

FW2.2 and cell cycle control in developing tomato fruit: a possible example of gene co-option in the evolution of a novel organ

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Abstract *fw2.2* is one of the few QTLs thus far isolated from plants and the first one known to control fruit size. While it has been established that FW2.2 is a regulator (either directly or indirectly) of cell division, FW2.2 does not share sequence homology to any protein of known function (Frery et al. *Science* 289:85–88, 2000; Cong et al. *Proc Natl Acad Sci USA* 99:13606–13611, 2002; Liu et al. *Plant Physiol* 132:292–299, 2003). Thus, the mechanism by which FW2.2 mediates cell division in developing fruit is currently unknown. In an effort to remedy this situation, a combination of yeast two-hybrid screens, in vitro binding assays and cell bombardment studies were performed. The results provide strong evidence that FW2.2 physically interacts at or near the plasma membrane with the regulatory (beta) subunit of a CKII kinase. CKII kinases are well-studied in both yeast and animals where they form part of cell cycle related signaling pathway. Thus while FW2.2 is a plant-specific protein and regulates cell division in a specialized plant organ (fruit), it appears to participate in a cell-cycle control signal transduction pathway that predates the divergence of single- and multi-cellular organisms. These results thus provide a glimpse into how ancient and conserved regulatory processes can be co-opted in the evolution of novel organs such as fruit.

Keywords Tomato fruit · *fw2.2* · Protein interactions · CKII β · Gene co-option

Introduction

fw2.2 was one of the first quantitative trait loci (QTLs) cloned in either plants or animals and holds biological interest for several reasons (Frery et al. 2000). First, it was through selection of mutation(s) at *fw2.2* that early humans began domesticating large-fruited tomatoes from their small-berried wild ancestors (Frery et al. 2000; Nesbitt and Tanksley 2002). Second, cumulative studies now indicate that *fw2.2* acts as a negative regulator of cell division during the very early stages of fruit development following pollination, and it was changes in the regulation of *fw2.2* expression, rather than in the *fw2.2* coding sequence that enabled tomatoes to enlarge (Frery et al. 2000; Cong et al. 2002; Liu et al. 2003). Thus, *fw2.2* is one of a number of growing cases, such as *achaete-scute*, *scabrous* and *Delta* QTL genes in fruit flies (Mackay 1996; Long et al. 1998), *teosinte-branched (tb1)* in maize (Wang et al. 1999) and *Hox* genes in animals (Carroll 2000) where morphological evolution is attributable to evolution in gene regulation, rather than evolution in the protein function—a prediction made by a small, but vocal group of evolutionary biologists more than 30 years (Ohno 1970; King and Wilson 1975).

While a clear picture is emerging of the genetic and developmental events underlying the *fw2.2* QTL and the evolution of large fruit, several key questions still remained unanswered, including: (1) From what ancestral protein did FW2.2 evolve? (2) Through what signal transduction pathway does it control cell division in developing fruit? (3) How does FW2.2 co-ordinately regulate cell division throughout the entire developing fruit such that the resultant fruit is uniform in shape? These questions might be more readily addressed if

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FW2.2 had detectable homology with proteins known to be involved in cell cycle control in other organisms. However, this is not the case. The only detectable homologs to FW2.2 are found in other plant species and none of these have known function (Frary et al. 2000). In an effort to begin unraveling the above questions, we embarked on a study to determine the cellular localization of FW2.2 and to identify its interactive partners—hoping that FW2.2 may interact with one or more proteins whose function has been established in other organisms. The results suggest that FW2.2 may have come to control cell division in developing fruit through gene co-option and recruitment of a cell cycle control pathway that predates the divergence of animals and plants.

Materials and methods

RNA extraction and RT-PCR

RNA was extracted from root tip, shoot meristem, cotyledons, leaves, small flower buds (about 2 mm long), large flower buds (about 6–8 mm long), anthesis flowers, 0, 6, 12, 18, 24, 30, 36, 42 days post anthesis (DPA) fruits (*S. lycopersicum* cv. TA1143). RT-PCR analyses were performed using gene-specific primers in 5' end and 3'UTR of *LeCKIIβ1*. The 5' primer was 5' ATG TAC AAA GAA AGA AGA GTT GGC GGT GGA 3'; the 3' primer was 5' CGC AGT ATT TGA GAG ATC CTC ACA GGG TAA 3'. 18S was amplified as an internal control. The primer sequences for 18S were 18SF: 5' CTT CGG GAT CGG AGT AAT GA 3', and 18SR: 5' TTA GCA GGC TGA GGT CTC GT 3'.

Yeast strains, expression plasmids and construction of a cDNA library from tomato fruit

Saccharomyces cerevisiae Y187 and AH109 strains, expression plasmids of pGBKT7 (containing GAL4-DNA binding domain), pGADT7-Rec (containing GAL4-activation domain) and pGADT7 (containing GAL4-activation domain) were obtained from BD Biosciences Clontech (Palo Alto, California, USA). An *fw2.2* cDNA, as well as its truncated versions were ligated into pGBKT7, respectively (Fig. 1b). Bait constructs were designated as pGBKT7-FW2.2, pGBKT7-E1-63, pGBKT7-E64-163, and pGBKT7-E90-163, respectively (Fig. 1b). All bait constructs were transformed into both Y187 and AH109 yeast cells. A series of 3-AT concentrations were tested to

optimize the amount needed to preclude spurious, leaky cell growth. 2.5 mM of 3-AT was thus determined to be optimum concentration. Further, none of the baits vectors were able to activate the reporter gene with the addition of 3-AT—eliminating the possibility of false positives due to leakiness. Expression of bait protein was confirmed by Western blots assays.

RNA was isolated from developing tomato fruit at following stages: 0, 2, 4, 6, 8, 10 and 12 days post anthesis (DPA) (*S. lycopersicum* cv. TA1143 & TA1144). Equal amount of RNA from each stage were then pooled and used for mRNA isolation. cDNAs were synthesized following the manufacturer's protocol (BD Biosciences Clontech, Palo Alto, CA). cDNAs fused to GAL4-DNA activation domain (pGADT7-Rec) were then transformed into yeast AH109 cells to generate a yeast cDNA library. The transformation efficiency, as measured by the number of transformants per 3 μg pGADT7-Rec, was approximately 7.5×10^6 . Transformants from 100 plates (150 mm) were harvested and pooled to total 350 ml (1.60×10^9 cells/ml). Insert size for the library averaged 1.05 kb.

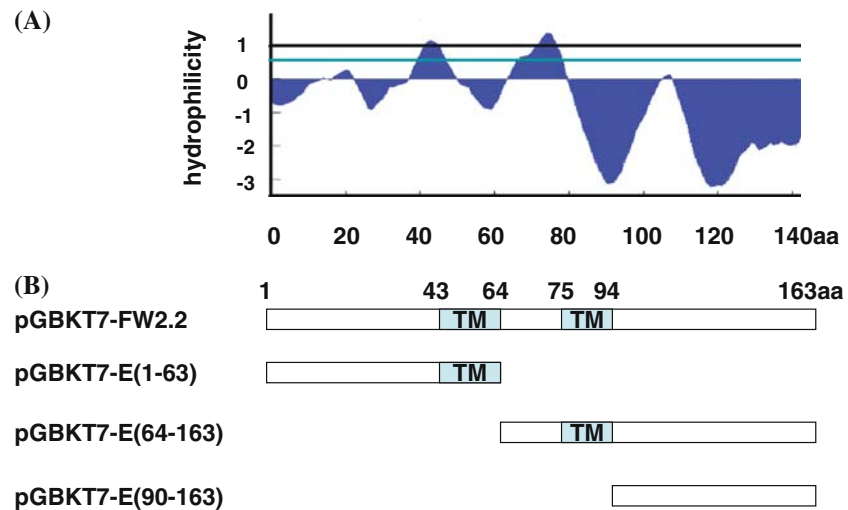
Yeast two-hybrid screen

For the yeast two-hybrid screen, the baits pGBKT7-FW2.2 and pGBKT7-E90-163 were used to screen the pGADT7-cDNA library, respectively. Plasmids of the putative positive clones were isolated, re-transformed into DH5α cells and the inserts sequenced.

Recombinant protein expression and purification

cDNAs of candidate clones (YBP45, TYBP43, TYBP55, TYBP9, TYBP34, and TYBP35), identified from the yeast two-hybrid system, were PCR amplified with specific primers containing *Bam*HI and *Eco*RI cutting sites. PCR products were then cloned into the pMAL-C2X plasmid (New England Biolabs, USA) and transformed into *E. coli* (DH5α). Cultures of the recombinant MBP-clones as well as MBP alone (as a negative control), grown overnight at 37°C in 3 ml Luria-Bertani broth containing 100 μg/ml ampicillin and 0.2% Glucose, were diluted with fresh medium (50 ml) and allowed to grow until an OD600 reading of 0.5. Expression of MBP and recombinant MBP was induced with 0.3 mM isopropyl thio-β-D-galactoside (IPTG, invitrogen, USA) for 3 h at 37°C. Bacterial cells were harvested and resuspended in 5 ml lysis buffer containing 40 mM Tris-Cl (pH7.4), 100 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol and a complete

Fig. 1 FW2.2 structure and constructs. **(a)** Hydrophilicity plot. **(b)** Schematic diagrams of different *fw2.2* cDNA constructs (fused to GAL4 DNA binding domain) used in two-hybrid screens



protease inhibitor tablet (Roche), and sonicated on ice at 6 μ A six times for 15 s with 45 s rest intervals. Samples were centrifuged at 12,000g (JA20 rotor) for 30 min at 4°C. About 1.5 ml of each supernatant was transferred to new tube, and 200 μ l of 50% amylose resins (New England Biolabs), which were washed 3–4 times using column buffer (20 mM Tris-Cl (pH7.4), 200 mM NaCl, 1 mM EDTA, 10 mM β -mercaptoethanol) just prior this step, was added. Samples were incubated at 4°C for 45 min and then set on ice for another 15 min, spun (500–600g, bench microcentrifuge) and washed with the column buffer four times. MBP and MBP fusion proteins were subjected to SDS-PAGE staining with Coomassie blue to check the quantity and quality of the purified proteins. Amylose resin conjugated proteins were aliquoted and stored at 4°C.

In vitro protein transcription and translation

Plasmid pGBKT7-FW2.2 (full length) was used for in vitro expression of FW2.2 by TNT[®] coupled reticulocyte lysate system (Promega, Madison, WI). The myc tag in the upstream of *fw2.2* insert allowed protein expression to be detected using myc antibody (9E10, Covance, Richmond, CA).

In vitro protein binding assays

Ten to 20 μ l of each MBP recombinant protein, or MBP alone (negative control) and 10 μ l of in vitro expressed myc-FW2.2 were added to 120–130 μ l of IPAB buffer (total 150 μ l, 20 mM HEPES at pH 7.4, 150 mM KCl, 0.1% Gelatin, 0.1% Triton \times 100, 0.1% NP-40, 5 mM MgCl₂, 2 mM DTT). Mixtures were rotated at 4°C for 3 h and then centrifuged 1 min at 550g.

Pelleted protein complexes were washed 4 times with 1 ml washing buffer (10 mM HEPES at pH 7.4, 150 mM NaCl, 0.25% NP-40). Bound proteins from pellets were eluted by boiling for 5 min in 1 \times SDS loading buffer (125 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 5% β -mercaptoethanol), separated on 12% SDS-PAGE gels, and transferred onto PolyScreen[®] PVDF membrane (NEN[®] Life Science Products, Boston, MA). Proteins were detected using chemiluminescence (PerkinElmer[™] Life Sciences, Inc. Boston, MA). The primary antibody was c-Myc monoclonal antibody (Covance, Richmond, CA); the secondary antibody was anti-mouse Ig, Horseradish Peroxidase linked whole antibody (NA 931V, from sheep, Amersham Biosciences UK limited, England).

Isolation of full-length genomic and cDNA clones of a tomato CKII β subunit 1

Genomic sequence corresponding to the tomato *CKII β* subunit 1 cDNA was amplified using Universal GenomeWalker kit (BD Biosciences Clontech, Palo Alto, CA). Aliquots of genomic DNA (*S. lycopersicum* cv. TA209) were extracted and digested with *Dra*I, *Eco*RV, *Stu*I, *Hpa*I, and *Scal*I, respectively. After purification, the digested DNAs were ligated to GenomeWalker adapters to construct libraries. Individual libraries were PCR amplified by using primers *LeCKII β 1* GSP1 (5' CGC AGT ATT TGA GAG ATC CTC ACA GGG TAA 3') and AP1 (5' GTA ATA CGA CTC ACT ATA GGG C 3'). PCR products were then used as templates for secondary nested PCR by using primers *LeCKII β 1* GSP2 (5' GAA ACC AAA AAC TCT GGG AAC GTA GCT CTG 3') and AP2

(5' ACT ATA GGG CAC GCG TGG T 3'). The PCR conditions were 5 cycles (94°C 25 s, 68°C 3 min), 20 cycles (94°C 25 s, 63°C 3 min), and 63°C 7 min. A 3 Kb genomic DNA sequence of *LeCKIIβ1* was thus isolated and sequenced.

5' RACE was employed to obtain a full length cDNA of *LeCKIIβ1* and identify the transcriptional start site. 500 ng of mRNA from 0 to 12 DPA fruit was mixed with *LeCKIIβ1* GSP₁ primer (5' CGC AGT ATT TGA GAG ATC CTC ACA GGG TAA 3') for the first-strand cDNA synthesis, SMART IIITM Oligo (BD Sciences Clontech, Palo Alto, CA) was used to add adapter to 5' end of the cDNA according to manufacturer's instructions. 5' RACE PCR was performed using 5' PCR primer (BD Sciences Clontech, Palo Alto, CA) and *LeCKIIβ1* GSP₂ primer (5' GAA ACC AAA AACTCT GGG AAC GTA GCT CTG 3'). Nested PCR was performed using the 5' PCR primer and *LeCKIIβ1* GSP₃ (5' GCT CTG AGA AAT CTT CTG TGG CTT GAG ATG 3'). The primary PCR condition was 94°C 30 s, 63°C 1 min 30 s, 72°C 2 min 30 s for 30 cycles. The nested PCR was 94°C 30 s, 63°C 45 s, 72°C 1 min 30 s for 30 cycles. After amplification, the PCR products were cloned into pCR vector (Invitrogen, USA) for subsequent characterization.

Yeast two-hybrid retesting

A full length clone of *LeCKIIβ1*, obtained from RT-PCR, was fused with GAL4 activation domain (AD) to generate pGADT7-*LeCKIIβ1*. pGADT7-SV40 and pGBKT7-Lam were generated as controls. Bait constructs pGBKT7-FW2.2, pGBKT7-E1-63, pGBKT7-E64-163, pGBKT7-E90-163, pGBKT7-Lam and pGBKT7 were transformed into yeast strain Y187, respectively; while prey constructs pGADT7-*LeCKIIβ1*, pGADT7-SV40, and pGADT7 were transformed into yeast strain AH109. Both yeast strains containing bait construct or prey construct were mated and spread onto SD/-Leu/-Trp medium, then streaked onto selective plates containing SD/-Ade/-His/-Leu/-Trp/X-α-gal/3-AT medium.

Mapping and southern blot hybridization

LeCKIIβ1 was probed onto genomic southern blots containing restriction-digested DNA from *S. lycopersicum* cv. LA925 and *S. pennellii* LA716 in order to determine copy number. Further, *LeCKIIβ1* was probed onto southern blots containing *EcoRV* digested DNA from F2 progeny derived from a cross of the above two genetic stocks. As this same population is the basis of the

high density tomato genetic map, it was possible to localize the precise map position of the *LeCKIIβ1* gene in the tomato genome (Frery et al. 2005) (<http://www.sgn.cornell.edu>). *LeCKIIβ2* (SGN-U219524) and *LeCKIIβ3* (SGN-U234196) were also mapped on the same F2 population by CAPs assay. Southern hybridization and mapping protocols can be found in Frery et al. (2005).

Subcellular localization experiments and confocal microscopy

Full-length *fw2.2* and *LeCKIIβ1* clones were fused with CFP and YFP fluorescent reporter genes (provided by Dr. Chua, Rockefeller University, New York), respectively. The *fw2.2* cDNA was also fused with GFP as a control. These plasmids were then bombarded into tomato young leaves and onion epidermal cells using the Biolistic PDS-2000/He Particle Delivery System (Bio-Rad, USA). The leaf and onion tissues were then held in the dark for 24 h at room temperature. Confocal images for CFP and YFP were obtained sequentially with Leica Spectral Confocal Microscope (Leica confocal TSC SP2, DMRE-7 Microscope). Images were processed using Leica lite and Adobe Photoshop software.

Phylogenetic analysis of tomato *CKIIβ* genes

CKIIβ gene sequences were retrieved by homology searches using the BLAST program from the Genbank (<http://www.ncbi.nih.gov>), SGN (<http://www.sgn.cornell.edu>) and TIGR (<http://www.tigr.org>) databases. Where possible, full length cDNAs were assembled from multiple EST reads. Otherwise, original cDNA clones were retrieved and subjected to complete sequencing. Deduced *CKIIβ* protein sequences were aligned using T-Coffee with default parameters, and regions that could not be unambiguously aligned were eliminated. Phylogenetic analysis was then performed and a neighbor-joining unrooted tree was constructed using the MEGA3 program package based on alignments of nucleotide sequences, which were guided from amino acid alignment.

Phenotypic evaluation of Arabidopsis T-DNA knockout lines

From the ABRC (Ohio) stock center, T-DNA knockout stocks were obtained for AT5G47080 (*CKIIβ1*, SALK_065565, SALK_134035, SALK_144967, SALK_030209, SALK_051327, and SALK_140691), and

AT3G60250 (*CKII β 3*, SALK_057852, SALK_085870, and SALK_108997). Homozygous mutants and wild type stocks were obtained from segregating progeny of each stock used for phenotypic evaluations.

Results

Identification of proteins putatively interacting with FW2.2 via yeast two-hybrid screens

Yeast two-hybrid screens were performed in the hope of illuminating the pathway by which FW2.2 modulates cell division in developing fruit. A cDNA library, synthesized from mRNA isolated from 0 to 12 DPA (days post anthesis) tomato fruit was screened with the full-length FW2.2 “bait” vector construct pGBKT7-FW2.2 (Fig. 1b). The 0–12 DPA stage of fruit development was chosen as it corresponds to the peak interval of *fw2.2* expression during fruit development (Cong et al. 2002). From the two-hybrid screen, approximately 400 clones, encoding putatively-interacting polypeptides, were thus identified (out of 7×10^6 total clones). In an effort to reduce potential false positives, a second screen was performed using a truncated version of FW2.2 (pGBKT7-E90-163) lacking the transmembrane domains which were predicted by TopPred (von Heijne 1992) (Fig. 1a). Previous research has shown that such membrane-anchored proteins give less reliable results in yeast two-hybrid screens, thus the logic was that a second screen with FW2.2 bait (minus transmembrane domains) might yield a subset of higher-confidence interacting proteins (Fields and Song 1989; Miller et al. 2005). From this second screen, 77 positive clones were identified, sequenced and annotated. All positive clones have been subjected to test autonomous reporter gene activation and performed mating to confirm interactions with their corresponding baits (data not shown). Sequence analysis of the putative positive clones, furthermore, eliminated some clones which were found not in frame with the GAL4 activation domain. Thus, six clones were chosen for further study because either their functions have been implicated in cell division, organ growth in other organisms or they were isolated multiple times. These were: YBP45, TYBP43, TYBP55, TYBP9, TYBP34, and TYBP35.

Testing candidate proteins for in vitro binding of FW2.2

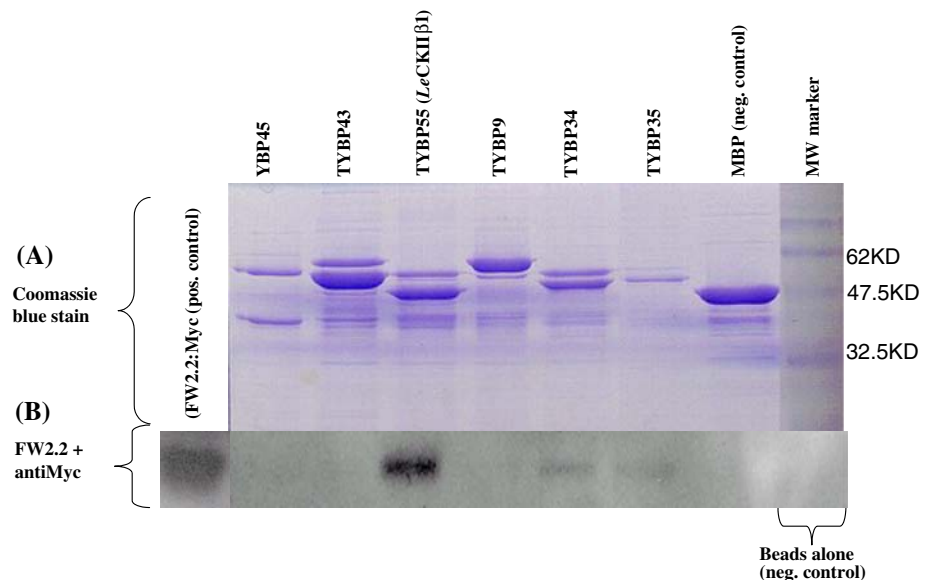
The six genes (YBP45, TYBP43, TYBP55, TYBP9, TYBP34, and TYBP35) identified in the yeast two-hybrid screens as encoding proteins with a putative

physical interaction with FW2.2 were subjected to further analysis via an in vitro binding assay. In vitro transcribed and translated myc-tagged FW2.2 protein was incubated with an equimolar amount of each of the corresponding MBP recombinant proteins which had been verified on acrylamide gels by Coomassie blue staining (Fig. 2a). Bound proteins were eluted, gel separated, blotted and subjected to immunoblot analysis (Fig. 2b). The results indicate that myc-tagged FW2.2 binds strongly, and thus co-precipitates, with TYBP55 in vitro (Fig. 2b). In contrast, in vitro binding of FW2.2 with the other MBP fused candidate proteins, MBP or beads alone could not be confirmed, with the possible exception of a very weak binding with TYBP34 and TYBP35 (Fig. 2b). Based on these results, TYBP55 was selected for further study.

Isolation and characterization of a full length *LeCKII β 1* clone

TYBP55 encodes a protein with high homology to the regulatory subunit of CKII kinase in various organisms (including *A. thaliana*, *Z. Mays*, *S. pombe*, *D. Melanogaster*, *C.elegans* and *H. sapiens*) and, for reasons to be discussed later, was chosen for our detailed study (Fig. 3). TYBP55 isolated in the two-hybrid screen above was discovered to cover only the last 90 (C-terminus) amino acids from what was predicted (based on homologous proteins from other species) to be a protein of approximately 250 amino acids. Therefore genomic and full length cDNA sequences of this gene (hereafter denoted as *LeCKII β 1*) were amplified using genome walking and 5'RACE. Sequence analysis revealed that *LeCKII β 1* encodes a putative protein of 286 amino acids, with all major functional domains associated with proteins shown to have *CKII β* function in other organisms, including *H. sapiens*, *D. Melanogaster*, *S. pombe*, *C. elegans*, *A. thaliana*, *N. tabacum*, *Z. Mays*. These include a destruction box conferring mitosis specific degradation to cyclin B (Zhang et al. 2002); a cysteine-/zinc-finger motif CPX₃C-X₂₂-CPX₁C mediating dimerization of CKII subunits (Chantalat et al. 1999); an acidic region binding to polyamines and regulating the interaction with the CKII α ; and a region involved in the interaction with the CKII α (GAYFGTXFP) (Boldyreff et al. 1996; Chantalat et al. 1999) (Fig. 3). Alignments of CKII β sequences from multiple species revealed that plant CKII β possess a less conserved N-terminal extension (about 90 amino acids), whereas CKII β from *H. sapiens*, *D. melanogaster*, *S. pombe*, and *C. elegans* carry a short C-terminal extension (about 20–30 amino acids). The bulk sequences in the middle are highly

Fig. 2 Results from in vitro interaction tests between FW2.2, candidate clones from two-hybrid screens. (a) SDS-PAGE stained with Coomassie blue verifying expression of each candidate protein fused with MBP (about 90 aas in C-terminus of TYBP55 was fused with MBP). (b) Immunoblot analyses of in vitro interactions between FW2.2 and candidate proteins. myc-tagged FW2.2 was used as an input control



conserved among all CKII β species, although the CKII β sequences from plant species share even higher similarities (Fig. 3).

LeCKII β 1 expression was measured in root tip, shoot meristem, cotyledons, leaves, small flower buds (about 2 mm long), large flower buds (about 6–8 mm long), anthesis flowers, and 0, 6, 12, 18, 24, 30, 36, and 42 DPA fruits (data not shown). The results indicated that *LeCKII β 1* is expressed in all of the above mentioned tissues in relatively similar amount and may thus be active in all tissues.

Testing the in vivo interaction of the full length *LeCKII β 1* polypeptide and FW2.2 in yeast cells

As mentioned earlier, none of the cDNA clones of *LeCKII β 1* isolated in the original yeast two-hybrid screen proved to be full length. Thus, to further confirm the potential physical interaction between *LeCKII β 1* and FW2.2, the full length *LeCKII β 1* clone was used as a “prey” in a yeast two-hybrid assay in which FW2.2 and its three truncated versions were used as “bait” (Fig. 1b). As shown in Fig. 4, all 30 strains containing GAL4-DB-FW2.2 or its truncated variants were able to interact with pGADT7-*LeCKII β 1* and conferred yeast growth on the selective medium and activated the reporter gene expression (Fig. 4E, H, K, N). As negative controls, no interaction was observed between “bait” GAL4-DB-FW2.2, or its truncated variants and the GAL4 activation domain alone (pGADT7) or in fusion with the SV40 large T-antigen (pGADT7-SV40) (Fig. 4D, G, F, I, J, L, M, O). Similarly, no interaction was detected between “prey” pGADT7-*LeCKII β 1* and

the GAL4 DNA binding domain alone (pGBKT7) or in fusion with an unrelated protein Lamin C (pGADT7-Lam) (Fig. 4B, Q). The results thus support the interaction of FW2.2 and *LeCKII β 1* in yeast cells. The strains containing GAL4-DB-p53 and GAL4-AD-SV40 (known to physically interact) were used as a positive control (data not shown).

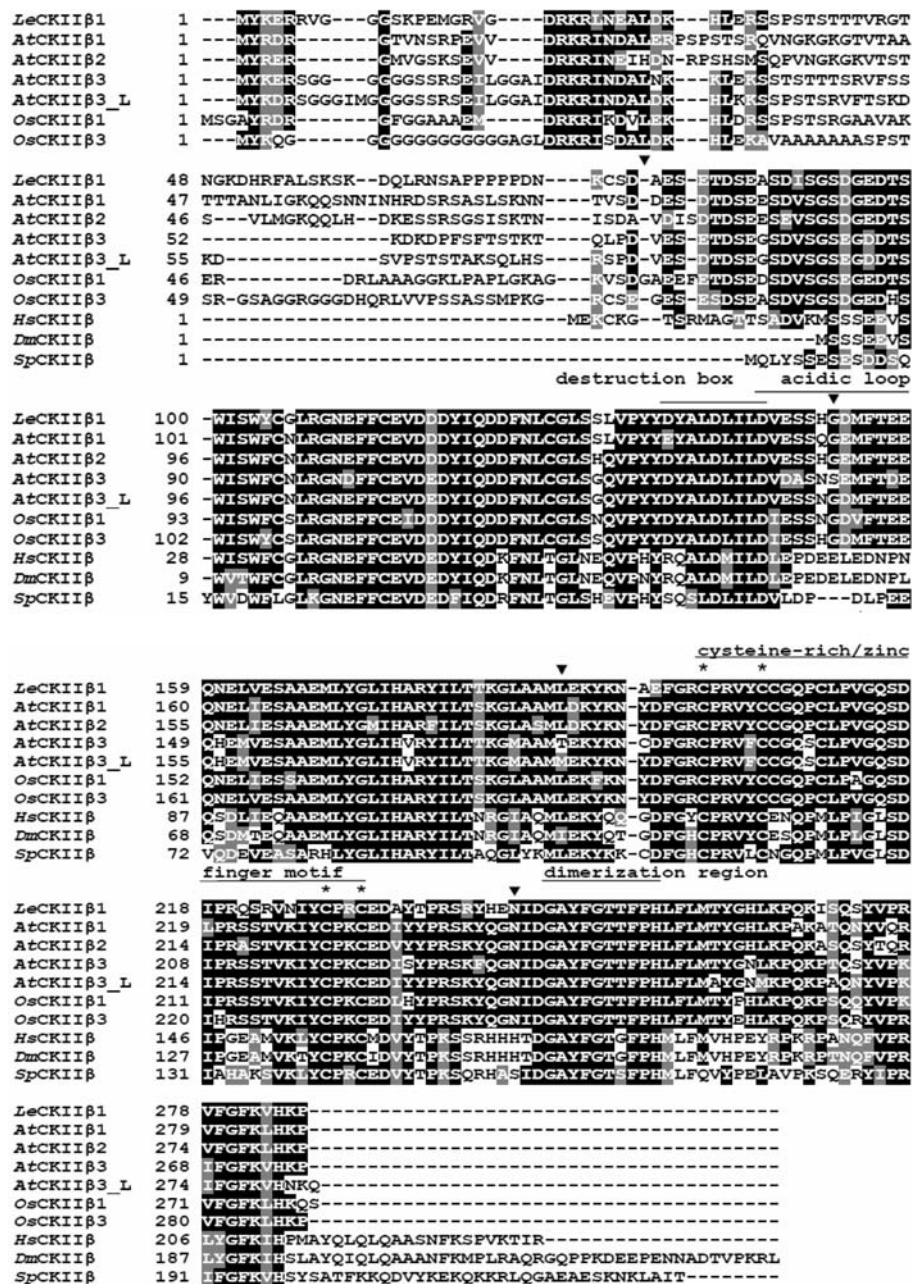
Subcellular localization of *LeCKII β 1* and FW2.2

To provide additional evidence that FW2.2 and *LeCKII β 1* may physically interact, the subcellular localizations of FW2.2 and *LeCKII β 1* were investigated using particle bombardment. *LeCKII β 1* tagged with yellow fluorescence protein (YFP) and FW2.2 tagged with cyan fluorescence protein (CFP) were transiently expressed in onion epidermal cells. In addition, FW2.2 tagged with green fluorescence protein (GFP) was transiently expressed in tomato leaf epidermal cells. In both onion and tomato leaf epidermal cells, FW2.2 localized proximal to or associated with the plasma membrane, with a plasma membrane association being more clearly defined with the tomato cells (Fig. 5a, b). *LeCKII β 1* localized to both the cytoplasm and the nucleus—suggesting that any physical interaction between FW2.2 and *LeCKII β 1* (as supported by previous evidence) is likely to occur in the cytoplasm near the plasma membrane (Fig. 5b).

Copy number and genetic mapping of *LeCKII β 1*

Southern hybridization (using *LeCKII β 1* as a probe) on restriction-digested tomato genomic DNA shows

Fig. 3 Alignments of the amino acid sequence *LeCKIIβ1* with other *CKIIβ* sequences from different species. The amino acid sequence of *LeCKIIβ1* was aligned with *CKIIβ* from *Arabidopsis thaliana* (*AtCKIIβ1*, AT5G47080.1; *AtCKIIβ2*, AT4G17640.1; *AtCKIIβ3*, AT3G60250.1; and *AtCKIIβ3_Like*, AT2G44680.1), *Oryza sativa* (*OsCKIIβ1*, NM_197780.1; and *OsCKIIβ3*, NM_187345.1), *Homo sapiens* (*HsCKIIβ*, CAI18393.2), *Drosophila melanogaster* (*DmCKIIβ*, AAS65321.1), and *Schizosaccharomyces pombe* (*SpCKIIβ*, CAA52330.1). The positions of the introns in *LeCKIIβ1* are indicated as black triangles. Acidic loop, cysteine-rich/zinc finger motif, and dimerization region are marked with lines on top of alignment. Residues of cysteine are indicated by stars



hybridization of multiple bands at a low stringency condition, raising the possibility that *LeCKIIβ1* is a member of a multigenic family (Fig. 6a). A screen of the tomato EST database (<http://www.sgn.cornell.edu>) confirmed this prediction, revealing two paralogs of *CKIIβ* in the tomato genome (SGN-U219524 designated as *LeCKIIβ2* and SGN-U234196 as *LeCKIIβ3*, Fig. 7a). Genetic mapping indicates that all three genes (*LeCKIIβ1*, *LeCKIIβ2*, and *LeCKIIβ3*) are located on different chromosomes (6, 10, and 1, respectively) and hence they are unlikely arose from tandem duplication events. Their exact map positions can be viewed at the

SGN website (<http://www.sgn.cornell.edu/>). Interestingly, *LeCKIIβ1* has been mapped to a position of a previously located fruit weight QTL on chromosome 6 (Grandillo and Tanksley 1999). It will be interesting to determine whether genetic variation at *LeCKIIβ1*, as with *fw2.2*, may modulate fruit size among tomato varieties/species (Frary et al. 2000; Liu et al. 2003).

Phylogenetic analysis of *LeCKIIβ* gene family

To determine the evolutionary relationship of the three tomato *LeCKIIβ* genes and their homologous

Fig. 4 Results of *in vivo* interactions of *LeCKIIβ1* and FW2.2 as determined by yeast two-hybrid assays. Top row, AH109 cell containing: pGADT7-*LeCKIIβ1* (full length *LeCKIIβ1* cDNA), pGADT7 and pGADT7-SV40 (empty vector and SV40 controls, respectively). Left column, Y187 cells containing: pGBKT7 (empty vector), pGBKT7-FW2.2 and its truncated versions (Fig. 1b), pGBKT7-lamin (negative control). Overnight cultures (from all row:column combinations) were spread on SD medium lacking Leu and Trp (SD/-Leu/-Trp). After 3d incubation at 30°C, colonies were patched onto SD medium plates as described in “Material and Methods”. Blue color indicates positive protein:protein interactions. The positive controls (pGBKT7-p53 and pGADT7-SV40) not shown

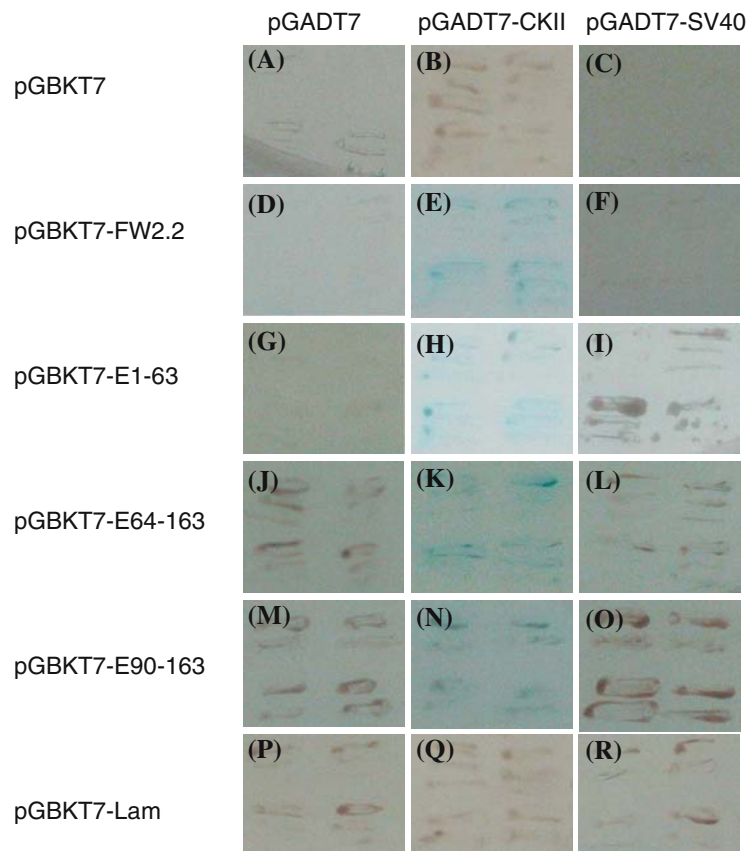
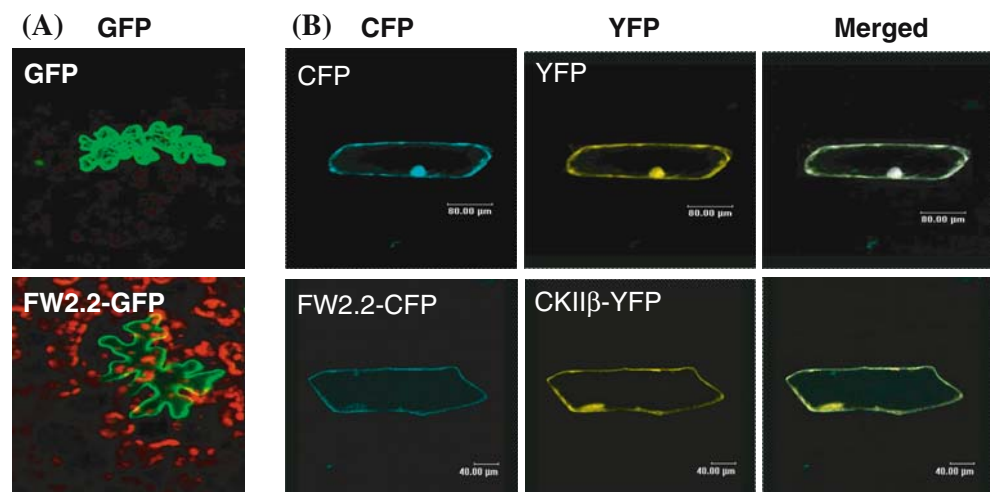


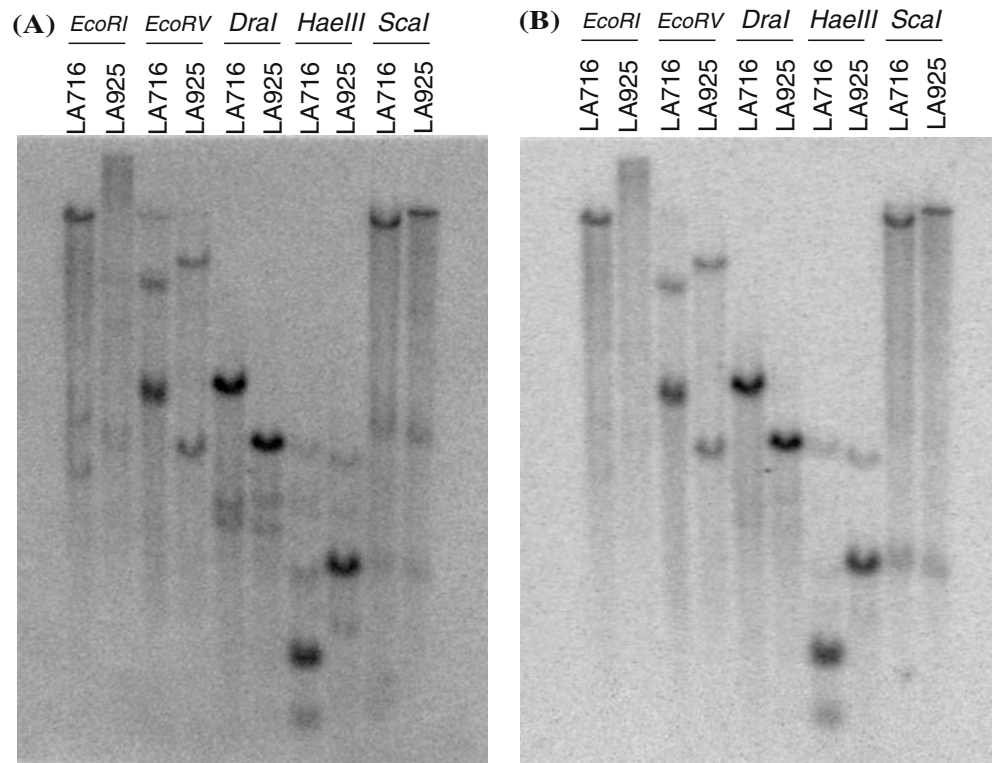
Fig. 5 Subcellular localization of FW2.2 and *LeCKIIβ1*. (a) Subcellular localization of FW2.2-GFP in tomato young leaf cells with GFP alone as control. (b) Co-localization of FW2.2-CFP and *LeCKIIβ1*-YFP in onion epidermal cells with CFP alone and YFP alone as controls



counterparts in other organisms, the tomato *CKIIβ* genes were aligned with homologs from *Arabidopsis* and rice and a phylogenetic tree was generated based on an overlap region of approximately 235 aas (Fig. 7b). *LeCKIIβ2* (SGN-U219524) and *LeCKIIβ3* (SGN-U234196) are more closely related to each other than they are to either *LeCKIIβ1* or the *Arabidopsis* and rice homologs. *LeCKIIβ1* stands out as being well differentiated from either of the other two tomato

homologs as well as the *Arabidopsis* and rice homologs—perhaps reflecting a past rapid divergence and specialization of this gene—possibly to perform a specialized function in controlling fruit development. However, RT-PCR analyses indicate that *LeCKIIβ1* gene is expressed in all tested tissues (root tip, shoot meristem, cotyledons, leaves, small flower buds, big flower buds, anthesis flowers, 0, 6, 12, 18, 24, 30, 36, 42 DPA fruits) of the tomato plant—suggesting that the

Fig. 6 *LeCKIIβ1* southern hybridization. Genomic DNA from tomato stocks LA716 and LA925 was digested with the restriction enzymes *EcoRI*, *EcoRV*, *DraI*, *HaeIII*, and *ScaI*. The filter was hybridized by using *LeCKIIβ1* cDNA as probe. (a) Hybridization in low-stringency conditions. (b) Hybridization in high-stringency conditions



CKIIβ proteins may participate in signaling pathways in many tissues in organs. In contrast, *fw2.2* is expressed only in early stages of fruit development (Cong et al. 2002). Thus, it is perhaps through *LeCKIIβ1* that *FW2.2* specialized to control cell division in developing fruit.

Shorter siliques associated with knockout mutation in *AtCKIIβ* homologs in *Arabidopsis*

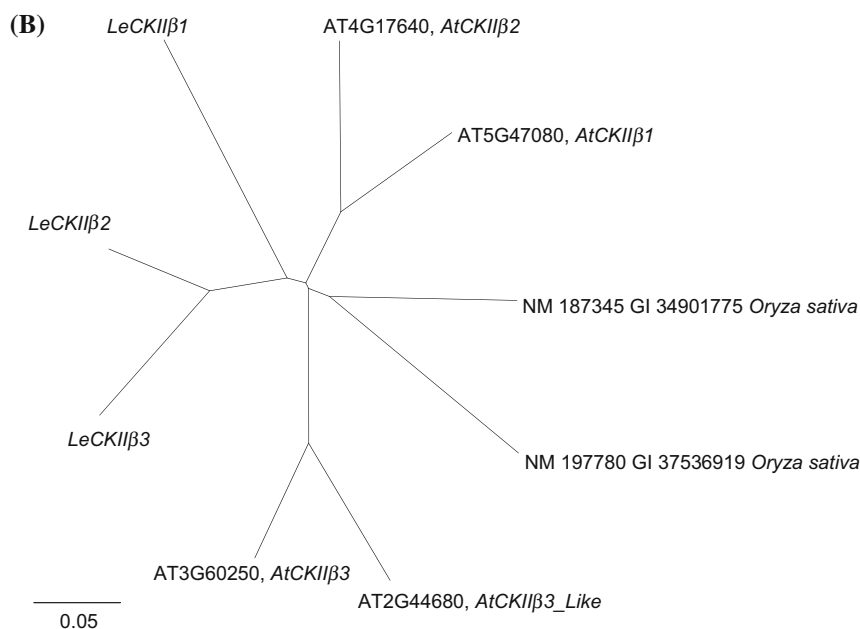
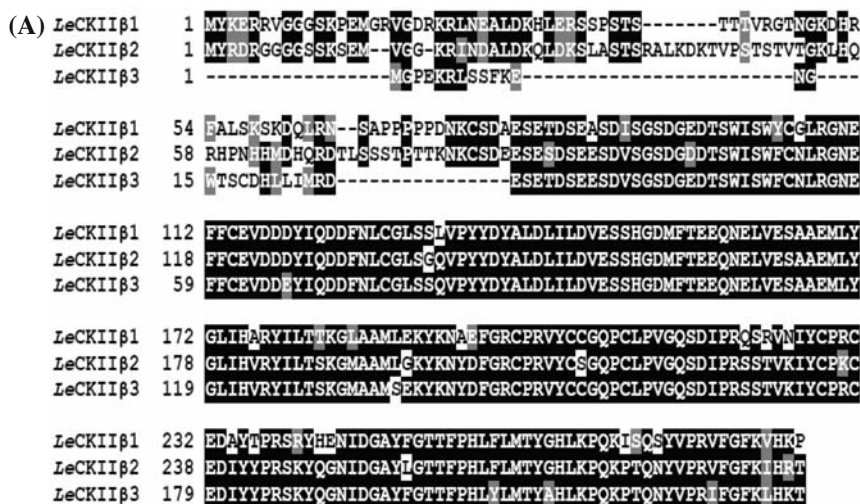
As indicated earlier, the tomato *LeCKIIβ1* gene maps to the same position as a previously described QTL for fruit size, supporting the possibility that genetic variation at *LeCKIIβ1* may contribute to fruit size variation in tomato. Unfortunately, there is no resource of gene knock out stocks for tomato which would provide a natural follow-up to this possibility. However, T-DNA knockout mutations are available for 2 of the 4 *LeCKIIβ* homologs in *Arabidopsis*—AT5G47080 (*CKIIβ1*) and AT3G60250 (*CKIIβ3*) (Fig. 7b). *Arabidopsis* does not produce the same kind of fruit as tomato; however, the anatomical counterpart to tomato fruit in *Arabidopsis* is silique. Homozygous T-DNA knockout lines were thus isolated for these two *Arabidopsis* homologs. All homozygous knockout stocks produced significantly shorter siliques ($P < 0.001$) than did the wild type Columbia (Fig. 8). Despite being shorter, the

siliques produced normal seeds and no other obvious changes in growth and development were observed in these mutants (data not shown). A similar inhibition in growth has also been reported with antisense *CKIIβ* in human fibroblasts cells and in *S. pombe* with the *CKIIβ* gene deletion (Pepperkok et al. 1991; Roussou and Draetta 1994). While these results do not prove the function of *LeCKIIβ1* in tomato, they do demonstrate that modulation of *LeCKIIβ1* homologs do affect the anatomical counterpart of fruit in *Arabidopsis* (siliques)—a finding consistent with *LeCKIIβ1* being involved in a signal transduction pathway, along with *FW2.2*, in the control of cell division (and hence fruit size) in tomato.

Discussion

fw2.2 is a major QTL controlling the size of fruit between domesticated and wild tomatoes (Grandillo and Tanksley 1999; Frary et al. 2000). Moreover, mutation(s) in the *fw2.2* promoter, which changed the timing of gene expression, are thought to have to constitute a first key step in the transition of wild tomatoes from small berries to the large fruit we now associated with modern agriculture (Frary et al. 2000; Cong et al. 2002). Not only is *fw2.2* key in the evolu-

Fig. 7 Paralogs of *LeCKIIβ1* in tomato and phylogenetic tree. **(a)** Alignments of the deduced amino acid sequences of tomato CKIIβ1, CKIIβ2 and CKIIβ3. **(b)** Phylogenetic analysis of CKIIβ homologs from tomato (*Le*), Arabidopsis (*AT*) and rice (*O. sativa*)



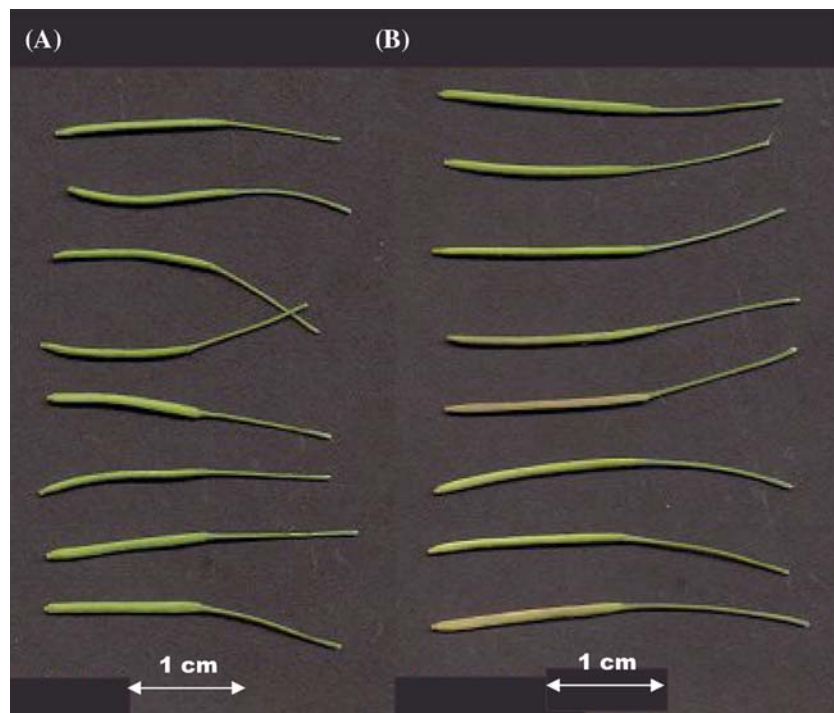
tion of fruit in tomato, mutations in an ortholog of *fw2.2* were likely causally associated with the increase of fruit size that accompanied the domestication of eggplants (Doganlar et al. 2002). Hence it seems likely that homologs of *fw2.2* may have played a key role in the domestication of a variety of fruit bearing crops (Doganlar et al. 2002). While *fw2.2* is known to control cell division in the early stages of fruit development, the molecular pathway by which it exercises this control is currently unknown (Liu et al. 2003). Deciphering the signal transduction pathway through which *fw2.2* exercises control of cell division is thus key to understand fruit development. In the larger context of developmental biology, it is also important to understand the origins of *fw2.2* and the pathway in which it is

embedded. It is only through such knowledge that we may begin to understand how new and/or modified organs (such as fruit) can be generated during evolution (True and Carroll 2002).

FW2.2 accumulates at cell membrane surface and interacts with the regulatory subunit of CKII kinase

Based on the results presented herein, *fw2.2* encodes a protein with two hydrophobic transmembrane domains—a result compatible with subcellular localization experiments showing that FW2.2 accumulates at or near the plasma membrane (Figs. 1a, 5). Of the putative FW2.2 interacting proteins identified by the yeast two-hybrid screens, follow-up studies confirmed that a

Fig. 8 Representative siliques of (A) homozygous *AtCKII β 3* T-DNA insertion line (At3G60250, SALK_108997), and (B) Columbia wild type



protein showed reproducible binding to FW2.2 in both in vivo and in vitro assays. That protein, *LeCKII β 1*, shows strong homology to the regulatory subunit of the CKII kinase, known from both animals and yeast studies to be involved in cell cycle control (Roussou and Draetta 1994; Chen and Cooper, 1997; Li et al. 1999; Tapia et al. 2004). The initial yeast two-hybrid screen resulted in the isolation of a *LeCKII β 1* cDNA clone encoding only the C-terminal most 90 amino acids of the 286 amino acids full length *LeCKII β 1* protein. Thus FW2.2 must physically interact with the C-terminal region of *LeCKII β 1*. This segment of the *LeCKII β 1* protein contains the CKII dimerization domain where CKII β has been shown to bind a variety of proteins involved in the cell cycle signal transduction pathways in animal and in yeast, including A-Raf, c-Mos, Chk1 and the catalytic subunit of the CKII kinase (Chen et al. 1997; Hagemann et al. 1997; Kusk et al. 1999; Guerra et al. 2003; Lieberman and Ruderman 2004).

Unlike FW2.2, *LeCKII β 1* localizes both to the cytosol and to the nucleus which is consistent with the localization of this protein in both animal and yeast cells (Faust and Montenarh 2000; Filhol et al. 2003). Together, these results suggest that FW2.2 physically interacts with *LeCKII β 1* at or near plasma membrane and that this interaction could be part of a signal transduction pathway through which FW2.2 controls cell division in developing fruit.

Co-option of an ancient cell-cycle signal transduction pathway to control development of fruit—organ unique to plant?

CKII is known to play an important role in cell proliferation in yeast and mammalian cells (Roussou and Draetta 1994; Glover 1998; Litchfield 2003; Daniel et al. 2004; Filhol et al. 2004; Lieberman and Ruderman 2004; Homma et al. 2005). Moreover, multiple comparisons across divergent species (*A. thaliana*, *Z. mays*, *S. pombe*, *D. melanogaster*, *C. elegans* and *H. sapiens*) have revealed that CKII β is highly conserved during evolution and shares all important domains among species during evolution (Nastainczyk et al. 1995). Moreover, previous studies have shown that plant CKII has a composition similar to its yeast and animal counterparts and it is regulated in a similar manner (Collinge and Walker 1994; Klimczak et al. 1995). There is also evidence that CKII in plants also participates in control of cell division (Espunya et al. 1999). However, it is in yeast and animals that the cell cycle signal transduction pathway involving CKII β is best characterized. In both yeast and animals, the CKII β -involved regulating signal transduction pathways share many similarities, suggesting that this CKII β involved cell cycle control pathway is ancient—predating the divergent of single cellular and multicellular eukaryotes (Munstermann et al. 1990; Bibby and Litchfield 2005).

The results presented in the current paper indicate that FW2.2, a plant specific protein that regulates cell division in developing tomato fruit, interacts with the highly conserved regulatory unit of CKII β and thus may be affecting regulation of cell division via CKII-mediated pathways in common with yeast and animals. Thus while FW2.2 regulates growth of a plant specific organ (fruit), it may do so by having co-opted an existing and ancient pathway for cell cycle control. In contemplating such a possibility, it is important to take note of the fact that FW2.2 exercises co-ordinate control of cell division throughout developing fruit. This can be inferred from previous studies which have shown that differential expression of *fw2.2* causes changes in overall fruit size but not in fruit shape (Frery et al. 2000; Liu et al. 2003). Lack of such co-ordinate control would result in fruit of various shapes, depending on the level of cell division in different parts of the fruit. This observation, combined with the fact that FW2.2 has transmembrane domains and accumulates at or near the plasma membrane surface, suggests that FW2.2 may be part of an upstream signaling system which is activated by an extra-cellular signal which co-ordinates cell division in all tissues throughout developing fruit. Such a signal may then be passed down through CKII kinase-mediated MAPK cascade which has been documented in multiple organisms or through CKII-interacted CDK inhibitors (p27KIP1, p21WAF1/CIP1) or an activator (Cdc25B) which in turn modulates cell cycle progression (Romero-Oliva and Allende 2001; Theis-Febvre et al. 2003; Tapia et al. 2004).

The unknown origins of FW2.2

While FW2.2 may affect control over cell division in developing fruit via CKII kinase mediated pathways, the FW2.2 protein itself appears to be unique to plants and it is only in tomato where any function of FW2.2-like proteins has been established. However, while there is no alignable homology detectable between FW2.2 and non-plant proteins, computational folding algorithms have classified FW2.2 as having a possible three-dimensional structure similar to RasP21 (Frery et al. 2000). Previous research has shown that proteins deriving from a common ancestral protein, can nonetheless retain conserved three-dimensional structure and function (Orengo and Taylor 1990; Holm and Sander 1996). Subsequent attempts to crystallize the FW2.2 and determine an X-ray structure have proven unsuccessful—apparently due to solubility problems stemming from the presence of hydrophobic domains (Fig. 1a, Clardy et al. unpublished data). It is interesting

to note that, in animals, CKII β phosphorylates Ras and physically interacts with and activates A-Raf (a cytoplasmic serine/threonine protein kinase) via phosphorylation in mammalian cells (Moyers et al. 1998; Hagemann and Rapp 1999). In turn, A-Raf kinase, known to be a mitogen to relay the signal from the Ras, which subsequently activates MEK, ERK in the cell cycle progression coupled MAPK signaling transduction pathway (Boldyreff and Issinger 1997). Given the predicted shared three-dimensional structure of Ras and FW2.2, combined with the fact that both appear to participate as part of a CKII kinase signal transduction pathway in cell cycle control raises the question of whether FW2.2 may have evolved from ancestral Ras-like protein a long ago, that has now taken on an upstream regulatory role in global control of cell division in developing fruit. If this hypothesis turns about to be correct, it will become one of a number of growing cases where gene co-option underlies evolution of new developmental structures, such as the fruit (True and Carroll 2002). Validation or refutation of this hypothesis also awaits further experimentation.

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