Metabolic Engineering Communications 2 (2015) 93–98

Contents lists available at ScienceDirect

Metabolic Engineering Communications

journal homepage: www.elsevier.com/locate/mec

Field trial evaluation of the accumulation of omega-3 long chain polyunsaturated fatty acids in transgenic *Camelina sativa*: Making fish oil substitutes in plants

Sarah Usher, Richard P. Haslam, Noemi Ruiz-Lopez [1], Olga Sayanova, Johnathan A. Napier *

Department of Biological Chemistry and Crop Protection, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK

ARTICLE INFO

Article history:
Received 22 December 2014
Received in revised form
12 April 2015
Accepted 28 April 2015
Available online 9 July 2015

Keywords:
Plant metabolic engineering
GM field trials
Omega-3 long chain polyunsaturated fatty acids
Fish oils
Camelina
Oilseeds

ABSTRACT

The global consumption of fish oils currently exceeds one million tonnes, with the natural *de novo* source of these important fatty acids forming the base of marine foodwebs. Here we describe the first field-based evaluation of a terrestrial source of these essential nutrients, synthesised in the seeds of transgenic *Camelina sativa* plants via the heterologous reconstitution of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway. Our data demonstrate the robust nature of this novel trait, and the feasibility of making fish oils in genetically modified crops. Moreover, to our knowledge, this is the most complex example of plant genetic engineering to undergo environmental release and field evaluation.

1. Introduction

The consumption of oily fish and the n-3 (also known as omega-3) long chain polyunsaturated fatty acids (LC-PUFA) they contain is now widely recognised by health professionals as conferring an important health benefit, reducing the risk of cardiovascular disease and related metabolic conditions (Saravanan et al., 2010; Swanson et al., 2012). However, the primary source of these nutrients (global oceanic fish stocks) are either at the limits of sustainable management and/or suffering from environmental pollution, precluding increased exploitation (Hixson, 2014). The combination of an expanding human population and changes in dietary consumption (driven by increased affluence) means that more people are consuming animal protein, a significant percentage of which is produced by fish farming (aquaculture) (Cressey, 2009) The aquaculture industry has expanded year-on-year for the last two decades, with 2013 marking the first year in which the majority (51%) of all fish consumed by humans was produced by fish-farming (as opposed to wild capture) (Cressey, 2009; Tacon and Metian, 2009). Thus, although aquaculture has a vital and growing role in the efficient production of food for human consumption, there remains a quandary in the cultivation of marine and salmonid species, namely the requirement for n-3 LC-PUFAs (since such fish, like other vertebrates, cannot effectively synthesise these fatty acids from shorter precursors). Thus, the paradoxical situation exists in which farmed fish must have a dietary source of n-3 LC-PUFAs, primarily derived from other oceanic sources. Supplementation of aquafeed diets with vegetable oils, which lack n-3 LC-PUFAs, results in finished fish devoid of these health-beneficial fatty acids and consumer confusion (Bell et al., 2010).

For all of these reasons, a new sustainable *de novo* source of n-3 LC-PUFAs is desirable, to break the cycle of capture-extraction-feed. We and others have produced genetically engineered plants in which endogenous fatty acid biosynthesis has been augmented with the capacity to synthesise the otherwise non-native n-3 LC-PUFAs (Qi et al., 2004; Wu et al., 2005; Petrie et al., 2012; Ruiz-Lopez et al., 2014). This metabolic engineering demonstrated the feasibility of making eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) in the seed oils of transgenic plants, and in particular, *Camelina sativa*. *C. sativa* seed oil has a favourable endogenous fatty acid composition, being rich in the C18 precursor α-linolenic acid (18:3n-3; ALA), and more pertinently, has been shown to be a well-accepted component of aquafeed diets for a range of important fish species (Morais et al., 2012; Hixson et al., 2014). Previously, we and others had shown that *C. sativa* can accumulate up to 20% of seed oil as

* Corresponding author.
E-mail address: johnathan.napier@rothamsted.ac.uk (J.A. Napier).
[1] Present address: Instituto de la Grasa (CSIC), Av. Padre García Tejero 4, 41012 Seville, Spain.

http://dx.doi.org/10.1016/j.meteno.2015.04.002

non-native EPA and DHA, by introducing a suite of algal genes under seed-specific promoters (Ruiz-Lopez et al., 2014; Petrie et al., 2014). These transgenic plants showed no phenotypic difference to their wild type (WT) controls when grown under glasshouse conditions (Ruiz-Lopez et al., 2014) and oil from such plants was an effective replacement for fish oils in salmon feeding trials (Betancor et al., 2015) but for such a potentially important trait to be validated, it is necessary to determine the stability and impact of the altered seed lipid metabolism on plants grown in the field, under real-world conditions. With this in mind, we carried out a field trial on the performance of a genetically modified (GM) *C. sativa* line engineered to accumulate EPA and DHA.

2. Materials and methods

2.1. Plant material and growth conditions

C. sativa grown in the glasshouse (GM-GH) was sown on the 1st of May 2014 and grown in controlled conditions at 25 °C day/16 °C night, 50–60% humidity, and kept under a 16 h photoperiod (long day), with supplemental light provided when ambient levels fell below 400 μmol m^{-2} s^{-1}. Harvest usually occurred 95 days after sowing (Supplementary Table 3).

2.2. Generation of transgenic plants

Transgenic *C. sativa* lines were generated as previously described (Ruiz-Lopez et al., 2014). The designed vectors were transferred into *Agrobacterium tumefaciens* strain AGL1. *C. sativa* inflorescences were immersed in the *Agrobacterium* suspension for 30 s without applying any vacuum. Transgenic seeds expressing the DHA pathway were identified by visual screening for DsRed activity. Seeds harvested from transformed plants were illuminated using a green LED light. Fluorescent seeds were visualised using a red lens filter. In all cases, no phenotypic perturbation was observed as a result of modification of the seed oil composition.

2.3. Vector construction

The construct used for plant transformation, contained an optimal set of genes for EPA and DHA synthesis (Fig. 1b): a Δ6-desaturase gene from *Ostreococcus tauri*[17] (OtΔ6) (Domergue et al., 2005a), together with a Δ6 fatty acid elongase gene from *Physcomitrella patens* (PSE1), a Δ5-desaturase gene from *Thraustochytrium* sp. (TcΔ5) (Wu et al., 2005), a Δ12-desaturase gene from *Phytophthora sojae* (PsΔ12) to enhance the levels oflinoleate-CoA (as substrate for the OtΔ6 enzyme) and an ω3-desaturase from *Phytophthora infestans* (Pi-ω3) (Bauer et al., 2012) to increase the conversion of ARA to EPA, OtElo5, an *O. tauri* Δ5 fatty acid elongase gene (Meyer et al., 2004) and EhΔ4, a Δ4-desaturase gene from *Emiliania huxleyi* (Sayanova et al., 2011) both flanked by conlinin promoters and OCS terminators, were added to the p5_EPA construct (Ruiz-Lopez et al., 2013, 2014). The destination vector contained a DsRed marker for visual selection via seed coat-specific expression of DsRed. All genes were individually cloned under the control of seed-specific promoters, and then combined into a single T-DNA transformation vector as previously described (Ruiz-Lopez et al., 2013). All open reading frames for desaturases and elongases were re-synthesised (GenScript Corporation, NJ; www.genscript.com) and codon-optimized for expression in *C. sativa*.

2.4. Field trial

Field experiments were conducted at Rothamsted Research (Harpenden, Hertfordshire, U.K.; grid reference TL 120130; Supplementary Fig. 1). The field trial site consisted of two 5×10 m^2 subplots of GM *C. sativa* separated by a 2×10 m^2 strip of WT. The two sub-plots were denoted GM East (GM-E) and GM West (GM-W) (Supplementary Fig. 1). The experimental plot was surrounded by a 7 m-wide WT *C. sativa* strip which served as a "buffer" to mitigate the dispersal of GM pollen. *C. sativa* is considered to be a self-pollinating species, with very low rates of outcrossing or cross-pollination (Walsh et al., 2012). The trial plot was sown on the 15th of May 2014, with T6 GM *C. sativa* seeds sown to create a standing plant density of 290/m^2, and 300/m^2 for the WT.

Seedlings were irrigated as necessary following emergence and an insect-proof net was erected around the central experimental plot prior to flowering in order to prevent insect-mediated pollen dispersal; (net removed following the cessation of flowering). Plants were allowed to set seed and monitored for seed maturation. Both WT and GM *C sativa* were harvested on the 5th of September 2014 (113 day growing season), when seeds were fully mature and following three consecutive dry days. In order to complete the harvest, the pollen barrier was removed. To prevent any potential harvest crossover, a 0.25 m-wide strip either side of the border between the GM *C. sativa* and the WT separation was marked and labelled border. Plants within these borders were harvested, but not used for further analysis. The harvested plants were transported to the GM facility glasshouse to further dry before threshing using a Haldrup LT-20 laboratory thresher. Cleaned seeds were tested for DsRed activity, stored, double bagged in paper bags inside locking plastic boxes prior to analysis.

2.5. Fatty acid analysis

Total fatty acids in seed batches were extracted and methylated (Garcés and Mancha, 1993). Methyl ester derivatives of total fatty acids extracted were analysed by Gas Chromatography-FID (flame ionisation detection) and the results were confirmed by GC–MS (Ruiz-Lopez et al., 2013). Values presented are from the analysis of single seeds.

2.6. Total lipid extraction

Three replicates each from GM-E, GM-W, and WT; consisting of one gram of seeds in 20 mL of chloroform:methanol (2:1) were homogenised using a mortar and pestle. The homogenate was then briefly centrifuged and the liquid phase removed to two separate glass tubes, each made up to a volume of 20 mL chloroform:methanol (2/1). The solvent phase was then washed with the addition of 4 mL of water. After vortexing for 30 s, the mixture was centrifuged at low speed (2000 rpm) to separate the two phases; the lower phase was removed, re-combined and dried under a stream of nitrogen.

2.7. Carbon and nitrogen determination

Total carbon and nitrogen were determined by combustion using a Combustion Analyser (LECO TruMac, Leco Corp, St. Paul, MN). This was performed by the in-house analytical unit at Rothamsted Research. Data is present as a percentage of 100% dry matter content.

2.8. Thousand grain weight

Three replicates of a thousand seeds from *C. sativa* GM-E, GM-W and WT were counted and the weight determined by mass balance.