Characterization and expression profiling of cucumber kinesin genes during early fruit development: revealing the roles of kinesins in exponential cell production and enlargement in cucumber fruit

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Abstract

Rapid cell division and expansion in early fruit development are important phases for cucumber fruit yield and quality. Kinesin proteins are microtubule-based motors responsible for modulating cell division and enlargement. In this work, the candidate kinesin genes involved in rapid cell division and expansion during cucumber fruit development were investigated. The morphological and cellular changes during early fruit development were compared in four cucumber genotypes with varied fruit size. The correlation between the expression profiles of cucumber kinesin genes and cellular changes in fruit was investigated. Finally, the biochemical characteristics and subcellular localizations of three candidate kinesins were studied. The results clarified the morphological and cellular changes during early cucumber fruit development. This study found that CsKF2–CsKF6 were positively correlated with rapid cell production; CsKF1 and CsKF7 showed a strongly positive correlation with rapid cell expansion. The results also indicated that CsKF1 localized to the plasma membrane of fast-expanding fruit cells, that CsKF2 might play a role in fruit chloroplast division, and that CsKF3 is involved in the function or formation of phragmoplasts in fruit telophase cells. The results strongly suggest that specific fruit-enriched kinesins are specialized in their functions in rapid cell division and expansion during cucumber fruit development.

Key words: Cell division, chloroplast, cucumber, expansion, fruit, kinesin, phragmoplast, plasma membrane.

Introduction

In most plants, early fruit development can be divided into three phases: development of the ovary, cell division, and subsequent cell expansion (Gillaspy et al., 1993). For cucumber fruit, the fruit weight could increase by ~200 times in 2 weeks through extremely rapid cell division [before anthesis to 5 days after anthesis (DAA)] and exponential cell enlargement (5 DAA to 14 DAA) (Marcelis and Baan Hofman-Eijer, 1993; Boonkorkaew et al., 2008; Fu et al., 2008, 2010). Unlike fruits that are eaten at a mature stage (such as tomato), cucumbers (Cucumis sativus L.), which are consumed as both a fresh product and a processed food, are typically harvested at an early phase of fruit development, namely the middle to late phase of the rapid fruit growth and ~2 weeks after anthesis (Ando et al., 2012). Therefore, the rapid cell division and expansion in early fruit development are important phases for cucumber fruit production. In addition to the crucial roles in fruit size, shape, and yield, recent research has also shown that the early stage is an important determinant of fruit quality traits, such as the...
accumulation of sugars and organic acids and the establishment of cutin (Lemaire-Chamley et al., 2005; Schlosser et al., 2008; Mounet et al., 2009; Ando et al., 2012). A number of studies have focused on fruit ripening and post-harvest physiology; however, the mechanism of early fruit growth, especially the mechanism of rapid cell division and expansion in cucumber, is not well understood. Recently, transcriptome analyses have indicated that complicated factors are involved in early cucumber fruit growth, and cytoskeletal proteins are suggested to play important roles in this process (Ando et al., 2012).

Controlling microtubule (MT) dynamics and spatial organization is a fundamental requirement of eukaryotic cell division and expansion. Kinesins constitute a superfamily of MT-dependent motor proteins and play critical roles in mitosis, meiosis, cell morphology, and the transport of vesicles and organelles (Lee and Liu, 2004; Lu et al., 2005; Vanstraalen et al., 2006; Hirokawa et al., 2009; Li et al., 2011; Zhou et al., 2011). Ultimately, kinesins contribute directly or indirectly to cell division and cell growth in various tissues. In plants, several kinesins, including ATK1, ATK5, AtKRP125c, AtPAKR1, and AtKINESIN-13A, have been reported to mediate cell mitosis and expansion in an MT-dependent manner (Lee and Liu, 2000; Chen et al., 2002; Lu et al., 2005; Ambrose and Cyr, 2007; Bannigan et al., 2007). Recent studies have revealed that cyclin and cyclin-dependent kinases, which are important facilitators of cell production during fruit development (Malladi and Johnson, 2011), phosphorylate a mitotic kinesin protein for the appropriate onset and/or progression of cytokinesis (Sasabe et al., 2011). However, it is still not known whether specific kinesin proteins are required for regulating exponential cell production and expansion during early fruit development.

Based on the interaction with cytoskeleton MTs, different kinesins have various roles in division and expansion: chromosome attachment to MTs, chromosome aggregation to the metaphase plate, sister chromatid segregation, spindle elongation, MT organization, organelle transportation for cell elongation, and control of cellulose microfibril order. Meanwhile, cell division and expansion in early cucumber fruit development have unique features, such as the rapid division and expansion speed (Marcelis and Baan Hofman-Berg.de/) was then performed, and all proteins without a kinesin motor domain were eliminated. This resulted in the identification of 47 predicted kinesins in cucumber.

RT-PCR and quantitative RT-PCR
Total RNA was isolated from various tissues of the plants using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA concentration was determined with a NanoDrop ND-2000 spectrophotometer. Reverse transcription was performed with 5 μg of total RNA using M-MLV reverse transcriptase (Promega) according to the manufacturer’s instructions. The specific primers were designed for reverse transcription–PCR (RT-PCR) and quantitative real-time PCR (RT-PCR) according to the cucumber kinesin sequences (Supplementary Table S2 available at JXB online). Quantitative RT-PCR was performed following the protocol of the Perfect Real-time PCR kit (TaKaRa) on an iQ™5 Multicolor Real time PCR Detection system (Bio-Rad). Amplification products were visualized by SYBR Green. Aliquots of the reverse transcription reaction products were used as templates for quantitative RT-PCRs. For relative quantification, the 2^(-ΔΔCT) method (Livak and Schmittgen, 2001) was used. A cucumber ACTIN gene (Csa06M484600) was detected as an internal reference, and each experiment was repeated three times (Ando et al., 2012).

Materials and methods

Plant materials
After seed germination, 100 plants of each cucumber (Cucumis sativus L.) variety, namely the ‘Chinese long’ inbred line 9930, the hybrid cultivar Zhong Nong 27, the Gherkin Nation Pickle, and 1972 B-2, were grown in pots containing mixed peat moss and vermiculite (1:1, v/v) in a greenhouse at the Institute of Vegetables and Flowers. These four varieties are of natural parthenocarpic capacity. The temperature was kept between 21 °C and 28 °C. Pest control was performed according to standard management practices.

Prior to the harvests, which were performed at 2, 0, 1, 2, 3, 5, 7, 8, 10, 12, 14, and 16 DAA, the fruits were measured for weight, length, and diameter, and were examined for changes in fruit firmness by using a ‘FHM-1’ (Japan) penetrometer with a 1 mm or 5 mm diameter plunger. Each sampling and measurement used five ovaries or fruits from the eighth to 10th nodes of the main vine from five plants with three replicates. The ovaries or fruits were simultaneously sampled, frozen quickly in liquid nitrogen, and stored at -80 °C for RNA or protein analysis.

Identification of kinesin genes from cucumber
All BLAST searches were conducted in the cucumber genome database by using three distinct motor domain sequences from the HKC (N-terminal motor in human), KIF2 (internal motor in mouse), and AtKCBP (C-terminal motor in Arabidopsis) as queries. An E-value cut-off of 1 was used, which yielded kinesins and many unrelated proteins. Domain analysis in SMART (http://smart.embl-heidelberg.de/) was then performed, and all proteins without a kinesin motor domain were eliminated. This resulted in the identification of 47 predicted kinesins in cucumber.

Cell number and cell area measurement
Ovaries or fruits were sampled at 0, 3, 5, 7, 12, and 16 DAA and fixed in a mixture of 70% ethanol, formaldehyde, and acetic acid (90:5:5, v/v/v). Slices 5 mm thick were cut from different parts of the fruit (outer, middle, and inner pericarp) and embedded in paraffin. Next, 8 μm thick cross-sections and longitudinal sections were prepared from the slices using a microtome. The sections were mounted, stained with haematoxylin–eosin, and photographed under a microscope (Ben-Cheikh et al., 1997; Yu et al., 2001). The cell number (X), cell area (A), and average cell area in a given section was calculated using infinity capture 6.0 and Image Plus 5.1 software. The area of the whole fruit cross-sections and longitudinal sections (A') was determined by measuring the ovary or fruit diameter and using the equation for the area of a circle (for a cross-section) or an ellipse (for a longitudinal section). The cell number in the whole fruit cross-sections and longitudinal sections (X') was calculated using the equation X'/A'=X'/A. The measurements were made at five sites of each tissue for five sections from each fruit.

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Plasmid construction

The full-length cDNA sequences of CsKF1 (CsaM446860), CsKF2 (CsaM3062600), and CsKF3 (CsaM002000) are available in the cucumber genome database. Constructs were generated using sequence-specific primers (Supplementary Table S1 at JXB online). For protein expression in vitro, the coding regions for KF1-N, KF2-C, KF3-C, KF1-motor, KF2-motor, and KF3-motor were amplified by PCR and inserted into either the pET28a vector or the pET30a vector. For protoplast transient expression assays, the full-length cDNAs of CsKF1 and CsKF2 were amplified by PCR. The corresponding green fluorescent protein (GFP) fusion constructs were made by fusing the target sequences to the N-terminus of GFP, and all of the fusion constructs above were cloned into a pUC vector under the control of the 3SS Cauliflower mosaic virus promoter.

Protein expression and antibody production

His-tagged KF1-N, KF2-C, KF3-C, KF1-motor, KF2-motor, and KF3-motor fusion proteins were expressed in the Escherichia coli BL21 (DE3) strain, induced with 0.1 mM isopropyl-β-D-thiogalactoside for 6 h at 22 °C, and affinity purified using an Ni2+-chelating Sepharose Fast Flow (Amersham Biosciences) column following the manufacturer’s instructions. Polyclonal anti-KF1, anti-KF2, and anti-KF3 antibodies were raised in rabbits using the purified His-KF1-N, His-KF2-C, and His-KF3-C protein as the antigens. Antiserum was then affinity purified using the AminoLink Plus kit (Pierce Chemical) with immobilized antigens according to the manufacturer’s instructions.

ATPase activity assay

MT-stimulated ATPase activity was assayed in PEM buffer (50 mM PIPES, 5 mM EGTA, and 1 mM MgSO4, pH 6.9). The reaction was initiated by adding 2 mM ATP to a mixture of 0–12 μM taxol-stabilized MTs and 5 μM KF1-motor or KF2-motor. The reaction lasted for 15 min at 25 °C and was terminated by adding 10% trichloroacetic acid (TCA) on ice for 10 min. The supernatant was collected after centrifugation at 4 °C, 20,000 g for 5 min. The inorganic phosphate (Pi) in the supernatant was determined as described previously (LeBel et al., 1978). As a control, TCA was added before the addition of ATP. The assays were repeated three times.

Microtubule binding assay

MT co-sedimentation assays were performed in PEM buffer plus 1 mM dithiothreitol (DTT) and 10 μM taxol. For each assay, 7 μM of highly purified KF1-motor or KF2-motor was incubated with 10 μM taxol-stabilized MTs for 10 min at 25 °C in the absence or presence of 5 mM Mg-ATP or 5 mM 5′-adenylylimidodiphosphate (AMP-PNP; a non-hydrolysable ATP analogue; Sigma). The samples were then centrifuged at 100,000 g for 30 min. The supernatants and pellets were separated, brought to equal volumes in the SDS sample buffer, analysed by SDS-PAGE, and visualized by staining the gel with Coomassie Brilliant Blue R-250.

Immunolabelling and protoplast transient expression assays

Fruit cells (0 DAA for CsKF2, 2 DAA for CsKF3, and 5 DAA for CsKF1) were harvested at 06:00 h, when cell division is rapid, and immunolabelled as described by Xu et al. (2009). Anti-KF1, anti-KF2, and anti-KF3 antibodies (1:500), and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories; diluted 1:200) were used to detect CsKF1, CsKF2, and CsKF3 proteins. Anti-α-tubulin monoclonal antibody (Sigma; diluted 1:500) and TRITC (tetramethylrhodamine-β-isothiocyanate)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories; diluted 1:200) were used to label MTs. DNA was stained with 0.5 μg ml−1 DAPI (4′,6-diamidino-2-phenylindole; Sigma).

The plasmids that were used for transient expression were purified using a TIANprep Midi Plasmid Kit (TIANGEN). The fusion constructs were introduced into Arabidopsis mesophyll protoplasts using PEG (polyethylene glycol)-mediated transformation (Yoo et al., 2007).

Results

Physiological changes, cell production, and cell expansion during early cucumber fruit development in 9930

The ‘Chinese long’ inbred line 9930 is a cucumber cultivar grown in northern China with an available genomic sequence (Huang et al., 2009). This cucumber cultivar was grown in a greenhouse as described in the Materials and methods. It was observed that 9930 grew rapidly during the early stage, from −2 DAA to 16 DAA (Fig. 1A). There was a slight change in fruit weight between −2 DAA and 5 DAA, but the fruit weight increased sharply after 5 DAA (Fig. 1B). The fruit length and fruit diameter showed similar growth curves, initiating from −2 DAA to 3 DAA and increasing almost exponentially between 3 DAA and 16 DAA (Fig. 1C, D). The fruit firmness was relatively constant before 3 DAA and then decreased rapidly (Fig. 1E). After 16 DAA, 9930 fruit showed no significant morphological changes. These results indicated that the period from 3 DAA to 5 DAA was an obvious boundary for fast growth of the fruit of 9930.

To clarify further the change in the cellular growth pattern during early fruit development, the cell number and average cell area were measured and calculated on the cross-section and longitudinal section of the fruit. As shown in Fig. 2, rapid increases in cell number were observed between 0 DAA and 3 DAA in both the cross-section and longitudinal section, with an ~200% increase. The growth rate then decreased between 3 DAA and 5 DAA. Although the cell number of the cucumber fruit continued to increase from 5 DAA to 16 DAA, this occurred at a lower rate (Fig. 2A, B). The average cell area increased by <2-fold from 0 DAA to 5 DAA. However, the majority of the exponential increase in cell size (almost 30-fold) occurred between 5 DAA and 16 DAA, after the cells exited from mitotic cell production (Fig. 2C). It was also found that the changes in average cell area were similar in both the cross-section and longitudinal section directions during early 9930 fruit development (data not shown), which suggests that 9930 fruit elongation is mainly caused by an increase in cell number in the longitudinal direction rather than increased cell expansion in the longitudinal direction.

Together, the above data indicated that early fruit growth was closely associated with cell production before 3 DAA and that the cell division rate decreased between 3 DAA and 5 DAA. At this time, cell expansion was initiated, and fruit growth was primarily driven by cell expansion after 5 DAA.

Identification of cucumber kinesin genes

To investigate the function of cucumber kinesin genes during early fruit development, 47 genes were identified in the cucumber genome by using the conserved kinesin motor
Fig. 1. Morphological and physiological changes in 9930 during early fruit development. (A) Developing cucumber fruits at –2, 0, 1, 2, 3, 5, 7, 8, 10, 12, 14, and 16 days after anthesis (DAA). (B) Increase in the weight of 9930 fruit during early fruit development. (C) Increase in the length of 9930 fruit during early fruit development. (D) Increase in the diameter of 9930 fruit during early fruit development. (E) Decrease in the firmness of 9930 fruit during early fruit development.

Fig. 2. Cell production kinematics and cell expansion during early fruit development in cucumber 9930 fruit. (A) Total cell number of the cross-section in fruits during the early developing stage. (B) Total cell number of the longitudinal section in fruits during the early developing stage. (C) Average cell area of the cross-section in fruits during the early developing stage.
Fruit-enriched kinesins are specialized in their functions in cucumber fruit development

domains of human, mouse, and Arabidopsis as BLAST queries in the cucumber genome database (http://www.icugi.org/cgi-bin/ICuGI/). All of the 47 kinesin genes have corresponding homologues in Arabidopsis (Supplementary Table S3 at JXB online). According to the phylogenetic analysis of the cucumber kinesins and the previously recognized family, all predicted cucumber kinesins were found to be distributed across all kinesin families except families 2, 3, 9, and 11, which was consistent with a previous report in the land plant (Zhu and Dixit, 2011). The results also showed that all of these cucumber families were smaller than those in Arabidopsis, with the exception of the kinesin-13 family. Cucumber has three members in this family, whereas Arabidopsis only has two (Supplementary Fig. S1). Moreover, the expression of 47 cucumber kinesin genes during vegetative (root, stem, tendril, true leaf, cotyledon, and hypocotyl) and reproductive development (dry seed, gminated seed, male flower, female flower, ovary, 0 DAA fruit, 2 DAA fruit, and 10 DAA fruit), measured by using RT-PCR, indicated that all of these predicted kinesins were expressed genes and were differentially expressed in various tissues (Supplementary Fig. S2).

Kinesin gene expression patterns in early cucumber fruit development

To find the potential candidate kinesin proteins that are involved in early cucumber fruit development, actively transcribed genes were measured using quantitative RT-PCR. The quantitative changes of kinesin gene expression were compared with changes in cell production and expansion, and it was possible to observe the expression patterns of kinesin genes in early 9930 fruit development (Fig 3; Supplementary Fig. S3 at JXB online). As shown in Fig. 3, hierarchical clustering gave a more visual analysis of kinesin gene expression. All of the cucumber kinesin genes, except Cs4g219350, showed significant changes in expression during early fruit development (from –2 DAA to 16 DAA). Further analysis indicated that 10 kinesin genes (labelled in red and blue) showed peak expression levels in the rapid cell division phase (before 3 DAA), while two kinesin genes showed peak expression levels during the rapid cell expansion (after 3 DAA) (Fig. 3; Supplementary Table S4). Eight of these kinesin genes that showed at least a 4-fold up-regulation were selected for further study (labelled by the red ticks; Fig. 3). Csα6M499030, Csα4M002000, Csα2M2509930, Csα5M157410, Csα3M062600, and Csα1M568560 displayed enhanced levels of expression during the rapid cell production phase, between –2 DAA and 3 DAA, while two kinesin genes showed peak expression levels during the rapid cell expansion (after 5 DAA) (Fig. 3; Supplementary Table S4). Eight of these kinesin genes that showed at least a 4-fold up-regulation were selected for further study (labelled by the red ticks; Fig. 3). Csα6M499030, Csα4M002000, Csα2M2509930, Csα5M157410, Csα3M062600, and Csα1M568560 displayed enhanced levels of expression during the rapid cell production phase, between –2 DAA and 3 DAA, while both Csα1M495290 and Csα7M446860 are typically characterized by the specific up-regulation at the initiation of exponential cell expansion, ~5 DAA during early fruit development.

Physiological and cellular characterization of fruit in four cucumber varieties with different fruit size

Because fruit cell number and cell size are important determinants of fruit size, it was decided to clarify further the relationship between the eight selected kinesin genes and rapid cell division or expansion. Therefore, the gene expression profiles were investigated in three other cucumber varieties with varied fruit size during early fruit development. Two of the cucumber varieties, 1972 B-2 and National Pickle, are pickling cucumbers with relatively smaller fruit, while the third variety, Zhong Nong 27, has relatively larger fruit and is a hybrid variety found in northern China.

The morphological and cellular changes in the three varieties were characterized during early fruit development. As shown in Fig. 4A, 1972 B-2 produced the smallest fruit, National Pickle produced a relatively larger fruit, and Zhong Nong 27 produced the largest fruit during early fruit development (from –2 DAA to 16 DAA). After 16 DAA, these varieties showed little change in fruit size. From the largest to the smallest, the cucumber varieties rank in fruit size as follows: Zhong Nong 27 > 9930 > National Pickle > 1972 B-2 (Figs 1, 4). Similar to the observations of 9930, it was found that the stage from 3 DAA to 5 DAA was also a period of transition in fruit development.
growth for 1972 B-2, National Pickle, and Zhong Nong 27 (Supplementary Fig. S4 at JXB online). However, the growth rates of fruit weight, length, and diameter were much higher in the variety with larger fruit (Supplementary Fig. S4).

To understand the cellular changes in the four cucumber varieties during early fruit development, the cell number and average cell area of the total cross-section of the fruit were observed and compared. Figure 4B shows that similarly to 9930, Zhong Nong 27 exhibits an exponential increase in cell number between 0 DAA and 3 DAA, resulting in a 200% increase in cell number. Between 3 DAA and 5 DAA, there was a small increase in the cell number, and the cell number increased very slowly from 5 DAA to 16 DAA (Fig. 4B). Both 1972 B-2 and National Pickle also showed very rapid increases in cell number by almost 200% between 0 DAA and 3 DAA; however, from 3 DAA to 16 DAA, the cell number of these varieties exhibited constant growth curves (Fig. 4B). The order of the final cell number at 16 DAA is 9930 $\approx$ Zhong Nong 27 $>$ 1972 B-2 $\approx$ National Pickle (A $\approx$ B $\approx$ C $\approx$ D; Fig. 4B; Table 1). Interestingly, three varieties, 9930, Zhong Nong 27, and National Pickle, showed a rapid increase in the average cell area from 5 DAA to 16 DAA, resulting in a 30-fold increase. However, the cell area of 1972 B-2 only increased by $\sim$3-fold, which could explain why 1972 B-2 bears the smallest fruit (Fig. 4C). The order of the final average cell area at 16 DAA is Zhong Nong 27 $>$ 9930 $\approx$ National Pickle $>>$ 1972 B-2 (B $>$ A $=\approx$ D $>>$ C; Fig. 4C; Table 1). These results indicate that both cell numbers and cell area correlate strongly with fruit size.

Identification of seven candidate kinesins involved in rapid fruit cell division and expansion

The expression of the eight predicted kinesins was further analysed in these four characterized varieties during early fruit development. As expected, seven of the eight kinesin genes,
Csa7M446806, Csa3M062600, Csa4M002000, Csa6M499030, Csa2M250930, Csa5M157410, and Csa1M495290, showed different expression levels that correlated with the differences in cell number or expansion among the four cucumber varieties (Fig. 5). According to the result from quantitative trait loci (QTL) mapping of domestication-related traits, these seven candidate kinesin genes were also identified to be the potential trait genes selected during cucumber domestication (data not shown). Csa3M062600, Csa4M002000, Csa6M499030, Csa2M250930, and Csa5M157410 were up-regulated before 3 DAA, and the expression levels of these genes correlated with the difference in cell numbers in these four varieties (Fig. 5B–F; Table 2). Additionally, Csa7M446806 and Csa1M495290 were up-regulated after 5 DAA, and the expression levels of these genes correlated with the order of cell area in these four varieties (Fig. 5A, G; Table 2). However, the expression levels of the remaining kinesin gene, Csa1M568560, showed no significant difference among these four varieties (Fig. 5H). Csa3M891640, an unrelated gene, was used as a control (Fig. 5I).

These results strongly suggest that these seven candidate kinesin genes are involved in early cucumber fruit development. Csa3M062600, Csa4M002000, Csa6M499030, Csa2M250930, and Csa5M157410 play roles in rapid cell production, while Csa7M446806 and Csa1M495290 function in exponential cell expansion.
Identification of CsKF1, CsKF2, and CsKF3

For a more concise description in the following research, the seven candidate kinesin genes were then designated as CsKF1, CsKF2, CsKF3, CsKF4, CsKF5, CsKF6, and CsKF7 (KF is an abbreviation for ‘kinesin in fruit’), as indicated in Table 3. The corresponding Arabidopsis homologues, families, and predicted functions are also indicated in Table 3. The functions of all seven candidate kinesins CsKF1–CsKF7 in cucumber fruit need further investigation. In this work, CsKF1 (Csa7M446860), CsKF2 (Csa3M062600), and CsKF3 (Csa4M002000), whose Arabidopsis homologues have unknown functions, were first selected for further study. The full-length CDS (coding sequences) of CsKF1 (2058 bp), CsKF2 (3420 bp), and CsKF3 (3066 bp) were obtained by using the primers that were specifically designed according to the gene sequences found in the Cucurbit Genomics Database (Fig. 6A). The deduced CsKF1 protein has a calculated molecular mass of 77 kDa and a pI of 6.4, the CsKF2 protein consists of 1139 amino acids with a calculated molecular mass of 128 kDa and a pI of 6.2, and the CsKF3 protein consists of 1022 amino acids with a calculated molecular mass of 112 kDa and a pI of 8.3. Schematic structure analyses of CsKF1, CsKF2, and CsKF3 indicate that these kinesins have conserved motor domains (Fig. 6B). Phylogenetic analyses with other representative kinesins in Arabidopsis and rice were performed to investigate how these polypeptides relate to other kinesins. CsKF1 belongs to the kinesin-13 family, CsKF2 belongs to the kinesin-12 family, and CsKF3 belongs to the kinesin-14 family (Supplementary Fig. S1 at JXB online). The secondary structure prediction showed the localization of coiled-coil regions in these three kinesins, which are required for oligomerization of kinesin proteins in their native forms (Fig. 6C). RT-PCR expression assays demonstrated that CsKF1 is expressed in all examined tissues but was enriched in developed fruit (Supplementary Fig. S2). CsKF2 and CsKF3 were expressed in all tissues except the seed, and were also enriched in developed fruit (Supplementary Fig. S2).

Biochemical identification of the CsKF1 motor and CsKF2 motor

Kinesins are molecular motors that hydrolyse ATP as they move along MTs, and these proteins exhibit MT-stimulated ATPase activity (Vale and Fletterick, 1997). To examine whether the motor domains of CsKF1, CsKF2, and CsKF3 have traditional biochemical characteristics (MT

Table 3. List of seven candidate cucumber kinesins involved in rapid cell division and expansion

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Name</th>
<th>Arabidopsis homologue</th>
<th>Function of Arabidopsis homologue</th>
<th>Predicted function in cucumber fruit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csa7M446860</td>
<td>CsKF1</td>
<td>KINESIN-13B</td>
<td>A mitosis-specific kinesin localizes in plasma membrane with unknown function</td>
<td>Rapid cell expansion</td>
</tr>
<tr>
<td>Csa3M062600</td>
<td>CsKF2</td>
<td>AT3G20150</td>
<td>Unknown</td>
<td>Rapid cell division</td>
</tr>
<tr>
<td>Csa4M002000</td>
<td>CsKF3</td>
<td>AT2G47500</td>
<td>Kinesin-14</td>
<td>Rapid cell division</td>
</tr>
<tr>
<td>Csa6M499030</td>
<td>CsKF4</td>
<td>KCA1/KAC1</td>
<td>For cytokinesis and actin-based chloroplast movement</td>
<td>Rapid cell division</td>
</tr>
<tr>
<td>Csa2M250930</td>
<td>CsKF5</td>
<td>MKRP2</td>
<td>Predicted to work in mitochondria</td>
<td>Rapid cell division</td>
</tr>
<tr>
<td>Csa5M157410</td>
<td>CsKF6</td>
<td>ARK3</td>
<td>Accumulates at the pre-prophase band (PPB) in a cell cycle</td>
<td>Rapid cell division</td>
</tr>
<tr>
<td>Csa1M495290</td>
<td>CsKF7</td>
<td>AT3G45850</td>
<td>A mitosis-specific kinesin localizes in plasma membrane with unknown function</td>
<td>Rapid cell expansion</td>
</tr>
</tbody>
</table>

The final seven candidate kinesins predicted to be involved in early fruit development were designated to CsKF1, CsKF2, CsKF3, CsKF4, CsKF5, CsKF6, and CsKF7, and the corresponding Arabidopsis homologues, families, and predicted functions are indicated.
binding ability and MT-stimulated ATPase activity), a histidine-tagged CsKF1 motor domain (from amino acid 168 to 499) and a histidine-tagged CsKF2 motor domain (from amino acid 105 to 452) were solubly expressed in *E. coli* and purified from the cell extract by Ni-NTA agarose affinity chromatography as 40 kDa polypeptides (Fig. 7A). Unfortunately, the soluble-expressed motor domain of CsKF3 could not be obtained in *E. coli* under several expression conditions. This could have been because two short coiled-coil domains located in the predicted motor of CsKF3 reduce the solubility of this protein (Fig. 6D).

Figure 7B shows the results of an MT-stimulated ATPase activity assay. In the absence of MTs, the basal steady-state ATPase activity of the CsKF1 motor and the CsKF2 motor was 13.89 ± 3.97 Pi mol min⁻¹ mol⁻¹ and 21.72 ± 1.18 Pi mol min⁻¹ mol⁻¹, respectively (Fig. 7B). Moreover, the ATPase activity of the CsKF1 motor was triggered by MTs in a concentration-dependent manner, whereas, surprisingly, the ATPase activity of the CsKF2 motor was independent of the MT concentration. MTs alone showed no detectable ATPase activity (Fig. 7B).

These fusion proteins were further used in MT co-sedimentation experiments. In the absence of an exogenous nucleotide, the His-CsKF1-motor largely sedimented with the MT pellet. In the presence of ATP, >50% of the fusion protein was found in the supernatant rather than in the MT pellet. When ATP was replaced by the non-hydrolysable ATP analogue adenylyl imidodiphosphate (AMPPNP), the fusion protein appeared almost exclusively in the MT pellet (Fig. 7C). However, the His-CsKF2-motor co-sedimented with the MT pellet regardless of the presence of exogenous nucleotides (Fig. 7C).

These results indicate that His-CsKF1-motor has an MT-stimulated ATPase activity and binds to MTs in an ATP-dependent manner, which is consistent with the traditional characterization of a kinesin protein. However, the ATPase activity of the His-CsKF2-motor is independent of MT concentration, and this protein binds to MTs in a nucleotide-independent manner.

CsKF1, CsKF2, and CsKF3 localizations in cucumber fruit cells and Arabidopsis protoplasts

To gain insight into the functions of CsKF1, CsKF2, and CsKF3 in early fruit development, the intracellular localization pattern was determined in fruit cells via immunofluorescence labelling. Antibodies were raised against the affinity-purified His-CsKF1-N-terminus (KF1-N; amino acids 1–167), His-CsKF2-C-terminus (KF2-C; amino acids 943–1139), and the His-CsKF3-C-terminus (KF3-C; amino acids 873–1022) (Fig. 8A). Affinity-purified anti-KF1-N,
Fig. 7. *In vitro* ATPase activities and microtubule (MT) binding abilities of CsKF1 and CsKF2 motors. (A) Prokaryotic expression and affinity purification of His-tagged CsKF1 and CsKF2 motor domain fusion proteins. (B) MT concentration dependence of CsKF1-motor and CsKF2-motor ATPase activities. (C) MT binding assay. The resulting supernatant (S) and pellet (P) were analysed by SDS–PAGE and visualized by Coomassie blue staining. For each assay, 7 μM motor proteins were used for MT binding in the absence or presence of 5 mM MgATP or AMP-PNP.
anti-KF2-C, and anti-KF3-C antibodies could specifically recognize their corresponding prokaryotic-expressed His-tagged antigens, but did not recognize the two other proteins (Fig. 8B–D). The anti-KF1 antibody recognized an ~80 kDa band, which is similar to the predicted size of CsKF1, in cucumber root, leaf, shoot, and fruit (5 DAA). The anti-KF2 antibody recognized an ~125 kDa band in cucumber leaf, shoot, and fruit (0 DAA). The anti-KF3 antibody recognized an ~110 kDa band in cucumber root, stem, male flower, female flower, and fruit (2 DAA) (Fig. 8E–G). As expected, each of the three kinesin proteins could be detected more clearly in cucumber fruit cells sampled at the time points at which each gene was up-regulated. An anti-α-tubulin antibody was used as a positive control, and the α-tubulin band was observed in all samples (Fig. 8H). These results indicated that these antibodies specifically recognize CsKF1, CsKF2, and CsKF3 at the predicted sizes in cucumber in vivo.

Next, these specific antibodies were used to localize CsKF1, CsKF2, and CsKF3 in fruit cells via immunofluorescence. Figure 9A–C shows that CsKF1 was detected at the plasma membrane in fruit cells at 5 DAA. To confirm this result, GFP–KF1 was constructed and transiently expressed in Arabidopsis mesophyll protoplasts. It was found that the KF1–GFP signal mainly co-localized with the plasma membrane (Fig. 9D–F). The plasma membrane localization of CsKF1 coincides with the predicted function of CsKF1 in rapid cell enlargement (Table 3).

Interestingly, immunofluorescence labelling showed that anti-KF2 antibodies detected particles (~1 μm) in a punctate pattern in the cytoplasm in fruit cells at 0 DAA. These punctate signals showed no significant co-localization with immunolabeled tubulin (Fig. 10A–D). This result suggests that CsKF2 might work specifically in an organelle.

Figure 10E–H showed that immunofluorescence of anti-CsKF2 antibodies merged with chloroplast autofluorescence. To confirm the chloroplast targeting of CsKF2, KF2–GFP was transiently expressed in Arabidopsis mesophyll protoplasts. The GFP–KF2 signal was localized in chloroplasts (Fig. 10F–K). To understand the detailed localization of CsKF2 in chloroplasts, its detailed localization was studied in cucumber fruit cells by combining the chloroplast fraction with immunoblotting detection. As shown in Fig. 11, CsKF2 proteins exist predominantly in chloroplasts, in which proteins from both membrane and stroma showed CsKF2 immunoblotting signals. The chloroplast envelope marker Tic40 and stroma marker Hcf101 were used as controls (Fig. 11). To gain insight into how CsKF2 enters the chloroplast, the full-length peptide sequence of CsKF2 was
analysed using ChloroP 1.1. The results indicated that a chloroplast transit peptide (cTP; amino acids 1–38) is located at the N-terminus of CsKF2 (Supplementary Table S5 at JXB online). To the authors’ knowledge, this is the first reported kinesin-related protein that can enter the chloroplast. It was also found that the CsKF2 fluorescence signals could predominantly be detected in fruit that was harvested at –2 DAA to 2 DAA during rapid cell division (Supplementary Fig. S5). The results suggest that CsKF2 might be involved in the segregation of chloroplast nucleoids and plays a role in chloroplast division during early fruit cell production.

Immunofluorescence with the anti-KF3 antibody indicated that CsKF3 localized to the central region of telophase cells in fruit cells at 2 DAA (Fig. 12). This result raised the question of whether the localization of CsKF3 changed dynamically during the cell cycle. Dual localizations of CsKF3 and tubulin were performed. During interphase, CsKF3 was present throughout the cytoplasm and in the nucleus. During the progression of mitosis, no fluorescence signal was detected in the MT pre-prophase band or in MTs on the nuclear envelope (data not shown). In metaphase cells, judged by their spindle MTs, only a few CsKF3 punctate signals could be detected by fluorescence microscopy in the cytoplasm (Fig. 12A–D), and this same staining pattern was observed during anaphase (Fig. 12E–H). When sister chromatids were separated completely during telophase, CsKF3 mainly localized at the midzone of the phragmoplast (Fig. 12I–L). These results indicate that CsKF3 is involved in the function or formation of dynamic phragmoplasts in telophase during rapid division.

**Discussion**

The early stages of cucumber fruit development, from initial set through rapid cell division and cell enlargement, are critical determinants of size, yield, and quality (Fu et al., 2008; Ando et al., 2012). Recent studies have indicated that the cytoskeleton has important roles in this process (Ando and Grumet, 2010; Ando et al., 2012). However, there has been little detailed analysis. In the plant cell, the dynamics of MTs have critical roles in the cell cycle, construction of the cell wall, and maintenance of cell morphology. In this study, seven candidate MT-based kinesin genes that are involved in the early exponential growth of cucumber fruit were identified by comparing fruit morphological and cellular analyses using quantitative RT-PCR. Further functional studies showed that CsKF1 localizes to the plasma membrane and may participate in exponential cell expansion; that CsKF2 functions in the chloroplast during the rapid cell division phase, which suggested a role in chloroplast division; and that CsKF3 is involved in the function or formation of dynamic phragmoplasts in telophase during rapid division.

**Early exponential growth of cucumber fruit**

Early fruit development is characterized by phases of rapid cell division and expansion (Gillaspy et al., 1993). Previous studies have shown that in cucumber fruit, which develops from an enlarged inferior ovary, cell division occurs most rapidly prior to anthesis and then continues from 0 DAA to 5 DAA (Gillaspy et al., 1993; Robinson and Decker-Walters,
Fruit-enriched kinesins are specialized in their functions in cucumber fruit development (Boonkorkaew et al., 2008). Fruit enlargement begins almost immediately after pollination, with the most rapid increase occurring at ~4–12 DAA (Ando and Grumet, 2010). Cell division and expansion are largely completed by 12–16 DAA, with some variation depending on the cultivar and season (Marcelis and Baan Hofman-Eijer, 1993; Ando and Grumet, 2010; Ando et al., 2012). Here, the morphological and cellular changes during early fruit development were compared in four different cucumber varieties with varied fruit size (Figs 1, 4). The results closely concur with earlier studies. It was found that ~3–5 DAA is the transition from rapid cell division to cell expansion in early cucumber fruit development. Before 3–5 DAA, the cell number increases rapidly, and, following exit from mitotic cell production, an exponential increase in cell size (30-fold) occurs from 5 DAA to 16 DAA (Figs 2, 4). Additionally, the fruits of 9930 and Zhong Nong 27, the varieties with larger fruits, show higher rates of cell division and cell growth than those of the 1972 B-2 and National Pickle fruits, the varieties with smaller fruits (Figs 2, 4). Moreover, the average cell area in 1972 B-2 fruit is almost 20-fold less than the cell area of the other varieties (Fig. 4C), which strongly suggests why 1972 B-2 bears the smallest fruit in the four varieties. To the authors’ knowledge, this study is the first to determine the morphological and cellular changes of fruit in different genotypes of cucumber with varied fruit size, and these changes mirror the basic patterns of early cucumber fruit development.

Kinesins are involved in early cucumber fruit development

It has been reported that the tubulin gene shows high expression levels in the early phase of cucumber growth, which suggests that tubulin has a critical role in this process (Ando...
The dynamics of MTs are modulated by MT-associated proteins (Olmsted, 1986). Kinesin superfamily proteins are important MT-based motor proteins, with a motor domain that is conserved among all eukaryotic organisms (Li et al., 2012). In addition to the responsibility for unidirectionally transporting various cargos, kinesins also play critical roles in mitosis and cell morphogenesis (Lee and Liu, 2004; Hirokawa et al., 2009; Li et al., 2012; Zhu and Dixit, 2012). It is unclear whether any specific kinesin genes are involved in the rapid cell division and cell enlargement during early cucumber fruit development. In this research, 47 kinesin genes found in 14 kinesin families and one ungrouped family were identified in the cucumber genome (Supplementary Table S3, Supplementary Fig. S1 at JXB online). All of these kinesin genes were found to be expressed in various cucumber tissues (Supplementary Fig. S2). All of the kinesin genes except Cs4g219350 showed a significant change in expression during early fruit development (from –2 DAA to 16 DAA), suggesting important roles for the kinesin gene family in facilitating cell production and growth in cucumber fruit (Fig 3; Supplementary Fig. S3).

Several studies have previously addressed genome-wide changes in gene expression during fruit development, such as in tomatoes and apples (Alba et al., 2004; Lemaire-Chamley et al., 2005; Janssen et al., 2008; Malladi and Johnson, 2011), but there has been little research conducted in cucumbers. Recently, through linking morphological and cellular analyses with 454 pyrosequencing, researchers have studied transcript-level changes occurring in young cucumber fruit at five ages from anthesis through to the end of exponential

Fig. 11. Immunoblotting of chloroplast fractions shows that CsKF2 predominantly localizes to both the membrane and stroma of chloroplasts. (A) SDS–PAGE of protein from the cytosol, chloroplast, chloroplast membrane, and stroma. (B) CsKF2 (detected by anti-CsKF2 antibody) localizes to both the membrane fractions including envelope and thylakoid membranes, and the stroma fraction.

Fig. 12. CsKF3 distributes in a cell cycle-dependent manner in cucumber fruit cells. CsKF1 (green), microtubules (red), and the nucleus (stained with DAPI; blue) are shown at different cell division stages. (A–D) In metaphase cells, few CsKF3 punctate signals could be detected by fluorescence microscopy. (E–H) During anaphase, only small CsKF3 punctate signals could be detected by anti-CsKF3 antibodies. (I–L) CsKF3 mainly localized at the midzone of the phragmoplast in telophase cells. Scale bar=5 μm.
growth, providing knowledge of early cucumber fruit development (Ando and Grumet, 2010; Ando et al., 2012). In this study, quantitative expression changes of kinesin genes were compared with the changes that occur during cell production and cell expansion during early fruit development in the four cucumber varieties with varied fruit size. It was found that five kinesin genes, Csa3M062600, Csa4M082000, Csa6M499030, Csa2M250930, and Csa5M157410 (CsKF2–CsKF6), were positively correlated with rapid cell production, and two kinesin genes, Csa7M468860 and Csa1M495920 (CsKF1 and CsKF7), showed strong positive correlation with rapid cell expansion (Fig. 5; Tables 1–3). These seven candidate kinesins are distributed across six different kinesin families. Additionally, the Arabidopsis homologues of CsKF1, CsKF2, CsKF3, and CsKF7 have unknown functions, while the Arabidopsis homologues of CsKF4, CsKF5, and CsKF6 have been reported to work in chloroplast movement, mitochondrial division, and cell mitosis, respectively (Table 3). The results suggest that the cucumber kinesins have various functions in rapid fruit growth.

Function of novel kinesins CsKF1, CsKF2, and CsKF3 in early cucumber fruit development

Among these seven candidate kinesin genes, CsKF1, CsKF2, and CsKF3, were chosen for further study. The functions of the Arabidopsis homologues of these genes are unknown. Expression assays in various cucumber tissues revealed that all the three kinesin genes are more highly enriched in developing fruit (Supplementary Fig. S2 at JXB online). Immunoblot with specific polyclonal antibodies against CsKF1, CsKF2, and CsKF3 showed that these proteins could be detected in cucumber fruit sampled at specific times during fruit development (Fig. 8E–G). These results suggest the involvement of these three kinesins during early cucumber fruit development.

Phylogenetic analyses showed that CsKF1 belongs to the kinesin-13 family and is orthologous to AtKINESIN13-B in Arabidopsis (Supplementary Fig. S1 at JXB online). AtKINESIN13-A and AtKINESIN13-B are a pair of counterparts in Arabidopsis (Lee and Liu, 2004). The function of AtKINESIN13-B has not been reported. AtKinesin13A mutants have an abnormal trichome phenotype, suggesting the roles of MTs and AtKINESIN13-A in the organization of Golgi stacks, trichome elongation, and cell morphogenesis (Lu et al., 2005). In rice, the kinesin protein SRS3 has an amino acid sequence that resembles that of AtKINESIN13-B in Arabidopsis and is involved in regulating cell area and rice seed length (Kitagawa et al., 2010). The present results showed that CsKF1 localizes to the plasma membrane of fruit cells in the rapid expansion stage (Fig. 9) and has a strong positive correlation with rapid cell expansion during early fruit development (Fig. 5A; Tables 1–3). These results provided experimental clues that a novel kinesin CsKF1 played a role in fruit cell expansion in cucumber. It would be interesting to establish the detailed role of CsKF1 by transgenic methods in the future.

Phylogenetic analyses showed that CsKF2, whose homologue in Arabidopsis has an unknown function, belongs to the kinesin-12 family (Supplementary Fig. S1 at JXB online). Biochemical analyses showed that the ATPase activity of His-CsKF2-motor is independent of the concentration of MTs, and this protein binds to MTs in a nucleotide-independent manner, which are uncommon characteristics of kinesins (Fig. 7). Immunolabelling, transient expression of GFP fusion protein, and chloroplast fractionation showed that CsKF2 predominantly localized in the membrane and stroma of chloroplasts in fruit cells (Figs 10, 11). Moreover, a CTP was found in the N-terminus of CsKF2, indicating that CsKF2 functions within the chloroplast (Supplementary Table S5). Previous studies have shown that lipoxygenase (LOX) and hydroperoxide lyase (HPL), which are essential for the production of the cucumber flavour (E,Z)-2,4-nonadienal, are known to be important for cucumber fruit development. As is known, the prokaryotic homologue of tubulin, ftsZ, is responsible for plant chloroplast division (Osteryoung et al., 1998; Reski, 2002). In prokaryotes, a kinesin homologue, MukB, interacting with FtsZ, is involved in chromosome partitioning (Niki et al., 1991; Lockhart and Kendrick-Jones, 1998a, b). It was inferred that CsKF2 might be involved in chloroplast division, and the partner of CsKF2 might be ftsZ but not tubulin, which could explain the uncommon biochemical properties of CsKF2 that were observed during research with porcine brain tubulins.

CsKF3 belongs to the kinesin-14 family (Supplementary Fig. S1 at JXB online). A localization assay showed that CsKF3 localizes mainly at the midzone of the phragmoplast in fruit telophase cells (Fig. 12). Previous studies have revealed several motor proteins in the phragmoplast, such as POK1, POK2, AtPAKR1, AtPAKR1L, AtPAKR2, and GhKCH2. These motor proteins were indicated to be necessary for the functions and formation of the dynamic phragmoplast, such as assisting vesicles and cell wall materials to be correctly oriented and accumulated at the midzone of the phragmoplast (Lee et al., 2001; Pan et al., 2004; Müller et al., 2006; Xu et al., 2009). In the near future, RNA interference lines for CsKF1, CsKF2, and CsKF3 should be constructed to observe the relative phenotypes in cucumber fruit. Also understanding the roles of CsKF1, CsKF2, and CsKF3 in MT regulation (or FtsZ regulation) would be also helpful to investigate their fruit-specific functionality.

**Supplementary data**

Supplementary data are available at JXB online.

Figure S1. Phylogenetic analysis of predicted cucumber kinesins and other representatives of kinesins in plants indicated that all predicted kinesins distribute in 14 families and one plant-specific ungrouped family.
Figure S2. Expression of 47 cucumber kinesin genes during vegetative (root, stem, tendril, true leaf, cotyledon, and hypocotyl) and reproductive development (dry seed, gminated seed, male flower, female flower, ovary, 0 DAA fruit, 2 DAA fruit, and 10 DAA fruit) by using RT-PCR.

Figure S3. Expression profiles of kinesin family genes during early fruit development in cucumber. Quantitative RT-PCR results of the expression of 47 kinesin genes during early 9930 fruit development.

Figure S4. Physiological changes in 1972 B-2, National Pickle, and Zhong Nong 27 during early fruit development.

Figure S5. Protein expression patterns of CsKF2 during cucumber early fruit development.

Table S1. Sequence-specific primers used for construction.

Table S2. Specific primers used for RT-PCR and quantitative RT-PCR.

Table S3. List of predicted kinesin genes in cucumber.

Table S4. Twelve cucumber kinesin genes showed peak expression in the rapid cell division phase (before 3 DAA) or cell expansion phase (after 5 DAA).

Table S5. A predicted chloroplast transit peptide (cTP) in the N-terminus of CsKF2.

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