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Inhibition of tomato fruit ripening by 1-MCP, wortmannin and hexanal is associated with a decrease in transcript levels of phospholipase D and other ripening related genes

Mohd Sabri Pak Dek[a,b], Priya Padmanabhan[a], Jayasankar Subramanian[a], Gopinadhan Paliyath[a,*]

[a] Department of Plant Agriculture, University of Guelph, Guelph, Ontario, N1G 2W1, Canada
[b] Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

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ABSTRACT

Membrane deterioration is an inherent aspect of the advancement in senescence and loss in fruit quality during storage. Postharvest technologies used for extending shelf life and quality are targeted to reduce membrane damage through downregulating or blocking ethylene action. In this study, mature green tomato fruit were treated with inhibitors of ethylene receptor (ETR), phosphatidylinositol 3-kinase (PI3K) and phospholipase D (PLD), all recognized to be targets of regulation of fruit ripening. The inhibitors used included 1-methylcyclopropene (1-MCP, an ethylene receptor blocker), wortmannin (an inhibitor of PI3K), and hexanal (a PLD inhibitor). Fruit were treated at optimal levels of the inhibitors and were stored at 21 °C for 10 days. Color development was strongly delayed in wortmannin treated tomatoes just as in 1-MCP treated fruit; while, changes in respiration, firmness and ethylene evolution were very similar to that of control fruit. Hexanal delayed the initiation of these changes; while 1-MCP and wortmannin blocked the ripening process. Changes in expression levels of key genes involved in ethylene signalling, phosphoinositide metabolism, and lycopene synthesis that occurred in response to inhibitors, suggested potential roles for PI3K and PLD in ethylene signalling. Furthermore, fruit treated with all the three inhibitors showed a marked reduction in *PLD* transcript levels; suggesting that, regulation of *PLD* gene expression is a common critical regulatory point that regulates ripening. Lowered PLD levels may reduce membrane lipid catabolism and the generation of phosphatidic acid (PA), an intermediate in ethylene signalling regulation through downstream components.

1. Introduction

Fruit ripening is a complex process that involves an array of irreversible physiological, biochemical and organoleptic changes that lead to development of color, texture, flavor, aroma and nutritional qualities. A key event of ripening and senescence is alteration of membrane structure induced by the catabolism of phospholipids and membrane proteins as well as by increased sterol levels, resulting in the loss of cellular compartmentalization and ultimately tissue structure (Paliyath and Droillard, 1992; Paliyath et al., 2008). Phospholipase D (PLD, 3.1.4.4) initiates the first reaction in this degradative process through hydrolysis of structural lipids such as phosphatidylcholine into phosphatidic acid (PA) and the respective head group (Paliyath and Thompson, 1987; Pinhero et al., 2003). Phosphatidic acid is then converted to diacylglycerol and subsequently to free fatty acids. If PLD activity could be inhibited, then the generation of other catabolites in the lipid catabolic pathway is reduced, and this may result in increased membrane stability and increased longevity of produce (Paliyath and Droillard, 1992; Tiwari and Paliyath, 2011a). Though the biochemical processes involved in membrane deterioration in plant senescence has been well demonstrated (Paliyath and Droillard, 1992; Paliyath et al., 2008), early events that link the activation of the ethylene receptor and the initiation of membrane lipid catabolism are less well understood.

Phosphatidylinositol-3-kinase (PI3K) is a key enzyme that regulates a multitude of physiological processes in animals and plants. PI3K phosphorylates phosphatidylinositol (PI) at 3′-hydroxy position using ATP to produce a phosphoinositide with a phosphate molecule at the 3′-position of the inositol (Phosphatidylinositol 3-phosphate, PI3P) (Bago

Abbreviations: CRTISO, carotenoid isomerase; CTR1, constitutive triple response 1; DXS, deoxyxylulose phosphate synthase; ETR, ethylene receptors; GGPS, geranylgeranyl pyrophosphate synthase; 1-MCP, 1-methylcyclopropene; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol 3 – phosphate; PLC, phospholipase C; PLD, phospholipase D; PI4K, phoshatidylinositol 4-kinase; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; PSY1, phytoene synthase 1; PIPh, phosphoinositide phosphatase

* Corresponding author.
E-mail address: gpaliyat@uoguelph.ca (G. Paliyath).

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et al., 2014). Multiple isoforms of PI3K have been identified in mammalian system categorized into class I, II and III according to the protein domain structure and substrate specificity (Wymann and Pirola, 1998). A class III PI3K termed as Vps34p (vacuolar protein sorting 34) was first characterized in *Saccharomyces cerevisiae* (Herman and Emr, 1990). Further studies on Vps34p showed that this protein is required for normal vacuolar protein sorting and vacuolar segregation in yeast, and regulation of intracellular protein trafficking processes (Schu et al., 1993; Stack et al., 1993). Interestingly, in plant genome, only one copy of *PI3K* belonging to class III has been identified (Welters et al., 1994). In *Arabidopsis thaliana*, PI3K is encoded by a single gene, *AtVPS34* and is required for the normal growth and development. Downregulation of the *AtVPS34* expression in *Arabidopsis thaliana* resulted in inhibition of plant growth and development (Welters et al., 1994).

Though phosphoinositides (PI and its phosphorylated derivatives such as PIP, PIP2) constitutes only a relatively small portion of phospholipids in the membrane; PI plays a critical role in signal transduction processes in plants. Conversion of PI to phosphorylated inositols such as phosphatidylinositol 3-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP2), and other phosphorylated intermediates by the respective kinases provides multiple means of substrate generation for downstream signal transduction (Cantrell, 2001). Phosphorylation of PI can also result in increased negative charges in the membrane, which can affect preferential binding of cytosolic proteins having affinity to negatively charged membrane domains. Proteins with a C2 domain (PLD, protein kinases), or PH fold (phospholipase C or PLC) that function in the signal transduction systems have demonstrated this property (Rebecchi et al., 1992; Lomasney et al., 1996; Pappan et al., 1997). Several studies have shown that plant PLDs are characterized by the presence of a calcium-/lipid binding C2 domain at their N-terminal region that enables its membrane translocation during senescence, or in response to ethylene (Tiwari and Paliyath, 2011b).

In a recent study (Pak Dek et al. 2017), the role of PI3K in ethylene action was investigated in transgenic tobacco plants overexpressing PI3K. Transgenic plants over-expressing PI3K showed modified floral characteristics compared to wild type plants. Enhanced ethylene production, wilting and senescence were also noticed in transgenic flowers indicating a role of PI3K in senescence process. Overexpression of PI3K also resulted in a “triple response” phenotype in dark grown transgenic tobacco seedlings indicating that overexpression of PI3K may lead to enhanced ethylene function. As a corollary to this, downregulation of PI3K through antisense methods resulted in a tobacco phenotype having flowers with an extended shelf life (Pak Dek, unpublished).

These studies suggest that ethylene signal perception and initiation of membrane lipid degradation are linked, potentially through PI3K and PLD. However, the nature of these interactions, and how these processes influence downstream signal transduction are not well understood. One way to understand these processes will be to inhibit the ethylene receptor, PI3K and PLD independently, and analyze how this affects the ripening related gene expression processes. Thus, the effects of various inhibitors; 1-MCP, hexanal and wortmannin, targeted to downregulate ethylene signal transduction (1-MCP as an inhibitor of ethylene receptor; hexanal as an inhibitor of PLD; and wortmannin as an inhibitor of PI3K), were analyzed to understand the common features of their mechanism of action during tomato fruit ripening, specifically in relation to postharvest quality and expression of genes such as those involved in carotenoid biosynthesis and PI metabolism.

2. Materials and methods

2.1. Treatments of tomato fruit

Mature green tomato fruit (*Solanum lycopersicum* cv De Ruiter) purchased locally (Elmira, Ontario) were sorted and randomly separated into four groups for control, wortmannin, hexanal and 1-MCP treatments, respectively. Each treatment had a total of 600 g fruit (~120 fruit) that were split into three replicates of 200 g each. Wortmannin (0.5 μM) and hexanal (0.01 mM) treatments of tomatoes were carried out by vacuum infiltration in aqueous compositions at 600 mm Hg, for 1 min. Stock solution of wortmannin (10 mM in dimethylsulfoxide, Sigma-Aldrich, Canada) was diluted with water (no precipitation was observed at 0.5 μM) to provide the desired concentration (200 g tomato takes in ~ 20 mL of the solution. Assuming a density of 1 for tomato fruits, this translated to 20 mL diluted to 200 mL of tomato by volume. Thus, the effective concentration of wortmannin within the fruits may be ~0.05 μM. Similarly, final hexanal concentration in fruit may be assumed as ~20 μM). Hexanal was dissolved in water containing Tween 20 (0.2% v/v). Vacuum infiltrated (1 min) fruit in the respective solutions without the inhibitor served as the control. Treated fruit were air-dried overnight in the dark and then stored at room temperature for sampling at various intervals. Treatment with 1-MCP (as vapour, 10 mg L^{-1}) was carried out according to Tassoni et al. (2006). Briefly, 20 mg of SmartFresh™ (0.14% of active ingredient w/w) was dissolved in 1 mL water to provide a final gas concentration at 10 μL L^{-1} in an air tight glass bottle containing 200 g fruit and the fruit were exposed to 1-MCP for 18 h in the dark at room temperature. After 1-MCP treatment, fruit were withdrawn from the container and stored at room temperature for further studies. Three biological replicates were maintained for each treatment. Samples were collected at 1, 3, 6 and 10 day of storage for analysis of various ripening related quality parameters including colour, fruit firmness, ethylene content, respiration rate, lycopene content, and weight loss. Fruit pericarp samples after removal of seeds were frozen in liquid nitrogen and stored at –80 °C until further use for RNA isolation and gene expression analysis.

2.2. Measurement of ripening-related quality parameters

Fruit pericarp firmness was measured by a FirmTech 2 Fruit Firmness Tester (BioWorks, KS, USA) from 2 sides of 10 fruit per replicate. Fruit color parameters such as brightness (*L*), green or red (*a*) and blue or yellow (*b*) were measured (Minolta CR-300 Chroma Meter, Minolta, Ramsey, NJ) from both sides of 10 fruit per replicate. The color change in tomato fruit was then expressed as colour index (TCI = 2000 a/L $(a^2 + b^2)^{1/2}$) according to Richardson and Hobson (1987). Tomatoes from various treatments were placed in 500 mL glass containers after weighing, containers were sealed for an hour and the amount of ethylene and carbon dioxide in the head-space was measured. For ethylene measurement, 1 mL of head-space gas sample was injected into a Varian CP-3380 gas chromatograph (Varian Inc., Mississauga, ON) equipped with a 0.5 mL sample loop and the sample was separated with a 15 mm x 0.32 mm Restek Rt-SPLOTTM capillary column (Chromatographic Specialties Inc., Brockville, ON). Ethylene was detected with a flame ionization detector and quantified by commercial ethylene standards (BOC Gases, Mississauga, ON) and normalized for fruit mass. For carbon dioxide detection, 3 mL of head-space gas sample was injected into an ADC infrared gas analyzer (Nortech Control Equipment Inc., Etobicoke, ON) and quantified using a commercial standard (BOC Gases, Mississauga, ON).

2.3. Measurement of lycopene content

Lycopene estimation was carried out according to the method of Fish et al. (2002). Tomato pericarp tissue (10 g) was homogenized in 10 mL of water using a Brinkmann™ Polytron™ Homogenizer fitted with PTA 10 probe (Kinematica, Luzen, Switzerland) and the slurry was incubated on ice in the dark. Then, 20 mL of hexane: acetone: ethanol (10:5:5 v/v) mixture was added to the slurry and mixed thoroughly for 30 min on an orbital shaker at 180 rpm. Distilled water (3 mL) was added to the slurry followed by incubation on ice for 30 min for phase separation. The absorbance of the top hexane layer was measured at 503 nm for lycopene estimation.