and ovule formation are not well understood. Remarkably, however, the same genes, including *WUS*, involved in regulating SAM activity seem to play a role, suggesting the operation of related mechanisms (Skinner et al., 2004). *CKX3* and *CKX5* may regulate the activity of meristematic cells in the placenta and thus influence organogenic capacity and ovule primordia formation. In particular, the distance between two ovule primordia is regulated by cytokinin. A second distinct mechanism may be relevant as well. Given that a defined number of cells in the floral meristem give rise to each carpel (Bossinger and Smyth, 1996), CKX activity in the floral meristem may affect the population of cells that are recruited into the gynoecium primordium and thus determine the final gynoecium size.

The results discussed above highlight the role of cytokinin in regulating shoot organ initiation and size. A recurrent theme is the regulation of the exit of cells from their meristematic phase. Cytokinin retards this differentiation step, resulting in an increased organ cell number. Interestingly, this activity in the shoot organs is opposite to that in the root, where cytokinin causes an earlier cellular differentiation of meristematic cells (Werner et al., 2003; Dello Ioio et al., 2007). Obviously, the final size of plant organs is influenced by numerous factors (Bögre et al., 2008; Krizek, 2009), including AINTEGUMENTA, KLUH, and ARGOS, mediating auxin activity (Mizukami and Fischer, 2000; Hu et al., 2003; Anastasiou et al., 2007), and BIG BROTHER and DA1, functioning in the ubiquitin proteasome pathway (Disch et al., 2006; Li et al., 2008). It will be interesting to study how cytokinin is linked to these other factors regulating organ size.

Cytokinin Is an Evolutionary Conserved Yield-Regulating Factor

The results reported here extend the concept of cytokinin as a factor regulating yield, which was first noted during the analysis of the *Gn1a* (Os-*CKX2*) gene in rice (Ashikari et al., 2005), to a dicotyledonous plant. In contrast with rice, where mutation of a single gene was sufficient for yield enhancement, mutation of two *CKX* genes was needed in *Arabidopsis*. One of these, *CKX3*, is the closest *Arabidopsis* homolog of Os-*CKX2*, but *CKX5* is more distantly related. There are some fundamental differences between the developmental events leading to flower or spikelet formation in *Arabidopsis* and monocotyledonous plants such as rice. *Arabidopsis* produces floral meristems directly from the inflorescence meristem with indeterminate growth habit. By contrast, in rice the inflorescence (rachis) meristem forms bracts and inflorescence branches, and eventually aborts. The flowers are then formed from spikelet meristems developed from primary and secondary branch meristems, which are axillary meristems (Itoh et al., 2005). An additional difference is that the highest expression of Os-*CKX2* was found in the vascular tissues of developing culms, suggesting that Os-*CKX2* plays a role in regulating cytokinin levels in the vascular system, hence cytokinin transport to the inflorescence meristems (Ashikari et al., 2005). By contrast, the expression of At-*CKX3* in the organizing center suggests its role in meristem-autonomous modulation of the cytokinin concentration. It will be interesting to study how the expression and regulatory functions of *CKX* genes have been adapted during evolution to cope with the specific developmental differences between the reproductive tissues of *Arabidopsis* and rice. Despite these differences, the role of *CKX* genes in regulating seed yield has been evolutionary conserved, suggesting functional relevance for all or most flowering plants.

Importantly, yield enhancement in *ckx* mutants was obtained without increasing the strength of the CO_2-fixing source but was dependent on the strength of the sink tissue (i.e., its capacity to make use of the fixed carbon). This result supports models considering the sink strength as an important factor in regulating allocation of fixed carbon (Marcelis and Heuvelink, 2007) and, more specifically, underpins the relevance of regulating the differentiation and activity of reproductive meristems as a factor determining sink strength.

The increase in seed yield obtained by *ckx* gene mutation is high compared with an average annual increase of 1 to 2% achieved by classical breeding strategies (Aizen et al., 2008) and may be instructive for application. Increasing plant yield is an important agronomical goal, in particular in view of the challenging future increase of demand for food and plant-derived products for industrial applications (Borlaug, 2007; Edgerton, 2009). The findings reported here might be especially relevant to achieve yield enhancement in closely related crop species such as oilseed rape (*Brassica napus*), one of the most important sources of vegetable oil. However, it should be noted that crop plants have undergone selection for yield enhancement, which is

not the case in *Arabidopsis*. Therefore, it could be that beneficial alleles of *CKX* genes have already been accumulated in crop plants during the breeding process.

METHODS

Plant Material and Growth Conditions

The Columbia-0 ecotype of *Arabidopsis thaliana* was used as the wild type. Line 35S:CKX1 was described previously (Werner et al., 2003). The T-DNA insertion mutants *ckx2-1* (SALK_068485), *ckx3-1* (SALK_050938), *ckx4-1* (SALK_055204), *ckx5-1* (SALK_064309), and *ckx6-2* (SALK_070071) were from the Salk Institute Genomic Analysis Laboratory (Alonso et al., 2003), and *ckx5-2* (line ID 332B10) was from the GABI-KAT collection (Rosso et al., 2003). The *ahp6-1* and *ahp6-3* mutants were described by Mähönen et al. (2006). Genotyping was performed after DNA isolation by PCR with gene-specific and T-DNA border primers listed in Supplemental Table 1 online or derived cleaved-amplified polymorphic sequence markers in the case of *ahp6-1* (Mähönen et al., 2006). Double and triple mutants were obtained by crossing. Plants were grown in the greenhouse on soil at 22°C under long-day conditions (16 h light/8 h dark). Plants were grown in growth chambers (Percival AR-66L) on soil at 24°C, a light intensity of ~100 μmol m^{-2} s^{-1}, and 65% humidity under long-day conditions for seed yield measurement.

Analysis of *CKX* Gene Expression

Total RNA was extracted from seedlings according to Verwoerd et al. (1989). The RNA was treated with RNase-free DNase I (Fermentas) at 37°C for 30 min. One microliter of 25 mM EDTA was added at 65°C for 10 min. RNA (0.5 μg) was used for RT-PCR. Primers used for the respective *CKX* gene are listed in Supplemental Table 1 online. All primer pairs span the respective T-DNA insertion site. In all RT-PCR reactions, the *Actin2* gene was used as a control. RT-PCR was performed with the One-Step RT-PCR kit (Qiagen) according to the manufacturer's instructions. The PCR comprised 35 cycles of 30 s at 94°C, 30 s at 57°C, and 2 min at 72°C.

Scanning Electron Microscopy

Scanning electron microscopy was performed as described (Krupková et al., 2007) using a LEO 430 microscope (Zeiss).

Determination of the Cytokinin Status

Plants were grown on soil until the main inflorescence was ~10 cm high (~30 d after germination). For each sample, ~0.5 g of inflorescences with stage 1 to stage 15 flowers (Smyth et al., 1990) were pooled, and five independent samples were collected and analyzed for each genotype. The cytokinin content was determined by ultraperformance liquid chromatography–electrospray tandem mass spectrometry (Novák et al., 2008).

Petal Surface and Cell Size Measurement

The area of petals was measured from digital images of dissected organs with the Scion Image program. Petals were cleared (Malamy and Benfey, 1997), and average cell sizes were calculated from the number of cells per unit area of digital micrographs.

In Situ Hybridization

The *CKX3* probe for in situ hybridization was amplified by RT-PCR from total mRNA of 10-d-old wild-type seedlings. RT-PCR was performed with the One-Step RT-PCR kit (Qiagen) according to the manufacturer's instructions. The *CKX5* probe was amplified from the clone RAFL24-08-K03 (GenBank number AK176378.1), and the *WUS* probe from the cDNA clone PENTR221-AT2G17950 (GenBank number DQ446515). Primer sequences are detailed in Supplemental Table 1 online. Antisense probes were produced by in vitro transcription with digoxigenin-11-UTP using T7 RNA polymerase (Roche). In situ hybridization was performed according to standard protocols (Jackson et al., 1994).

Light Microscopy

Gynoecia were cleared and mounted as described (Malamy and Benfey, 1997) for ovule counting and observation. Hand-cut cross sections of the base of stems from 5-week-old plants were stained for 5 min in 0.02% aqueous toluidine blue O, rinsed, and mounted in water. All samples were viewed with an Axioskop 2 plus microscope (Zeiss).

CKX3 Complementation Construct

The 35S promoter of plasmid pBinSMGFP (Werner et al., 2003) was replaced by a synthetic oligonucleotide containing *Eco*RI, *Avr*II, *Sma*I, and *Kpn*I restriction sites (Werner et al., 2010). A *CKX3* genomic fragment was subcloned via *Kpn*I/*Xho*I restriction sites from pBS-CKX3 (Werner et al., 2001) to generate an N-terminal fusion with green fluorescent protein (GFP). Then, a 2088-bp promoter fragment of the *CKX3* gene was amplified by PCR from DNA of *Arabidopsis* Columbia-0 using appropriate primers (see Supplemental Table 1 online) and inserted into the *Avr*II/*Kpn*I sites upstream of the *CKX3-GFP* fusion gene, resulting in the vector pCKX3:CKX3-GFP, which was used for transformation of the *ckx3 ckx5* mutant.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *CKX1* (At2g41510), *CKX2* (At2g19500), *CKX3* (At5g56970), *CKX4* (At4g29740), *CKX5* (At1g75450), *CKX6* (At3g63440), and *AHP6* (At1g80100).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Complementation of the *ckx3 ckx5* Mutant Phenotype by the *CKX3* Gene.

Supplemental Figure 2. Developmental Aberrations of Floral Organs and Ovules in the *ckx3 ckx5* Mutant.

Supplemental Table 1. Primers Used in This Study.

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REFERENCES

Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., and Klein, A.M. (2008). Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. Curr. Biol. **18:** 1572–1575.

Alonso, J.M., et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana.* Science **301:** 653–657.

Anastasiou, E., Kenz, S., Gerstung, M., MacLean, D., Timmer, J., Fleck, C., and Lenhard, M. (2007). Control of plant organ size by *KLUH/CYP78A5*-dependent intercellular signaling. Dev. Cell **13:** 843–856.

Ashikari, M., Sakakibara, H., Lin, S., Yamamoto, T., Takashi, T., Nishimura, A., Angeles, E.R., Qian, Q., Kitano, H., and Matsuoka, M. (2005). Cytokinin oxidase regulates rice grain production. Science **309:** 741–745.

Bögre, L., Magyar, Z., and López-Juez, E. (2008). New clues to organ size control in plants. Genome Biol. **9:** 226.

Borlaug, N. (2007). Feeding a hungry world. Science **318:** 359.

Bossinger, G., and Smyth, D.R. (1996). Initiation patterns of flower and floral organ development in *Arabidopsis thaliana.* Development **122:** 1093–1102.

Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M., and Simon, R. (2000). Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by *CLV3* activity. Science **289:** 617–619.

Carles, C.C., Lertpiriyapong, K., Reville, K., and Fletcher, J.C. (2004). The *ULTRAPETALA1* gene functions early in *Arabidopsis* development to restrict shoot apical meristem activity and acts through *WUSCHEL* to regulate floral meristem determinacy. Genetics **167:** 1893–1903.

Chaudhury, A.M., Letham, S., Craig, S., and Dennis, E.S. (1993). Amp1 - A mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. Plant J. **4:** 907–916.

Dello Ioio, R., Linhares, F.S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., and Sabatini, S. (2007). Cytokinins determine *Arabidopsis* root-meristem size by controlling cell differentiation. Curr. Biol. **17:** 678–682.

Disch, S., Anastasiou, E., Sharma, V.K., Laux, T., Fletcher, J.C., and Lenhard, M. (2006). The E3 ubiquitin ligase BIG BROTHER controls *arabidopsis* organ size in a dosage-dependent manner. Curr. Biol. **16:** 272–279.

Edgerton, M.D. (2009). Increasing crop productivity to meet global needs for feed, food, and fuel. Plant Physiol. **149:** 7–13.

Endrizzi, K., Moussian, B., Haecker, A., Levin, J.Z., and Laux, T. (1996). The *SHOOT MERISTEMLESS* gene is required for maintenance of undifferentiated cells in *Arabidopsis* shoot and floral meristems and acts at a different regulatory level than the meristem genes *WUSCHEL* and *ZWILLE.* Plant J. **10:** 967–979.

Ferrándiz, C., Pelaz, S., and Yanofsky, M.F. (1999). Control of carpel and fruit development in *Arabidopsis.* Annu. Rev. Biochem. **68:** 321–354.

Galuszka, P., Popelková, H., Werner, T., Frébortová, J., Pospísilová, H., Mik, V., Köllmer, I., Schmülling, T., and Frébort, I. (2007). Biochemical characterization of cytokinin oxidases/dehydrogenases from *Arabidopsis thaliana* expressed in *Nicotiana tabacum* L. J. Plant Growth Regul. **26:** 255–267.

Giulini, A., Wang, J., and Jackson, D. (2004). Control of phyllotaxy by the cytokinin-inducible response regulator homologue *ABPHYL1.* Nature **430:** 1031–1034.

Gordon, S.P., Chickarmane, V.S., Ohno, C., and Meyerowitz, E.M. (2009). Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem. Proc. Natl. Acad. Sci. USA **106:** 16529–16534.

Heyl, A., Ramireddy, E., Brenner, W.G., Riefler, M., Allemeersch, J., and Schmülling, T. (2008). The transcriptional repressor ARR1-SRDX suppresses pleiotropic cytokinin activities in *Arabidopsis.* Plant Physiol. **147:** 1380–1395.

Higuchi, M., et al. (2004). *In planta* functions of the *Arabidopsis* cytokinin receptor family. Proc. Natl. Acad. Sci. USA **101:** 8821–8826.

Hu, Y., Xie, Q., and Chua, N.-H. (2003). The *Arabidopsis* auxin-inducible gene *ARGOS* controls lateral organ size. Plant Cell **15:** 1951–1961.

Itoh, J., Nonomura, K., Ikeda, K., Yamaki, S., Inukai, Y., Yamagishi, H., Kitano, H., and Nagato, Y. (2005). Rice plant development: from zygote to spikelet. Plant Cell Physiol. **46:** 23–47.

Jackson, D., Veit, B., and Hake, S. (1994). Expression of maize KNOTTED1 related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development **120:** 405–413.

Jasinski, S., Piazza, P., Craft, J., Hay, A., Woolley, L., Rieu, I., Phillips, A., Hedden, P., and Tsiantis, M. (2005). KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. Curr. Biol. **15:** 1560–1565.

Krizek, B.A. (2009). Making bigger plants: Key regulators of final organ size. Curr. Opin. Plant Biol. **12:** 17–22.

Krupková, E., Immerzeel, P., Pauly, M., and Schmülling, T. (2007). The T*UMOROUS SHOOT DEVELOPMENT2* gene of *Arabidopsis* encoding a putative methyltransferase is required for cell adhesion and co-ordinated plant development. Plant J. **50:** 735–750.

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., Sakakibara, H., and Kyozuka, J. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature **445:** 652–655.

Laufs, P., Grandjean, O., Jonak, C., Kiêu, K., and Traas, J. (1998). Cellular parameters of the shoot apical meristem in *Arabidopsis.* Plant Cell **10:** 1375–1390.

Leibfried, A., To, J.P.C., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J.J., and Lohmann, J.U. (2005). WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature **438:** 1172–1175.

Li, Y., Zheng, L., Corke, F., Smith, C., and Bevan, M.W. (2008). Control of final seed and organ size by the *DA1* gene family in *Arabidopsis thaliana.* Genes Dev. **22:** 1331–1336.

Lindsay, D.L., Sawhney, V.K., and Bonham-Smith, P.C. (2006). Cytokinin-induced changes in *CLAVATA1* and *WUSCHEL* expression temporally coincide with altered floral development in *Arabidopsis.* Plant Sci. **170:** 1111–1117.

Long, J.A., Moan, E.I., Medford, J.I., and Barton, M.K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis.* Nature **379:** 66–69.

Mähönen, A.P., Bishopp, A., Higuchi, M., Nieminen, K.M., Kinoshita, K., Törmäkangas, K., Ikeda, Y., Oka, A., Kakimoto, T., and Helariutta, Y. (2006). Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. Science **311:** 94–98.

Mähönen, A.P., Bonke, M., Kauppinen, L., Riikonen, M., Benfey, P. N., and Helariutta, Y. (2000). A novel two-component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root. Genes Dev. **14:** 2938–2943.

Malamy, J.E., and Benfey, P.N. (1997). Organization and cell differentiation in lateral roots of *Arabidopsis thaliana.* Development **124:** 33–44.

Marcelis, L.F.M., and Heuvelink, E. (2007). Concepts of modelling carbon allocation among plant organs. In Functional-Structural Plant Modelling in Crop Production, J. Vos, L.F.M. Marcelis, P.H.B. de Visser, P.C. Struik, and J.B. Evers, eds (Dordrecht, The Netherlands: Springer), pp. 103–111.

Matsumoto-Kitano, M., Kusumoto, T., Tarkowski, P., Kinoshita-Tsujimura, K., Václavíková, K., Miyawaki, K., and Kakimoto, T. (2008). Cytokinins are central regulators of cambial activity. Proc. Natl. Acad. Sci. USA **105:** 20027–20031.

Mayer, K.F.X., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G., and Laux, T. (1998). Role of WUSCHEL in regulating stem cell fate in the *Arabidopsis* shoot meristem. Cell **95:** 805–815.

Mizukami, Y., and Fischer, R.L. (2000). Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. Proc. Natl. Acad. Sci. USA **97:** 942–947.

Nieminen, K., et al. (2008). Cytokinin signaling regulates cambial development in poplar. Proc. Natl. Acad. Sci. USA **105:** 20032–20037.

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S., and Ueguchi, C. (2004). Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in *Arabidopsis.* Plant Cell **16:** 1365–1377.

Novák, O., Hauserová, E., Amakorová, P., Doležal, K., and Strnad, M. (2008). Cytokinin profiling in plant tissues using ultra-performance liquid chromatography-electrospray tandem mass spectrometry. Phytochemistry **69:** 2214–2224.

Riefler, M., Novak, O., Strnad, M., and Schmülling, T. (2006). *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell **18:** 40–54.

Rosso, M.G., Li, Y., Strizhov, N., Reiss, B., Dekker, K., and Weisshaar, B. (2003). An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol. Biol. **53:** 247–259.

Rupp, H.M., Frank, M., Werner, T., Strnad, M., and Schmülling, T. (1999). Increased steady state mRNA levels of the *STM* and *KNAT1* homeobox genes in cytokinin overproducing *Arabidopsis thaliana* indicate a role for cytokinins in the shoot apical meristem. Plant J. **18:** 557–563.

Sakakibara, H. (2006). Cytokinins: Activity, biosynthesis, and translocation. Annu. Rev. Plant Biol. **57:** 431–449.

Schneitz, K., Hülskamp, M., and Pruitt, R.E. (1995). Wild-type ovule development in *Arabidopsis thaliana*: A light microscope study of cleared whole-mount tissue. Plant J. **7:** 731–749.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F.X., Jürgens, G., and Laux, T. (2000). The stem cell population of *Arabidopsis* shoot meristems in maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. Cell **100:** 635–644.

Skinner, D.J., Hill, T.A., and Gasser, C.S. (2004). Regulation of ovule development. Plant Cell **16**(Suppl): S32–S45.

Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in *Arabidopsis.* Plant Cell **2:** 755–767.

Steeves, T.A., and Sussex, I.M. (1989). Patterns in Plant Development. (Cambridge, UK: Cambridge University Press).

To, J.P.C., Haberer, G., Ferreira, F.J., Deruère, J., Mason, M.G., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2004). Type-A *Arabidopsis* response regulators are partially redundant negative regulators of cytokinin signaling. Plant Cell **16:** 658–671.

Tucker, M.R., and Laux, T. (2007). Connecting the paths in plant stem cell regulation. Trends Cell Biol. **17:** 403–410.

Veit, B. (2009). Hormone mediated regulation of the shoot apical meristem. Plant Mol. Biol. **69:** 397–408.

Verwoerd, T.C., Dekker, B.M.M., and Hoekema, A. (1989). A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res. **17:** 2362.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmülling, T. (2003). Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15:** 2532–2550.

Werner, T., Motyka, V., Strnad, M., and Schmülling, T. (2001). Regulation of plant growth by cytokinin. Proc. Natl. Acad. Sci. USA **98:** 10487–10492.

Werner, T., Nehnevajova, E., Köllmer, I., Novak, O., Strnad, M., Krämer, U., and Schmülling, T. (2010). Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in *Arabidopsis* and tobacco. Plant Cell **22:** 3905–3920.

Werner, T., and Schmülling, T. (2009). Cytokinin action in plant development. Curr. Opin. Plant Biol. **12:** 527–538.

Yanai, O., Shani, E., Dolezal, K., Tarkowski, P., Sablowski, R., Sandberg, G., Samach, A., and Ori, N. (2005). *Arabidopsis* KNOXI proteins activate cytokinin biosynthesis. Curr. Biol. **15:** 1566–1571.

Zhao, Z., Andersen, S.U., Ljung, K., Dolezal, K., Miotk, A., Schultheiss, S.J., and Lohmann, J.U. (2010). Hormonal control of the shoot stem-cell niche. Nature **465:** 1089–1092.