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Seed development and hydroxy fatty acid biosynthesis in *Physaria lindheimeri*

Grace Q. Chen[a,*], Timothy J. Riiff[a], Kumiko Johnson[a], Eva Morales[a], Hyun Uk Kim[b], Kyeong-Ryeol Lee[c], Jiann-Tsyh Lin[a]

[a] Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, CA 94710, United States
[b] Department of Bioindustry and Bioresource Engineering, Plant Engineering Research Institute, Sejong University, Seoul 05006, Republic of Korea
[c] Department of Agricultural Biotechnology, National Institute of Agricultural Sciences, Rural Development Administration, Jeonju 54874, Republic of Korea

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ABSTRACT

Hydroxy fatty acids (HFAs) are valuable industrial raw materials used in many industries. *Physaria lindheimeri* accumulates over 80% HFA, in the form of lesquerolic acid (20:1OH), in its seed oil. Understanding the seed development of *Physaria lindheimeri* is an important step to utilizing this unique wild species as a genetic source of HFAs biosynthesis. The changes of seed growth, lipid accumulation and fatty acid composition during seed development of *P. lindheimeri* were examined from 14 days after pollination (DAP) to desiccation (56 DAP). The seed development could be divided into three periods. During the early period (14 and 21 DAP), seed rapidly increased in size and fresh weight. In mid-maturation period (28, 35, and 42 DAP), lipids and dry weights accumulated steadily. When seeds developed to late-maturation/desiccation stages (49 and 56 DAP), fresh weight dropped significantly due to water loss, and the dry weight and lipid accumulation reached their maximums. Seed color remained green up to 42 DAP and turned to orange-brown at 49 and 56 DAP. The major fatty acid 20:1OH started accumulation when seeds developed into mid-maturation stage (28 DAP) and the accumulation continued thereafter up to 56 DAP, eventually reaching up to 77% of the total seed oil. The HFA accumulation indicates embryonic storage tissue formation, thus 28 DAP defines a critical time point for seed development entering reserve synthesis and accumulation. The information and knowledge obtained from this study are essential to the success of HFA production using metabolic pathway engineering approaches in commodity oilseed crops.

1. Introduction

Castor (*Ricinus communis*) seed oil is a commercial source of hydroxy fatty acid (HFA) with numerous industrial applications (Caupin, 1997). However, castor seeds contain lethal toxin (Chen et al., 2005; Lord et al., 1994) and potent allergens (Chen et al., 2004; Machado and Silva, 1992; Youle and Huang, 1978). A safe source of HFA is from lesquerella (*Physaria fendleri*) seed oil, which contains a major HFA, lesquerolic acid (20:1OH), at 55–60% (Chen et al., 2010; Dierig et al., 2011; Hayes and Kleiman, 1996; Smith et al., 1961; Von Cruz and Dierig, 2015). Compared with castor oil which contains 90% HFA, ricinoleic acid (18:1OH) (Thomas, 2000), lesquerella remains to be improved for its HFA level to be cost-competitive. A related species, *Physaria lindheimeri* (formerly *Lesquerella lindheimeri*), accumulates up to 85% 20:1OH in its seed oil (Dauk et al., 2007; Jenderek et al., 2009), which makes *P. lindheimeri* a valuable genetic resource. *P. lindheimeri* was crossed with lesquerella, but there was no significant increase of HFA level in the hybrid off-springs of lesquerella (Dauk et al., 2007). However, genes and regulatory elements from *P. lindheimeri* are excellent targets for biotechnology using *Agrobacterium*-mediated transformation to improve HFA levels in lesquerella (Chen, 2011; Chen et al., 2016).

Seed oil or storage lipids are in the form of triacylglycerols (TAGs) where fatty acids (FAs) are esterified to each of the three hydroxyl groups of a glycerol backbone. Plastid is the organelle for *de novo* FAs biosynthesis. The newly synthesized FAs are then exported and converted to acyl-coenzyme As (acyl-CoAs) for the synthesis of TAG in the endoplasmic reticulum (ER) (Bates et al., 2013; Chapman and Ohlrogge, 2012), through a glycerol-3-phosphate (G3P) pathway or Kennedy pathway (Barron and Stumpf, 1962; Bates et al., 2013; Weiss et al., 1960). A glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first acylation at the *sn*-1 position of G3P yielding lysophosphatidic acid (LPA), which in turn is acylated by a lysophosphatidic acid acyltransferase (LPAT) at the *sn*-2 position to produce phosphatidic acid

* Corresponding author.
E-mail address: grace.chen@ars.usda.gov (G.Q. Chen).

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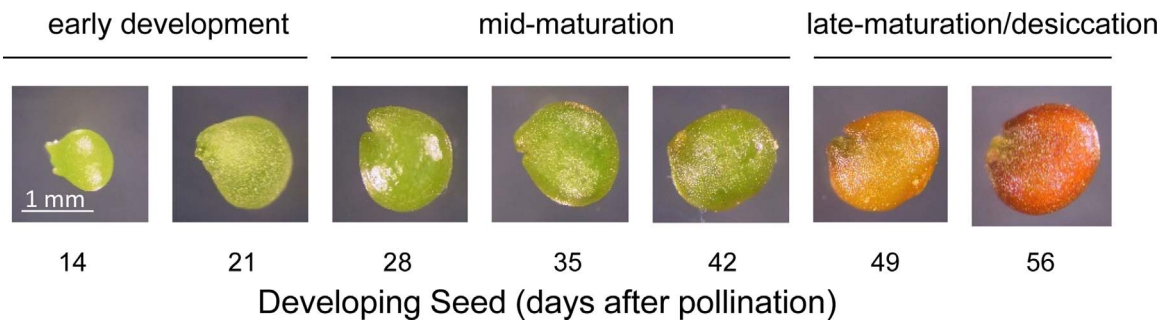

Fig. 1. The morphological changes of *Physaria lindheimeri* during seed development. Mature female flowers were individually hand-pollinated and tagged, and the tagging dates were recorded as 0 day after pollination (DAP). Silique were harvested at 7-day intervals from 14 DAP to 56 DAP. Seed were dissected out of the silique, and seed images were taken under a Leica dissecting microscope equipped with a digital camera.

(PA). PA is then converted to 1,2-sn-diacylglycerol (DAG) by PA phosphatase (PAP). A third FA is esterified to the *sn*-3 position of DAG by 1,2-sn-diacylglycerol acyltransferase (DGAT) to produce TAG. Also, the newly synthesized FA can also be incorporated into *sn*-2 position on phosphatidylcholine (PC) by the reactions of the acyl editing cycle (Lands, 1965; Li-Beisson et al., 2013). PC is the site for FA-modifying enzymes such as desaturases and hydroxylases, so rapid de-acylation and re-acylation of PC causes the acyl-CoA pool to be enriched with modified FAs (mFA) such as linoleic (18:2) and linolenic (18:3) and 18:1OH etc., which can subsequently be used for TAG synthesis. Besides, FAs on *sn*-2 PC can be transferred to the *sn*-3 position of DAG by phospholipid: DAG acyltransferase (PDAT) (Dahlqvist et al., 2000). Furthermore, the head groups of DAG and PC can be inter-exchanged by PC:DAG phosphocholine transferase (PDCT) (Hu et al., 2012; Lu et al., 2009) generating PC-derived DAG, which can be utilized for TAG synthesis. The enzymatic steps of 20:1OH biosynthesis have been elucidated. 18:1OH is first synthesized by hydroxylation of oleic acid 18:1 on *sn*-2 PC and then released and activated to 18:1OH-CoA (Bafor et al., 1991; Moreau and Stumpf, 1981). Subsequently, 18:1OH-CoA is elongated to 20:1OH-CoA for TAG synthesis (Engeseth and Stymne, 1996; Moon et al., 2001; Reed et al., 1997). Schematic pathways for 20:1OH-containing TAGs biosynthesis in lesquerella has been proposed (Chen et al., 2016).

Knowledge of *P. lindheimeri* seed development and HFA synthesis is required in implementing a biotech approach. Assessment of the seed development of *P. lindheimeri* is essential in the selection of seed samples and the construction of libraries for acquiring genes and information needed in *Agrobacterium*-mediated transformation. Yet, the precise seed development stages in *P. lindheimeri* have not been established. Here, we describe the time course of the entire seed development and present the changes of seed growth, lipid accumulation and fatty acid (FA) profile during the development. The relationships among these changes, and genes involved in HFA synthesis are discussed.

2. Materials and methods

2.1. Plant material

The *P. lindheimeri* seeds (accession number PI 643174) were obtained from United States Department of Agriculture, National Plant Germplasm System (http://www.ars-grin.gov/npgs/). Ten plants were grown in a greenhouse as described (Chen et al., 2009). Developing seeds from 14 DAP to 56 DAP were generated and harvested as described (Chen et al., 2009). Seeds were pooled from ten plants for each sample per time point. Fresh weight was measured on 300-seed replicates for 14 DAP samples, and 100-seed replicates for the remaining samples. Dry weight was measured after drying the fresh seeds at 56 °C for 24 h.

2.2. Lipid extraction and measurement

Triplicates samples were prepared by heating the dried 300-seeds or 100-seeds samples of each stage in 1 mL of isopropanol with 0.01 percent butylated hydroxytoluene, then grinding the seeds using mortar and pestle, and transferring the sample into a 15-mL glass tube. The lipids were extracted by adding 2 mL chloroform, 3 mL methanol, and 1 mL of isopropanol into the 15-mL tube, followed by adding 1.6 mL water, 2 mL chloroform, and 2 mL of 0.88% KCl. After vortexing and spinning the 15-mL tubes, the lipids, together with the solvent were split into the lower phase which was then transferred to a filter containing sodium sulfate for eliminating any residual water. The solvent was dried under nitrogen, and the lipid was weighted.

2.3. FAME preparation and GC analysis

FA methyl esters (FAMEs) were prepared for each sample by dissolving 1 mg of lipids in 1 mL hexanes, adding 1 mL 5% sulfuric acid in methanol solution, and then heating the sample at 95–100 °C for 30 min. The FAMEs were extracted by adding 1.5 mL water, vortexing and centrifuging. After centrifuging, the top, hexane layer containing FAMEs was collected and transferred into a vial for analysis on an Agilent 7890A gas chromatograph – Flame Ionization Detector (GC-FID) using a Restek RTX-2330 column as described (Chen et al., 2016). FAMES were quantified using the ratios of their peak areas to the sum of the total peak areas.

3. Results

3.1. Seed growth

Developing seeds of *P. lindheimeri* were examined for various changes from 14 to 56 DAP. Based on the changes of seed color, size, three major periods of seed development, early, mid-maturation, and late-maturation/desiccation periods, were exhibited in *P. lindheimeri*. Up to 21 DAP, developing seeds were at an early period, where seeds had translucent light green color and grew fast showing rapid increase in size (Figs. 1 2 A). (Numeric data for Fig. 2A is included in supplementary Table S1). During the mid-maturation period, represented by seeds at 28 DAP, 35 DAP and 42 DAP, the seeds' color turned to an opaque green and seed growth slowed down and reached a maximum size (Fig. 1). When seeds entered late-maturation/desiccation period (49–56 DAP), the seed coat color become orange-brown and the seeds' size remained almost unchanged (Fig. 1).

The increase of seed fresh weight showed distinct patterns among the three periods, and it corresponded well with the seed growth. During 14–21 DAP when young seeds are at the fastest growth phase, fresh weight gain was at the highest rate, showing 27 mg/day (Figs. 1, 2 A). After 21 DAP, seed growth slowed down (Fig. 1), and the rate of seed fresh weight gain was also decreased to 12 mg/day at 28 DAP and