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Reconstitution of EPA and DHA biosynthesis in *Arabidopsis*: Iterative metabolic engineering for the synthesis of *n*–3 LC-PUFAs in transgenic plants

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ABSTRACT

An iterative approach to optimising the accumulation of non-native long chain polyunsaturated fatty acids in transgenic plants was undertaken in *Arabidopsis thaliana*. The contribution of a number of different transgene enzyme activities was systematically determined, as was the contribution of endogenous fatty acid metabolism. Successive iterations were informed by lipidomic analysis of neutral, polar and acyl-CoA pools. This approach allowed for a four-fold improvement on levels previously reported for the accumulation of eicosapentaenoic acid in *Arabidopsis* seeds and also facilitated the successful engineering of the high value polyunsaturated fatty acid docosahexaenoic acid to 10-fold higher levels. Our studies identify the minimal gene set required to direct the efficient synthesis of these fatty acids in transgenic seed oil.

1. Introduction

Alternative sources of *n*–3 (also called omega-3) long chain polyunsaturated fatty acids (LC-PUFAs) have received considerable interest in recent years, based on the reduced availability of primary stocks (fish oils) and the clear evidence of health benefits from a diet that contains these fatty acids (Cressey, 2009; Saravanan et al., 2010). One approach to the sustainable supply of LC-PUFAs is the metabolic engineering of transgenic plants with the capacity to synthesise *n*–3 LC-PUFAs, since the global requirements for these fatty acids far exceeds what could be produced by algal or (recombinant) microbial fermentation (Domergue et al., 2005a). The "scalability" of agriculture-based production systems, combined with low input costs, make the development of a transgenic plant platform for the terrestrial synthesis of *n*–3 LC-PUFAs very appealing, although realising this goal has proved technically challenging to date (Sayanova and Napier, 2004; Robert, 2006; Dyer et al., 2008). This is due, in part, to the biochemical nuances of the *n*–3 LC-PUFA biosynthetic pathway as exemplified by the "substrate-dichotomy" bottleneck which exists between the phospholipid-dependent desaturases and the acyl-CoA-dependent elongases (Abbadi et al., 2004; Napier et al., 2004). Moreover, since higher plants have no endogenous capacity to synthesise these fatty acids, reconstruction (introduction) of the biosynthetic pathway demands the addition of multiple genes (for both primary synthesis and to direct the flux of substrate and biosynthetic intermediates towards final compartmentalisation in triacylglycerol) requiring co-ordinated tissue-specific expression in the developing seeds of a transgenic host (Cheng et al., 2010).

It is for this reason that the reconstruction of the *n*–3 LC-PUFA pathway represents the leading edge of metabolic engineering in transgenic plants—currently up to ten different genes have been stably introduced to different host species (Wu et al., 2005; Ruiz-Lopez et al., 2012). However, the accumulation of the target fatty acids such as eicosapentaenoic acid (20:5*n*–3; abbreviated to EPA) and especially docosahexaenoic acid (22:6*n*–3; abbreviated to DHA) have often proved disappointing, despite many attempts to optimise their synthesis (Venegas-Calerón et al., 2010). On one level, this is perhaps unsurprising, since the genes assembled to direct the synthesis of EPA and DHA (represented schematically by the pathway in Fig. 1C) are derived from a diverse set of *n*–3 LC-PUFA accumulating organisms, predominantly marine microbes such as diatoms and microalgae which form the base of the aquatic food-web. Early attempts to engineer the synthesis of EPA initially resulted in very low levels (<1% of total) of this fatty acid in seeds of transgenic linseed (Abbadi et al., 2004), but subsequent iterations increased the levels by at least 10-fold (though also increasing the undesired accumulation of biosynthetic intermediates)

Abbreviations: ALA, α-Linolenic acid; ARA, Arachidonic acid; DAG, Diacylglycerol; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; GLA, γ-Linolenic acid; LA, Linoleic acid; LC-PUFA, Long chain polyunsaturated fatty acid; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; PS, Phosphatidylserine; SDA, Stearidonic acid; TAG, Triacylglycerol

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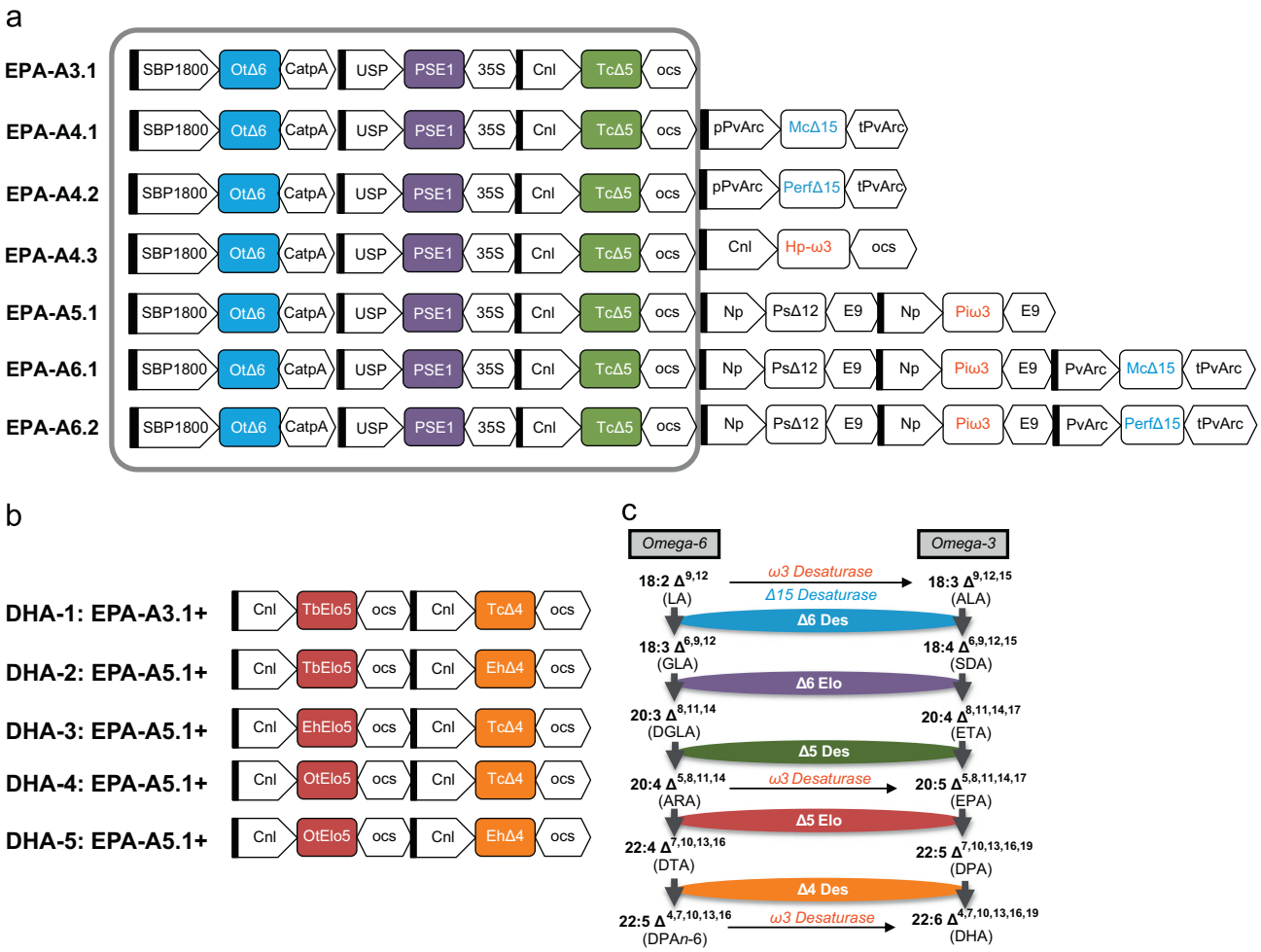

Fig. 1. Representation of the constructs used for Arabidopsis transformation. (A) EPA contructs; (B) DHA constructs. Cnl=conlinin 1promoter for the gene encoding the flax 2S storage protein conlinin; USP=promoter region of the unknown seed protein of *V. faba*; SBP1800=the sucrose binding protein 1800 promoter; NP=napin; OtΔ6, a Δ6-desaturase from *O. taurii*; TcΔ5- a Δ5-desaturase from *Thraustochytrium* sp.; Pi ω3 and Hpω3—ω-3 desaturases from *P. infestans and H. parasitica*, respectively; PsΔ12- a Δ12-desaturases from *P. sojae*; PerfΔ15 and McΔ15–Δ15-desaturases from *P. fruticosa* and *M. chthonoplastes*, respectively; EhΔ4and Tc4- Δ4-desaturases from *E. huxleyi and Thraustochytrium sp.*; PSE1, a Δ6-elongase from *P. patens*, OtElo5- Δ5-elongase from *O. tauri* TbElo5, aΔ5-elongase from *T. brucei* and EhElo5, a bifunctional Δ5/Δ6-elongase from *E. huxleyi*; OCS, 35S, E9, PvARC and CatpA—represent terminators. The biosynthetic pathway for LC-PUFAs in shown schematically in C, with the different enzyme activies shown in different colours (also reflected in (A) and (B)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Wu et al., 2005; Cheng et al., 2010). In both cases, these increases were achieved through the presence of additional enzyme activities, beyond the three primary biosynthetic activities used initially (Abbadi et al., 2004)—for recent consideration of these and other relevant studies, please consult these reviews (Ruiz-Lopez et al., 2012; Venegas-Calerón et al., 2010). Certainly, the predictive manipulation of plant seed oil compositions remains still in its infancy.

One significant advance in elevating the accumulation of LC-PUFAs in transgenic plants is the use of acyl-CoA-dependent desaturases, which bypass the above-mentioned substrate-dichotomy bottleneck. Elegant studies from Heinz and colleagues demonstrated the critical difference between phospholipid-dependent- and acyl-CoA-dependent desaturases in the heterologous reconstitution of LC-PUFA biosynthesis in yeast (Domergue et al., 2003) and these same researchers subsequently identified an acyl-CoA-dependent Δ6-desaturase from the picoalga *Ostreococcus tauri* which showed high activity in yeast towards LA-CoA and ALA-CoA (Domergue et al., 2005b). We and others have hypothesised that the use of acyl-CoA-dependent desaturases should result in increased synthesis and accumulation of EPA (Kajikawa et al., 2004; Graham et al., 2007; Petrie et al., 2010), although until recently, evidence for this has been lacking (Robert et al., 2005, Hoffmann et al., 2008). However, we have now demonstrated the benefits of using the *O. tauri* acyl-CoA dependent Δ6-desaturase, not only to elevate the accumulation of EPA, but also to avoid the accumulation of unwanted C18 biosynthetic intermediates (such as GLA and SDA), which are often associated with the expression of phospholipid-dependent Δ6-desaturases (Ruiz-Lopez et al., 2012; Sayanova et al., 2011; cf. Abbadi et al., 2004).

In this study, we build on those earlier observations to further optimise the accumulation of EPA, exclusively via the acyl-CoA-dependent desaturase pathway. Specifically, we wished to determine the contribution of both transgene-derived and endogenous activities on the accumulation of this target fatty acid. In addition, having generated a significant level of EPA in transgenic seeds, we systematically evaluated a number of different gene combinations to direct the synthesis of DHA. Using *Arabidopsis* as a well-established model system for the metabolic engineering of the *n*–3 LC-PUFA pathway (Qi et al., 2004; Robert et al., 2005; Hoffmann et al., 2008; Sayanova et al., 2011), we have successfully iterated the accumulation of DHA to 10-fold that of the levels previously reported in this species.