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PaKRP, a cyclin-dependent kinase inhibitor from avocado, may facilitate exit from the cell cycle during fruit growth

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ABSTRACT

Previous studies using 'Hass' avocado cultivar showed that its small-fruit (SF) phenotype is limited by cell number. To explore the molecular components affecting avocado cell production, we isolated four cDNAs encoding: an ICK/KRP protein, known to play cell cycle-regulating roles through modulation of CDK function; two CDK proteins and a D-type cyclin, and monitored their expression patterns, comparing NF (normal fruit) versus SF profiles. The accumulation of *PaKRP* gradually deceased during growth in both fruit populations. Despite these similarities, SF exhibited higher levels of *PaKRP* accumulation at early stages of growth. Moreover, in NF, augmented *PaKRP* expression coincided with a decrease in *CDK* and *PaCYCD1* levels, whereas in SF, enhanced *PaKPR* expression was coupled with an earlier decline of *CDK* and *PaCYCD1* levels. For both NF and SF, enhanced mesocarp *PaKRP* transcript accumulation, was associated with elevated abscisic acid (ABA) and ABA catabolites content. Nevertheless, the collective ABA levels, including catabolites, were substantially higher in SF tissues, as compared with NF tissues. Finally, additional expression analysis revealed that in cultured cells, *PaKRP* could be induced by ABA. Together, our data links *PaKRP* with exit from the fruit cell cycle and suggest a role for ABA in controlling its expression.

1. Introduction

Fruit growth depends on the relationship between cell division and cell expansion processes, both affecting final fruit size. In most fleshy fruits, cell production mainly takes place during a short period before and after fruit set, followed by a prolonged phase of cell expansion [1,2]. Avocado fruit growth is exceptional in this manner in that cell production continues throughout a relatively long development period [3,4].

A particular 'Hass' avocado cultivar (*Persea americana* Mill. cv Hass) produces two populations of fruit, namely normal (NF) and phenotypically small fruit (SF), without any particular pattern of distribution on the tree [5,6]. Typically, phenotypically small 'Hass' fruits are characterized by early seed coat senescence and cessation of growth [5,7–9]. Previous studies have shown that SF are limited in cell number yet not in cell size, a phenomenon that encouraged the use of this cultivar as an attractive model to study mechanisms affecting avocado fruit cell division [5,7–9]. Specifically, studies focused on the characterization of metabolic changes believed to affect fruit cell division revealed that changes in carbohydrate content and hormone homeostasis 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 impaired sugar metabolism, reduced capacity for cytosolic isoprenoid biosynthesis, and increased levels of abscisic acid (ABA) [5,7–9].

It is well established that in plants, as in animals, the production of new cells relies on the synthesis and destruction of cyclins, inducing waves of activity of cyclin-dependent kinases (CDKs), triggering cell cycle transition from G1 to S (replication phase) and from G2 to M (mitosis phase) [10–12]. Plants possess a diverse set of cyclins that can be classified into ten different groups based on phylogenetic considerations [13]. Among the major plants cyclins, A- and B-type cyclins are generally of importance for the control of S/G2/M cell-cycle transitions, while D-type cyclins are thought to regulate G1/S transitions [12,14]. Plant genomes also encode a wide range of CDKs that can be classified into seven groups (A–F) on the basis of their cyclin-binding domains [15,16]. The functions of CDKAs and CDKBs are perhaps the best documented. Several studies have shown that CDKAs are essential for cell division activity, regulating both G1/S and G2/M transitions, while CDKB-type proteins constitute a class of plant-specific proteins, presumed to regulate S/G2/M transition [11,12,16]. The initial commitment of cells to the cell cycle depends principally on CDKA kinase activity and its association with D-type cyclins (CYCDs). It is proposed that as is the case with their animals counterparts, the primary target of the CDKA/CYCD complex is the retinoblastoma

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(Rb)-related protein, itself a repressor of E2F transcription factors. Phosphorylation of the Rb-related protein releases it from E2F, leading to the activation of S-phase-related genes and to progression of the cell cycle [12]. The complex control of CDK activity is achieved both by modulating the levels of CDK transcripts and by post-translational events, including phoshorylation, degradation processes and interactions with different CDKs partners [10,12].

Plant ICKs (Inhibitors of CDKs) were originally described as a group of low molecular weight nuclear proteins able to bind CDK/CYC complexes and negatively modulate their activities [17,18]. The first plant ICK was identified in *Arabidopsis* using yeast two-hybrid (Y2H) screen analysis in which the *AtCDKA1;1* and *AtCYCD3;1* genes served as bait. It was subsequently reported that the sequence encoding the *Arabidopsis* ICK belongs to a gene family comprising seven members (*AtICK1-7*) [19–21]. Since than, ICK homologues have been cloned and characterized in several plant species, including tobacco [22], alfalfa [23], tomato [24], and rice [25]. It should be noted that while plant ICKs identified thus far share very little sequence similarity, all display a short conserved domain in the C-terminal region, homologous to a domain in the N-terminal region of mammalian KIP/CIP CDK inhibitors. As such, the revised nomenclature now refers to the plant proteins as ICK/KRPs (Kip-related proteins) [19,20,26]. The importance of the conserved C-terminal region of ICK/KRPs was established by domain-mapping studies demonstrating that ICK/KRPs interact with D-type cyclins and CDKA proteins via this conserved domain [20,27]. Further detailed analysis of ICK/KRP sequences allowed for identification of other conserved motifs, including those defining the nuclear and sub-nuclear localization patterns of the protein [17,18,28].

At the transcriptional level, the expression of plant ICK/KRP genes varies in different tissues and developmental stages. For example, it was shown that in *Arabidopsis*, *AtICK1*/*KRP1* is relatively highly expressed in mature leaves, as compared with young leaves, suggesting that the gene product might inhibit cell proliferation following differentiation [17]. Other studies performed in *Arabidopsis*, tobacco and rice have shown that over-expression of distinct ICK/KRP genes resulted in common phenotypes, including reduction in plant size, cell number, and in some cases, increased cell ploidy, supporting the concept that ICK/KRP genes regulate exit from mitosis [25,26,29,30]. More recent developmental studies also linked enhanced expression of distinct *ICK*/*KRP* genes with exit from the cell cycle during the growth of fleshy fruits, such as apple and tomato [24,31]. Finally, since various pieces of evidence correlate elevated levels of *ICK*/*KRP* transcription with reduced cell production, it is possible that plant ICK/KRPs integrate unfavorable environmental and/or growth signals, ultimately modulating cell division activity. In line with this assumption, it was shown that in some cases, *ICK*/*KRP* genes could be induced by ABA [23,27].

In a previous study aimed at exploring molecular mechanisms regulating avocado fruit cell division, we reported the isolation of distinct avocado cell proliferation-related genes and described their expression characteristics, comparing 'Hass' NF and SF developmental patterns [32]. Using three isolated cell-proliferation-related marker genes, including *PaCYCA1*, *PaCYCB1* and a proliferating cell nuclear antigen (*PaPCNA*), coupled with anatomical analysis, we showed that phenotypically small 'Hass' fruit growth is limited by cell division activity [32]. Additionally, we showed that throughout fruit development, SF expressed higher levels of the *Pafw2.2-like* gene, encoding a putative FW2.2 (fruit-weight)-like protein postulated to function as a negative regulator of cell division in tomato fruit [33,34]. Based on these findings, we proposed a conserved role for avocado FW2.2 as a negative regulator of cell division, affecting fruit final size [32].

Despite these advances, key questions regarding the regulation of avocado cell production remain unanswered. For example, it has yet to be established whether other factor(s), such as *ICK*/*KRP* genes, are also involved in the regulation of avocado fruit cell production, whether these are differently expressed in NF vs. SF tissues and whether their expression and/or activity is linked to the expression and/or activity of *Pafw2.2-like*. With these points in mind, we have isolated an avocado cDNA encoding an ICK/KRP, together with two cDNAs encoding CDKs and a fourth gene encoding a D-type cyclin. We first monitored the developmental expression patterns of the isolated genes, comparing NF vs. SF profiles. Next, we quantified the ABA and ABA catabolites content of selected fruit tissue samples and used avocado cell-suspension cultures to investigate the influence of ABA on the expression of the cloned transcripts. Based on our finding, we propose roles that *PaKRP* might assume in facilitating and/or accelerating exit from the cell division process.

2. Materials and methods

2.1. Plant material

'Hass' avocado fruit were harvested from commercial 'Hass' avocado trees growing in Kibbutz Yad-Hanna orchard, located in the central region of Israel. NF and SF were harvested from nine trees at various intervals throughout fruit development periods. All fruit collections were re-ordered according to days after full bloom (DAFB). Fruits, collected in the early morning, were transported to the laboratory where mass, diameter and length were determined. Subsequently, fruit seed and mesocarp tissues were dissected, frozen in liquid nitrogen and kept at −80 °C until further analysis. Upon dissection, each type of fruit tissue (i.e., seed and mesocarp) was divided into three experimental units, each composed of tissues from three trees. In addition, leaves and root tissues were collected from 'Hass' avocado seedlings grown under greenhouse conditions at Bet-Dagan, Israel.

2.2. Tissue cultures

Callus from mesocarp sections were started essentially as described [35]. Mesocarp sections, dissected from 'Hass' fruits harvested 210 DAFB, were dipped in 70% ethanol and immersed in 3.5% sodium hypoclorite for 15 min. Following a 1 min 80% ethanol rinse and three successive changes of ultra-pure water, fruit sections were blotted on sterile filter paper and placed on 0.8% agar plates containing MSB medium (Dushefa Biochemie, Haarlem, The Netherlands) and supplemented with NAA (5 mg/L), iP (5 mg/L) and 3% sucrose. The plates were then incubated in darkness at 25 °C, until callus tissues appeared. Cell suspensions were next initiated by transferring approximately 5 g 'wet' callus tissue into 20 mL liquid MSB medium containing NAA (5 mg/L), iP (5 mg/L) and 3% sucrose. The tissue cultures were maintained on a rotary shaker (120 rpm) in 125 mL Erlenmyer flasks at 25 °C in darkness and subcultured every two-three weeks. For experiments, 20 mL of a day 14 liquid culture were added to 180 mL fresh medium and cultivated in a 250 mL Erelnmeyer flask. The day on which dilution of the tissue culture was conducted was set as time zero.

2.3. Kinetic analysis of cell suspension cultures

Cell suspensions were initiated as described above. In some cases, following dilution of the cell suspension (at the time point set as zero), ABA was added to the culture at a final concentration of 100 μM. At least three different cell suspension cultures were subjected to ABA or control treatments and changes in biomass (mg/mL) were followed over 17 days. Aliquots (8 mL) were removed at various intervals, centrifuged (4000 rpm) and weighed. Relative growth rates (RGR: mg produced mg^{-1} and d^{-1}) were determined essentially as described [36] by calculating the slope (at time x) of the relationship between the logarithm of the biomass (B) and time