Cloning of distinct cell-proliferation-related genes from avocado and molecular characterization of their expression, as related to small and normal 'Hass' fruit phenotype development

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Abstract
Previous studies using 'Hass' avocado and its small fruit (SF) phenotype as a model have shown that SF is limited by cell number, not cell size. To explore the molecular mechanisms regulating cell division in avocado fruit, we isolated distinct avocado cell-proliferation-related genes and investigated their expression characteristics, comparing normal fruit (NF) and SF developmental patterns. Two cDNAs—*PaCYCA1*, *PaCYCB1*—encoding two mitotic cyclins were first isolated from young NF tissues. A third full-length cDNA, designated *Pafw2.2-like* and encoding a FW2.2 (fruit-weight)-like protein, was isolated from SF tissues. FW2.2 is postulated to function as a negative regulator of cell division in tomato fruit. Remarkably, Northern analysis revealed that accumulation of the mitotic cyclin transcripts gradually decreases in NF tissues during growth, whereas in SF, their levels have already decreased at earlier stages of fruit development, concomitant with earlier arrest of fruit cell division activity. In contrast, parallel semi-quantitative RT-PCR analysis showed that *Pafw2.2-like* mRNA accumulation is considerably higher in SF tissues than in the same NF tissues, essentially at all examined stages of fruit growth (Dahan et al, Planta, 2010). The possibility that *Pafw2.2-like* activity is similar to that of *fw2.2* in tomato is discussed. Finally, we have recently isolated a cDNA designated *PaICK,* encoding a cyclin-dependent kinase (CDK) inhibitor. ICK (CDK inhibitor) proteins have important functions in cell-cycle regulation. The expression profile of *PaICK* during avocado fruit growth is currently being examined in our laboratory. Questions related to the mechanism controlling cell-proliferation-related gene expression, in and its link to plant hormones and/or carbohydrate content are discussed.

Key words: Avocado, Cell division, Cyclins, *Fw2.2* (fruit-weight 2.2), Fruit growth

Introduction

The 'Hass' avocado (*Persea americana* Mill.) cultivar dominates the world avocado industry. Despite its popularity, 'Hass' avocado is known to be problematic with regard to fruit size. 'Hass' trees produce two populations of fruit—normal fruit (NF) and phenotypically small fruit (SF)—with no obvious pattern of distribution on the tree (Zilkah & Klein 1987; Cowan, Moore-Gordon, Bertling & Wolstenholme 1997). Remarkably, even under favorable conditions, a high proportion of 'Hass' SF are produced, a phenomenon which has facilitated the use of this cultivar as an attractive experimental model to study mechanisms controlling avocado fruit growth and development (Cowan et al 1997; Cowan, Cripps, Richings & Taylor 2001; Cowan, Taylor & van Staden 2005).

The avocado fruit depends on a viable seed for growth, which influences both fruit shape and final fruit size, as best reflected by size differences observed between small, seedless fruits and normal-sized seeded fruits (Blumenfeld & Gazit 1974; Tomer, Gazit & Eisenstein 1980; Lovatt 1990). Notably, small seedless fruits ('cukes') are effectively seedless due to early seed degeneration, representing a type of stenospermocarpy (Tomer et al 1980). Unlike seedless avocado fruits, the phenotypically small 'Hass' fruit contains a viable seed but is characterized by early seed-coat senescence and loss of vascular contact between the seed and the mesocarp tissue. All other physiological aspects of the fruit, such as maturation and ripening, seem to be unaffected. Previous studies have shown that small 'Hass' fruit growth is limited by cell number and not by cell size, indicating that events controlling cell division play a key role in fruit-size determination (Cowan et al 1997). Further studies characterizing the metabolic changes believed to affect avocado fruit cell division revealed that changes in hormone homeostasis and carbohydrate content, among others, are linked to 'Hass' SF appearance. For example, it was reported that at early stages of fruit development, small-sized 'Hass' fruits are characterized by decreased auxin (indole-3-acetic acid, IAA) content, yet present elevated levels of abscisic acid (ABA), a plant hormone implicated in stress-induced cell-cycle arrest (Cowan et al 1997; Taylor, & Cowan 2001; Cowan et al 2005).

It is generally accepted that the determination of an organ's final size requires coordination of its growth with the cell-cycle machinery. The basic activity of the plant cell-cycle oscillator depends on the synthesis and destruction of cyclins, inducing waves of cyclin-dependent kinase (CDK) activity and triggering the transition of the cell cycle from G1 to S (replication phase) and from G2 to M (mitotic phase). Notably, the identification of cell-proliferation-related genes and characterization of their developmental expression patterns have been shown to be a useful tool in the study of cell-cycle progression during fruit organogenesis in various fruit crops (Inze & Veylder 2006). Moreover, in recent years, considerable progress has been made in unraveling the mechanisms controlling fruit cell division and growth. For instance, in tomato, various quantitative trait loci (QTLs) for fruit weight have been detected. One of them, *FW2.2*, accounts for an as much as 30% difference between the domesticated fruit and its wild small-size relatives. Pioneering studies led to the cloning of *FW2.2* and revealed that the major QTL effect is caused by a single gene (*fw2.2*), proposed to function as a negative regulator of fruit cell division (Frary, Nesbitt, Grandillo, Knaap, Cong, Liu, Meller, Elber, Alpert & Tanksley 2000; Cong & Tanksley 2006).

Given the current state of knowledge related to avocado 'Hass' fruit growth, it was of interest to further study the molecular mechanisms controlling fruit cell division in NF and SF phenotypes. To this end, we isolated several cell-proliferation-related genes from avocado. In a first attempt to explore the molecular mechanisms regulating avocado fruit growth, we investigated the expression characteristics of these genes, and compared NF and SF developmental patterns. In parallel, we performed developmental studies of cell division (i.e. cell numbers) and cell expansion (i.e. cell area). Taken together, our data suggest essential roles for the different cell-proliferation-related genes in regulating avocado fruit development.

Materials and methods

Plant material

'Hass' avocado fruit were harvested from commercially bearing 'Hass' avocado trees grown in Kibbutz Schiller's orchard, located in the central coastal region of Israel. NF and SF were harvested from 12 trees prior to and at first appearance of the SF phenotype, and at various intervals throughout fruit development. Fruit collections were recorded as days after full bloom (DAFB). Fruits, collected in the early morning, were transported to the laboratory where fruit mass and diameter were determined. Fruit seed and mesocarp tissues were then dissected, frozen in liquid nitrogen, and kept at -80^{o}C for further analysis.

Cell counts and cell-size measurements

Equatorial fruit sections were prepared using a rotary microtome. Histological samples were examined and photographed using a Leitz Dialux 20 light microscope. To determine average cell size, the number of cells present in representative unit areas of 90,000 μm^2 was counted from microscopic images. Cell counts were used to calculate average cell area. Cell numbers in the equatorial mesocarp and seed tissue zones were estimated essentially as described by Cowan et al (1997), using the following expression: $n = l*\sqrt{X}$ where n is number of cells, l is mesocarp width and/or seed radius, and x is the number of cells in the sampled area.

RNA isolation and cDNA synthesis

Total RNA was extracted from mesocarp and seed tissues using the phenol-SDS method described by Or, Vilozny, Eyal & Ogrodovitch (2000). RNA was quantified with a NanoDrop spectrophotometer, while quality was verified by gel electrophoresis. Following confirmation of RNA integrity, 4 µg of total RNA, pretreated with 1 unit of DNase, was used as template in the synthesis of first-strand cDNA, using an oligo dT primer and M-MuLV reverse transcriptase (RT) (Fermentas). PCRs were then performed using 2-µl aliquots of the RT reaction products (see below).

RNA gel-blot analysis

For RNA gel-blot analysis, 15 µg of total RNA isolated from NF and SF tissues was resolved in 1% agarose/6% formaldehyde gels and transferred to nylon membranes (Hybond N^+, Amersham Biosciences). Hybridization with radiolabeled probes was performed using Church-Gilbert hybridization buffer (Church & Gilbert 1984). Following hybridization, radiolabeled bands were visualized using a Storm scanner and quantified using IMAGEQUANT software (both from Amersham Pharmacia Biotech).

Semi-quantative (sq) RT-PCR analysis

For sq-RT-PCR, 4 µg total RNA from seed or mesocarp NF and SF tissues pretreated with 1 unit of DNase was used to synthesize first-strand cDNAs. PCRs were performed with gene-specific primers and 2-µl aliquots of the RT reaction products (Dahan, Rosenfeld, Zadiranov & Irihimovitch 2010).The PCR products were resolved in 2% agarose gels and their identities confirmed by sequencing. Densitometric quantification of the PCR bands was performed using AlphaEaseFc software (Alpha Innotech).

Results

Developmental expression analysis of *PaCYCA1* and *PaCYCB1* in NF and SF tissues

Rapid amplification of 5' and 3' cDNA ends (RACE) was adopted to amplify full-length cDNA clones encoding two cell-proliferation-related proteins. The isolated cDNAs encoding cyclin-A and B-like proteins were 1,477 and 1,963 nucleotides long, encoding 479- and 446-deduced aa products, respectively (GenBank accession numbers GU272024 and GU272023, respectively). The developmental expression patterns of the two mitotic cyclins were next monitored in NF and SF tissues. NF and SF were collected from 'Hass' avocado trees at different stages of fruit development. However, since the SF phenotype can only be visually detected between 60 and 90 DAFB (Cowan et al 2005), at the first sampling intervals only NF populations were harvested. In accordance with previous reports (Cowan et al 1997, 2005), measurements of fruit-growth parameters showed that the larger size of the NF is reflected in both increased fruit width and fresh weight (Figure 1A,B).

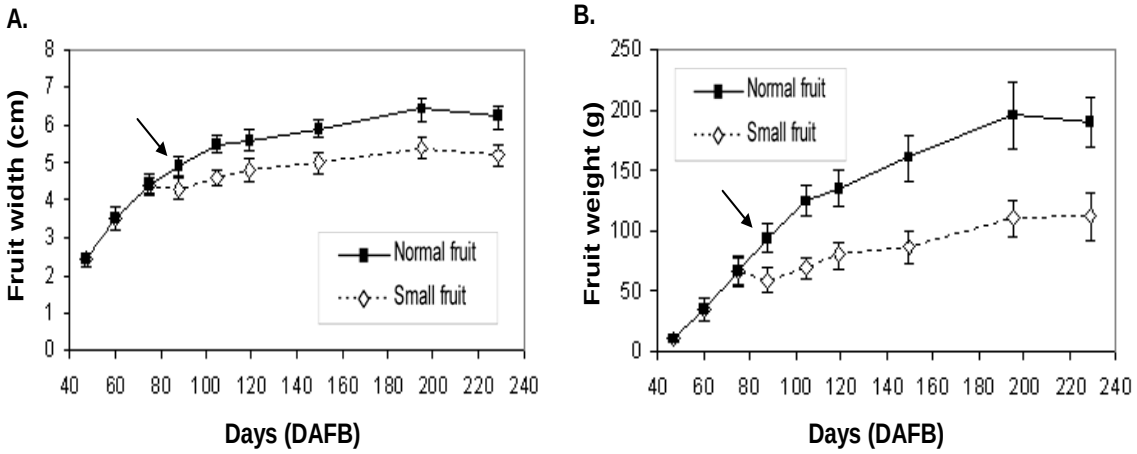

Figure 1. Fruit growth curves. Increases in normal fruit (NF) and small fruit (SF) width (A) and fresh weight (B) were measured during fruit growth. Arrows indicate visible appearance of the SF phenotype. Fruit collections were recorded as days after full bloom (DAFB). Values at each time point represent mean ± SE of 12 detached fruits. Adapted from Dahan et al (2010).

Next, total RNA extracted from NF and SF tissues was subjected to Northern blot analysis. Figure 2 shows representative RNA gel blot data revealing developmental differences in the accumulation patterns of the two cyclin genes between NF and SF seed tissues. As shown in Figure 2, cyclin A1 and B1 transcripts were highly

expressed in NF seeds at early stages of fruit development, gradually declining in abundance at middle stages of fruit development (88-120 DAFB) and becoming undetectable in the last stages of fruit growth. In contrast, in SF seed tissues (Figure 2, right panel), the levels of the two transcripts were already low by 88 DAFB, becoming undetectable as the fruit reached maturity. Parallel expression analysis of mesocarp tissues showed essentially similar developmental expression patterns (not shown). Thus, assuming that mRNAs of the mitotic cyclins are preferentially expressed during the S-G2-M cell-cycle transition states (Dewitte & Murray 2003; Inze et al 2006), the obtained data indicate that during fruit growth, cell division activity is attenuated and/or blocked earlier in SF seed and mesocarp tissues, as compared with the same NF tissues.

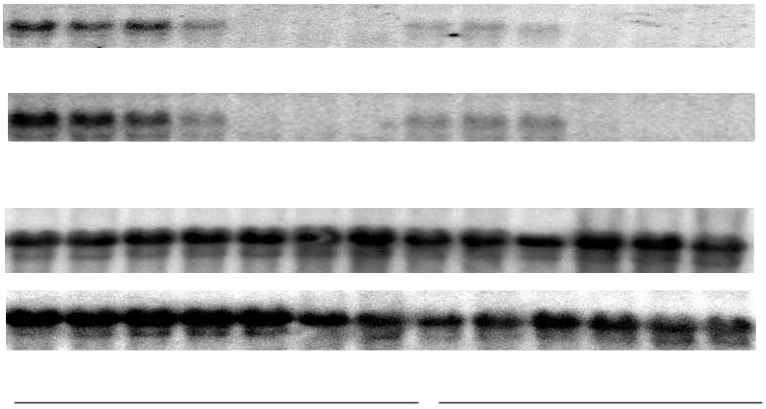

Figure 2. Northern blot analysis of *PaCYCB1* and *PaCYCA1* transcripts in seed tissues collected from normal fruit (NF) and small fruit (SF) at different stages of fruit development. Total RNA (15 µg) isolated from the indicated samples was separated by electrophoresis, blotted onto a membrane and initially hybridized with the probes shown on the left. The signals corresponding to the mRNAs were normalized to 18S rRNA levels, and the initial sampling time points were set to 1.0. The number beneath each lane shows the ratio of mRNA accumulation for each track. Two filters were used, and the 18S rRNA reprobing of each sample is shown. The identity of each filter is denoted by one or two asterisks to the right of the blots. DAFB, days after full bloom. Adapted from Dahan et al (2010).

Cell size and numbers in developing fruits

To further correlate the developmental expression patterns of the two isolated cyclin genes with fruit cell division activity, cell numbers across fruit tissues, as well as mean cell areas, were determined during NF and SF growth. Throughout the fruit's developmental period, seed cell number was found to be higher in NF than SF, with no significant difference in calculated mean cell area (Figure 3A,B). Moreover, in agreement with Cowan et al (1997), it was noted that during fruit growth, the calculated mesocarp cell number across the fruit was significantly higher in NF, yet no significant difference in mean cell area between NF and SF was detected (not shown). Taken together, and in agreement with the above RNA gel blot data, these results indicate that during fruit growth, cell division activity is reduced and arrests earlier in both seed and mesocarp tissues of SF as compared to the same NF tissues.

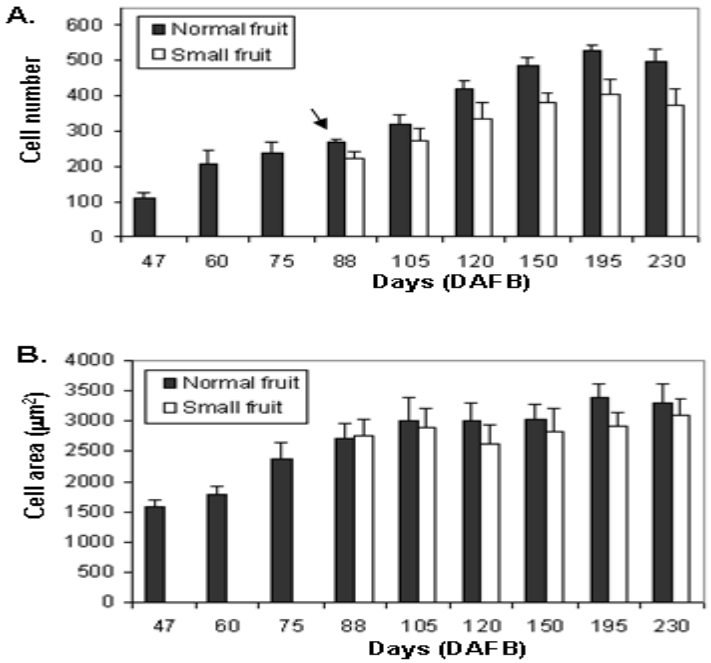

Figure 3. Changes in mean calculated seed cell number (A) and cell area (B) during normal fruit (NF) and small fruit (SF) development. Cell number and area were calculated as described in Materials and Methods. Values represent mean ± SE of 54 counts collected at each time point. DAFB, days after full bloom. Adapted from Dahan et al (2010).

Developmental expression analysis of *Pafw2.2-like* in NF and SF tissues

To determine whether a similar *fw2.2* gene product is found in avocado and to explore its function in relation to reduced cell division, a RT-PCR strategy was adopted. Using cDNA of SF mesocarp tissue and degenerate primers designed to conserved regions of *Lefw2.2* from tomato, a 306-bp fragment was amplified. The DNA sequence of the obtained fragment showed homology to *Lefw2.2* and was designated *Pafw2.2-like*. Further, 5'/3' RACE-PCR strategy was used to amplify the full-length gene. The identified *Pafw2.2* sequence contains an open reading frame (ORF) of 540 bp encoding a 180-aa protein (deposited in GenBank under accession no. GU272026). ClustalW-based alignment of the putative translated PaFW2.2-like protein revealed that the predicted protein shares 62% identity with LeFW2.2 (Figure 4A). Structural conservation between the *fw2.2-like* cDNA isolated from avocado and *fw2.2* suggested that they act in a similar fashion, negatively regulating cell division. As a first step to validating this assumption, the developmental expression profile of *Pafw2.2-like* was monitored in NF and SF seed and mesocarp tissues. Figure 4B shows a representative sq-RT-PCR analysis of *fw2.2-like* mRNA, demonstrating that in SF seed tissues, *Pafw2.2-like* transcript levels were highly abundant at 88 DAFB, declined slightly during fruit growth but resumed by 195 DAFB. A similar trend was observed in SF mesocarp tissues (not shown). Taking the evaluated periods of fruit development as a whole, it was shown that *Pafw2.2-like* mRNA levels are considerably higher in SF tissues, as compared to the same NF tissues.

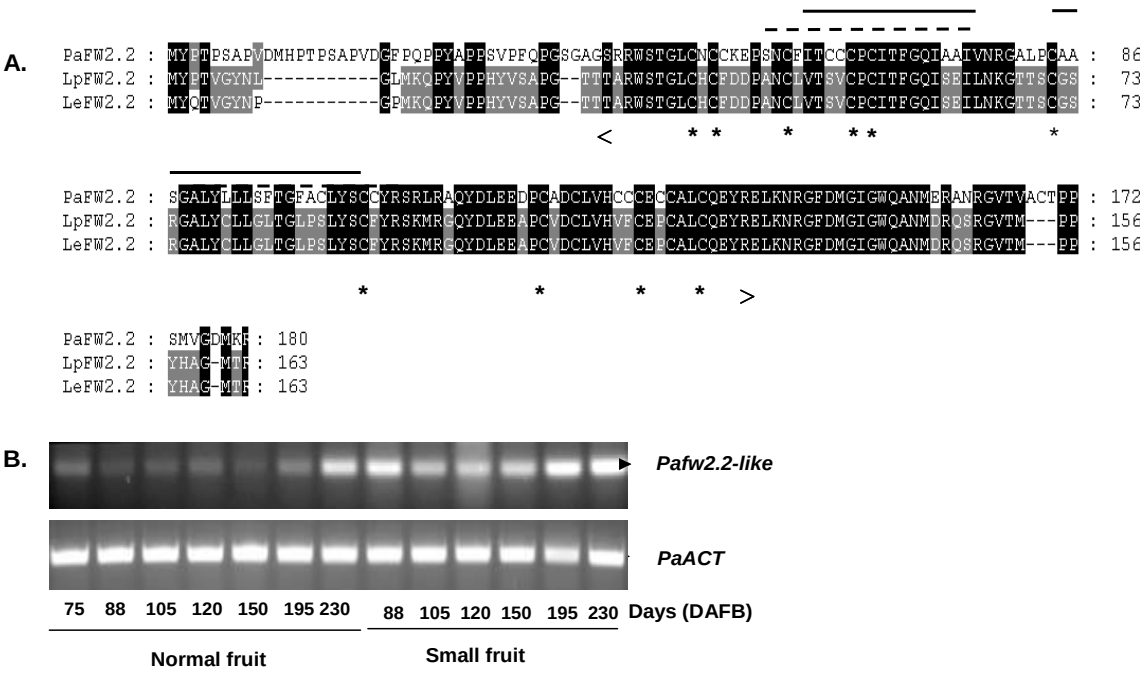

Figure 4. (A) Amino acid sequence comparison and structural features of avocado FW2.2-like. Comparison of the deduced amino acid sequence of PaFW2.2-like (acc. no. ADA70360) with two representative FW2.2 proteins from tomato, LeFW2.2 (acc. no. AAF74286.1) and LpFW2.2 (acc. no. AAF74287.1). Identical residues are shaded in black, conserved residues are shaded in gray. (B) Expression profile of *Pafw2.2-like* during development of normal and small fruit. RT-PCR analysis of *Pafw2.2-like* in seed tissues. *Pafw2.2*-like cDNA was amplified by 42 PCR cycles. Actin cDNA (*PaACT*) was used as an internal standard, using 25 PCR cycles. DAFB, days after full bloom. Adapted from Dahan et al (2010).

Discussion

Cell division plays a critical role in determining the final size of the fruit. However, although several aspects of avocado fruit growth regulation have been studied (Cowan et al 1997, 2005; Richings, Cripps & Cowan 2000), the molecular mechanisms controlling avocado cell division remain essentially unknown. As a first step to remedying this situation, we isolated two avocado genes encoding A and B mitotic cyclins, together with a third gene encoding a FW2.2-like protein. Using specific probes for *PaCYCA1* and *PaCYCB1* mRNAs, we confirmed and expanded upon a previous anatomical study suggesting that phenotypically small 'Hass' fruit growth is limited by cell division activity (Cowan et al 1997). Our results indicate that the premature arrest of SF cell division activity coincides with an accelerated decrease in cyclin mRNA accumulation in SF tissues, suggesting that factors contributing to premature down-regulation of these mRNAs might provoke early cessation of cell division in the fruit.

Repression of distinct cell-proliferation-related genes might be associated with changes in carbohydrate and/or hormone availability. Considering previous studies arguing that the occurrence of phenotypically small 'Hass' fruits might be linked to changes in sink strength and sugar content, and knowing that SF are characterized by decreased IAA content but elevated ABA levels (Cowan et al 1997, 2005; Richings et al 2000), it is tempting to speculate that limiting sucrose supply and/or changes in fruit hormone homeostasis might control the accelerated transcription repression of the two mitotic cyclins in SF tissues. Alternatively, in the case studied here, we can hypothesize that the regulation of cyclin mRNA accumulation is coupled to an adjustment in the expression of a negative regulator of fruit cell division, such as the FW2.2 homologous protein. Indeed, we also identified a third cDNA encoding a putative avocado FW2.2 protein, termed *Pafw2.2-like*. Furthermore, analysis of the developmental expression profile of *Pafw2.2-like* revealed that at almost all evaluated stages of fruit growth, its transcript levels are markedly higher in SF tissues than in NF tissues. It should be noted that cell division in tomato is restricted to a relatively short period of time (i.e., before and after anthesis). Accordingly, it was shown that during this time period, *fw2.2* is highly expressed in ‘small fruit’ lines, decreasing slowly as the fruit reach maturity

(Cong et al 2002). In contrast, in avocado, where cell division occurs throughout a longer period of fruit development, the finding that *Pafw2.2-like* is relatively highly expressed in SF tissues in the middle stages fruit development (88-120 DAFB), combined with the observation that at those time points its accumulation negatively correlates with fruit cell division activity, fits, in principle, with the predicted role for *Pafw2.2-like*. This may reflect *Pafw2.2-like* assuming a function similar to that of tomato *fw2.2*, namely, negative regulation of fruit cell division. It is worth noting that despite intensive studies on *fw2.2* in tomato, the connection between *fw2.2* and the cell-cycle machinery remains unclear. Nevertheless, Baldet, Hernould, Laporte, Mounet, Just, Mouras, Chevalier & Rothan (2006) suggested that *fw2.2* may function upstream of cell-proliferation-related genes in a signaling pathway linking the modulation of cell-cycle activity with sugars or hormonal signals. Whether up-regulation of *Pafw2.2* in phenotypically small 'Hass' avocado fruit is imposed by sugars or hormonal changes, e.g. modified levels of cytokinin, auxin or abscisic acid, remains an open question. Finally, it is also worth mentioning that for other plant genes, such as *AtCDK1* (Wang, Qi, Schorr, Cutler, Crosby & Fowke 1998), and *NtKIS1a* (Jasinski, Riou-Khamlichi, Roche, Perennes, Bergounioux & Glab 2002) encoding repressors of cell division, higher accumulation levels have been correlated with reduced cell division and smaller or narrowed organ size. Thus, the possibility that *Pafw2.2-like* might be linked either directly or indirectly to the expression and/or activity of such CDK inhibitors in avocado can be envisioned. We have recently isolated a cDNA termed *PaICK,* encoding a CDK inhibitor, and its expression pattern during avocado fruit growth is currently being examined.

Conclusions

Our data offer first insights into the cell-proliferation machinery of avocado and provide evidence that distinct cell-proliferation-related genes display different temporal accumulation patterns during growth of NF and SF avocado phenotypes. In particular, if one considers our results suggesting that in avocado, PaFW2.2-like function might be associated with regulation and/or repression of fruit cell division, further elucidation of PaFW2.2-like action represents an exciting future challenge. We predict that current efforts toward sequencing the entire avocado genome (Chanderbali, Albert, Ashworth, Clegg, Litz, Soltis & Soltis 2008) will facilitate such studies, allowing for a deeper understanding of the mechanism controlling the growth of this important fruit crop. Our hope is that a better understanding of processes controlling avocado cell division activity will lead to the establishment of improved PGRs treatments to increase 'Hass' avocado yield and fruit size.

Acknowledgments

We thank Dr. Gad Ish-Am and Dr. Leo Winner for kindly helping us determine the full bloom period and helpful suggestions, Mr. Moshe Goren for technical assistance and Kibbutz Shiller for giving us access to their 'Hass' avocado orchard, and allowing us to collect avocado fruits. This work was supported by grant no. 203-0708 from the Chief Scientist of the Ministry of Agriculture, Israel.

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