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Identification of target genes and processes involved in erucic acid accumulation during seed development in the biodiesel feedstock Pennycress (*Thlaspi arvense* L.)

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ABSTRACT

We studied erucic acid accumulation in the biodiesel feedstock Pennycress (*Thlaspi arvense* L.) as a first step towards the development of a sustainable strategy for biofuel production in the EU territory. To that end, two inbred Pennycress lines of European origin, "NASC" and "French," were cultivated in a controlled chamber and in experimental field plots, and their growth, seed production and seed oil characteristics analyzed. Differences in some agronomical traits like vernalization (winter-French versus spring-NASC), flowering time (delayed in the French line) and seed production (higher in the French line) were detected. Both lines showed a high amount (35–39%) of erucic acid ($22{:}1^{\Delta 13}$) in their seed oil. Biochemical characterization of the Pennycress seed oil indicated that TAG was the major reservoir of $22{:}1^{\Delta 13}$. Incorporation of $22{:}1^{\Delta 13}$ to TAG occurred very early during seed maturation, concomitant with a decrease of desaturase activity. This change in the acyl fluxes towards elongation was controlled by different genes at different levels. *TaFAE1* gene, encoding the fatty acid elongase, seemed to be controlled at the transcriptional level with high expression at the early stages of seed development. On the contrary, the *TaFAD2* gene that encodes the Δ12 fatty acid desaturase or *TaDGAT1* that catalyzes TAG biosynthesis were controlled post-transcriptionally. *TaWRI1*, the master regulator of seed-oil biosynthesis, showed also high expression at the early stages of seed development. Our data identified genes and processes that might improve the biotechnological manipulation of Pennycress seeds for high-quality biodiesel production.

1. Introduction

Field Pennycress (*Thlaspi arvense* L.) is a winter annual that belongs to the Brassicaceae family. As many other members of this family, such as Miscanthus (Robson et al., 2013), Etiopian mustard (*Brassica carinata*; Bouaid et al., 2009) and Camelina (*Camelina sativa*, Frohlich and Rice, 2005), Pennycress has attracted the attention of researchers as a promising alternative oilseed feedstock for biodiesel production, that accomplishes the land use and sustainability criteria and displaces other plant species like Jatropha (*Jatropha curcas*) or Crambe (*Crambe abyssinica*) that are not well adapted to the temperate climate conditions of Europe and North America (Moser, 2012). Native of Eurasia, Pennycress is widely distributed across temperate regions all around the world and is highly adapted to a wide variety of climatic conditions (Warwick et al., 2002; Vaughn et al., 2005; Moser et al., 2009a). Because of its growth cycle, it can be successfully planted at the end of the summer and germinates in the fall developing a low-growing rosette that protects the plant from low temperatures and cold winds during the winter. Pennycress is an extreme cold tolerant plant (Sharma et al., 2007). The plant resumes its growth in the spring, sets seeds and is harvested at the beginning of the summer. Because of its culture cycle it can be used in rotations, not displacing existing agricultural production (Moser, 2012). It does not need any agricultural inputs like fertilizers or pesticides and has no specific water requirements. Furthermore, Pennycress could be planted in lands not otherwise suited for agricultural production (Moser, 2012). Therefore, it does not compete with food chain cultures. Pennycress has been signaled for its potential to produce biomass for renewable biofuel production (Moser et al., 2009a,b; Moser, 2012), being a prolific seed producer (Fan et al., 2013). Harvested Pennycress seeds contain around 36% oil (w/w), which is

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twice the amount present in other oil commodities like soybean or sunflower and very similar to that found in camelina (Moser, 2012). Because of its high oil content and fatty acid profile, with high amounts of unsaturated fatty acids, particularly erucic acid ($22{:}1^{\Delta 13}$), Pennycress oil can be used for biodiesel and biojet production with excellent characteristics like high cetane number of 59.8 and low temperature properties (Moser et al., 2009a; Moser, 2012; Fan et al., 2013). These results indicate that Pennycress oil could qualify as a biomass-derived diesel according to the Renewable Fuels Standard (Fan et al., 2013). Despite these interesting characteristics, Pennycress is still a wild plant that requires much research to evaluate its actual agronomical potential. In the USA, test plots from Illinois reported seed yields that varied from 900 to 2352 kg ha^{-1} (Evangelista et al., 2012; Fan et al., 2013). In our first experimental campaign in Spain, productions around 1300 kg ha^{-1} were obtained with European varieties. Several traits, including seed dormancy and size, erucic acid content, pot shatter and flowering time have been identified as potential targets of Pennycress breeding to improve its potential as a dedicated bioenergy crop (Sedbrook et al., 2014). To the best of our knowledge, although it is well adapted to the European climatic conditions, and there is botanical register of its presence all through the EU, very few experiments on Pennycress cultivation have been performed, all of them using Pennycress seeds of American origin, and the agronomic conditions required for its successful cultivation have not been still established (Groeneveld and Klein, 2014, 2015).

In addition to the research efforts to be held at the agronomical level, understanding the biochemical and molecular pathways involved in oil biosynthesis in Pennycress seeds is necessary to guide future crop improvement efforts directed towards an increase in oil yield and quality as well as other important agronomic traits (dormancy, vernalization, etc). At the molecular level, a transcriptome assembly of Pennycress genes has been reported that might provide tools for the breeding of Pennycress (Dorn et al., 2013). More recent studies have performed a metabolite profiling of Pennycress seed embryos to determine the biochemical pathways active during oil synthesis (Tsogtbaatar et al., 2015). However, although the enzymes (fatty acid elongases and desaturases) and compounds (TAG, DAG) that participate in seed oil biosynthesis have been characterized in other plant species, mainly *Brassica napus* and *Arabidopsis thaliana* (Von Wettstein-Knowles, 1982; James et al., 1995; Millar and Kunst, 1997; Katavic et al., 2001, 2004), this information is still lacking for Pennycress seeds. This knowledge is necessary to avoid metabolic bottlenecks that usually appear during the manipulation of seed oil biosynthetic pathways (Cahoon et al., 2007). An example of these bottlenecks related to erucic acid manipulation was detected in genetically engineered *Crambe abyssinica* (Guan et al., 2014).

As a first step for the introduction of Pennycress as an alternative feedstock for biofuel production in the Mediterranean regions of the EU, we studied erucic acid accumulation both at the biochemical and molecular levels in two different *Thlaspi arvense* L. strains of European origin that showed differences in some agronomically interesting traits such as vernalization, flowering time, and seed size and production. Our data showed a rapid accumulation of $22{:}1^{\Delta 13}$ into TAG in developing Pennycress seeds. At the molecular level, several genes involved in $22{:}1^{\Delta 13}$ biosynthesis like *TaFAE1*, *TaFAD2* or in the regulation of seed oil biosynthesis like *TaDGAT1* or *TaWRI1*, were also characterized. The *TaFAE1* gene from *T. arvense* showed a high phylogenetic correlation with other FAE1 enzymes from plants that also accumulated high amounts of erucic acid in their seed oil. The expression analysis of the *TaFAE1* gene suggested that the accumulation of $22{:}1^{\Delta 13}$ was controlled at the transcriptional level at the earlier stages of seed development and that a change in the acyl fluxes towards elongation versus desaturation took place to favor $22{:}1^{\Delta 13}$ accumulation in Pennycress seeds. These data may help to improve its biotechnological manipulation for high-quality biodiesel production.

2. Materials and methods

2.1. Plant growth and characterization of plant lines

Pennycress seeds (*Thlaspi arvense* L.) were obtained from different sources. "NASC" seeds were obtained from the *Nothingham Arabidopsis Stock Centre*, UK (NASC). "French" seeds were obtained from the *B&T World Seeds* company (France). Seeds were germinated in plates on wet Whatman paper without addition of any other supplement. Once germinated, seeds were transferred to pots containing a 75:25 mixture of substrate (peat moss, Kekkilä white 420W): vermiculite and grown in a bioclimatic chamber under a light intensity of 120–150 μmol m^{-2} s^{-1}, with a 16 h/8 h light/dark photoperiod at 22 °C and a relative humidity of 45%. When grown in the culture chamber, French plants required a vernalization treatment to fully develop into flowers and seeds. To that end, upon emergence of the first pair of true leaves, the plants were transferred to a cold chamber at 6 °C for 5 weeks with a light intensity of 120 μmol m^{-2} s^{-1} and 10/14 h day/night cycle, respectively. The plants were then placed back into the growth chamber under normal growth conditions and allowed to fully develop.

Pennycress was also grown under field conditions in small experimental plots (10 m^2) located at the research farm of the Estación Experimental de Aula Dei (Consejo Superior de Investigaciones Científicas), in the Aragon region (NE Spain) (41°44′N, 0°46′W, 259 m alt.). Sowing was done at the end of September with a density of 6–8 kg ha^{-1}. The area is characterized by a semiarid Mediterranean climate with an average annual rainfall of 355 mm and an average annual air temperature of 14.5 °C. The soil of the experimental plots is representative of the soils in semiarid Aragon with a medium texture, alkaline and generally with low organic carbon content. Specifically, for the 0–40 cm depth, this soil is a loam soil (23% sand, 53% silt and 24% clay) with pH = 8.3, 13.6 g kg^{-1} of organic matter content and 345 g kg^{-1} of $CaCO_3$. Mean soil nutrient contents (0–40 cm depth) were 11, 130, 28 and 193 mg kg^{-1} for P, K, N, and Mg, respectively. No herbicide or biocide treatments were applied during the crop growth. Seeds were manually harvested at the end of May. The weight and size of seeds, number of seeds per pod, number of pods per plant, and number of seeds per plant were obtained from at least 10 different plants from each Pennycress lines. The amount of oil per seed dry weight basis was obtained gravimetrically by the method of Li et al. (2006) from 1 g of seeds.

2.2. Lipid and fatty acid composition analysis

Total lipids were extracted from *Thlaspi arvense* seeds (0.5 g) in four successive maturation phases (green, green-yellow, yellow-green and dry phase) with chloroform/methanol (2:1, v/v) as described by Bligh and Dyer (1959). Fatty acid methyl esters of total lipids or individual lipid classes were produced by acid-catalyzed transmethylation (Garcés and Mancha, 1993) and analyzed by gas chromatography (GC), using a 7890A (Agilent, Santa Clara, CA USA) fitted with a capillary column (60-m length; 0.25-mm inner diameter; 0.2-μm film thickness) of fused silica (Supelco, Bellafonte, PA, USA) and a FID detector. Helium was used as a carrier gas with a linear rate of 1.2 ml min^{-1} and split ratio of 1/100. The injector temperature was 250 °C and the detector temperature was 260 °C. The oven temperature was modified as follows: 170 °C for 30 min, then raising the temperature by 5 °C/min to 200 °C. 17:0 was used as an internal standard. Data from fatty acid analysis were obtained from three biological experiments in the case of plants grown in culture chamber and two independent biological experiments for plants