Available online at www.sciencedirect.com
SCIENCE DIRECT
Plant Science 170 (2006) 170–177
PLANT SCIENCE
www.elsevier.com/locate/plantsci

*FAD*2-silencing has pleiotropic effect on polar lipids of leaves and varied effect in different organs of transgenic tobacco

Mingfeng Yang, Guiling Zheng, Fayun Zhang, Yinong Xu *

Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Nanxincun 20, Xiangshan, Beijing 100093, China

Received 20 June 2005; received in revised form 24 August 2005; accepted 30 August 2005
Available online 19 September 2005

Abstract

A microsomal ω-6 fatty acid desaturase gene (*FAD*2) of tobacco (*Nicotiana tabaccum*) was cloned by PCR-based method and a partial coding sequence of the putative *FAD*2 gene was used to create intron-containing construct expressing hairpin RNA for silencing endogenous *FAD*2 gene. In addition to a marked increase of oleic acid in phosphatidylcholine and phosphatidylethanolamine, the main lipid components of the extrachloroplastic membranes of plant cells, the silencing of *FAD*2 resulted in pleiotropic effect on polar lipids of leaves, i.e., a significant increase of oleic acid levels in sulfoquinovosyldiacylglycerol, phosphatidylglycerol, digalactosyldiacylglycerol and monogalactosyldiacylglycerol, located predominantly in the chloroplast, and a significant reduction of palmitic acid levels in some individual polar lipids. The significant increase of oleic acid only found in lipids of leaves and seeds of transgenic lines proved the diversity of the silencing effect in different organs. The possible mechanisms involved in the control of lipid unsaturation level in different organs of transgenic tobacco were discussed.

Keywords: Gene silencing; *FAD*2; Lipid; Tobacco; Organ

1. Introduction

Plant oils represent a vast renewable resource of highly reduced carbon and those with a high content of oleic acid are of interest for nutritional and industrial purposes [1]. In fact, oils rich in monounsaturated fatty acids and poor in palmitic acids are more suitable and beneficial for improved oil stability, flavor, and nutrition [1]. A thorough understanding of desaturases involved in lipid synthesis is necessary to cultivate crops with oils containing suitable fatty acids. However, isolation and biochemical characterization of most fatty acid desaturases has proven difficult due to their membrane-bound nature that a considerable amount of knowledge about plant desaturases comes from the characterization of a series of *Arabidopsis* mutants with defects in fatty acid desaturation [2].

There are two distinct pathways (''prokaryotic'' pathway, ''eukaryotic'' pathway) in plant cells for the biosynthesis of glycerolipids and the associated production of polyunsaturated fatty acids [2,3]. Two pathways coordinate in glycerolipid synthesis in plants and the balance of fluxes through these pathways may be altered to compensate for the effects of mutations that block steps in one of the pathways [2,4]. At least for the endoplasmic reticulum (ER) and the plastid, lipid traffic between the membranes is bi-directional and most of the mutations affect the composition of both chloroplast and extrachloroplast membranes even though the enzymes are located in one compartment or the other [4]. Genes of lipid synthesis have been cloned in many plant species among which the ω-6 desaturase gene is of particular interest for it is the enzyme that places the second double bond in the fatty acid and catalyzes the first step in polyunsaturated fatty acid biosynthesis. All higher plants contain one or more microsomal ω-6 desaturase(s) (also named microsomal Δ12 desaturase, oleoyl-PC desaturase or *FAD*2) that insert a double bond between carbon 12 and 13 of monounsaturated oleic acid to generate polyunsaturated linoleic acid and control the most of polyunsaturated lipid synthesis in nonphotosynthetic plant tissues [5,6]. The *FAD*2 gene appears to be also important in the chilling sensitivity of plants, as polyunsaturated membrane phospholipids are important in maintaining cellular function

* Corresponding author. Tel.: +86 10 62836504; fax: +86 10 62590833.
E-mail address: yinongxu@ibcas.ac.cn (Y. Xu).

0168-9452/$ – see front matter
doi:10.1016/j.plantsci.2005.08.022

and plant viability at low temperatures [6–8]. Many details about *FAD*2 remain elusive, since *FAD*2 is an ER membrane-bound desaturase [9,10], such as the relations between prokaryotic and eukaryotic pathways, the regulation of *FAD*2 activity in a wide range of environmental conditions, the development- and tissue-specific *FAD*2 modulation etc. It has been demonstrated that hpRNA-mediated gene silencing, a kind of post-transcriptional gene silencing (PTGS), results in high efficiency and efficacy of gene silencing in plants [11–13]. A clearer understanding of the expression patterns of the *FAD*2 gene and associated fatty acid composition in *FAD*2-silenced plant cells is crucial in understanding the function of *FAD*2 gene, and in applying such a PTGS technology to manipulate the polyunsaturated fatty acid composition of plant membranes, predictably to improve the seed oil value, vigor and viability of crop plants.

In this report, we used a partial coding sequence of tobacco (*Nicotiana tabaccum*) *FAD*2 gene to make an hpRNA-producing construct to specifically silence endogenous *FAD*2 gene. We demonstrated that the silencing of *FAD*2 gene had pleiotropic effect on fatty acid composition of lipids in transgenic plants. To our knowledge, this is the first report on the tissue-specific effect of *FAD*2 gene silencing in transgenic plant.

2. Materials and methods

2.1. FAD2 cloning

A list of *FAD*2 sequences identified from different plant species was aligned to design a pair of degenerated oligonucleotide primers. The sense primer was 5′-CGTCGCCA (CT) CA (CT) TC (CT) AACAC-3′, and the antisense primer was 5′-CCCCTAA (AG) CCA (AG) TCCCA (CT) TC-3′. A 452 bp sequence was amplified by PCR from tobacco (Wisconsin 38) cDNA prepared from leaves. The procedure of PCR is 5 min at 94 °C, 35 cycles for 30 s at 94 °C, 45 s at 54 °C, 1 min at 72 °C, and extension 10 min at 72 °C. Both 3′-RACE and 5′-RACE were performed using RACE kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.2. Gene-silencing constructs

The 452 bp *FAD*2 fragment was inserted into pKANNIBAL vector in inverted repeat configuration essentially as described [11]. The correct orientation of silencing construct was confirmed by sequencing. A fragment containing the silencing construct was subcloned as *Not*I fragments into binary vector pART27 [14] as shown in Fig. 2, and then introduced into *Agrobacterium tumefaciens* strain LBA4404 by tri-parental mating for transformation of tobacco by the leaf-disc method [15]. Seedlings that survived in MS medium containing kanamycin (150 mg/L) were grown in growth chamber under 16 h 80 μmol m^{-2} s^{-1} light/8 h dark conditions at 24 °C.

2.3. Southern blot analysis

Tobacco genomic DNA was isolated from T0 generation and wild-type seedlings according to Porebski et al. [16]. Approximately 20 μg of DNA was digested with *Eco*RI and separated on a 0.8% (w/v) agarose gel by electrophoresis and transferred to a Hybond N^+ nylon membrane according to Sambrook et al. [17]. Southern blot analyses were carried out by hybridizing with a [^{32}P]dCTP-labeled *FAD*2 452 bp fragment got by PCR using the purified *FAD*2 cDNA 452 bp fragment as template. Prehybridization and hybridization was carried out using hybridization cocktails I and III (Sangon, Shanghai, CN) following the manufacturer's instructions.

2.4. RT-PCR

RNA was prepared from tissues of tobacco using the Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. After digestion with RNase-free DNase I (Promega, Madison, WI, USA), the reverse transcription was carried out using reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The PCR procedures were same to that used in *FAD*2 cDNA 452 bp fragment cloning, except that the repeat cycles were 25 instead of 35. Tobacco actin Tac9 was used as a parallel control.

2.5. Northern blot analysis

Fifteen micrograms of total RNA was separated on a denaturing formaldehyde gel and transferred to Hybond N^+ nylon membrane (Pharmacia) according to Sambrook et al. [17]. The probes used for *FAD*2 mRNA were the same to that used in Southern blot analysis. To confirm that lanes were equally loaded, nylon membrane was stripped by washing in 10 mM Tris–Cl (pH 7.4), 0.2% (w/v) SDS at 75 °C for 1 h, then re-hybridized with an α-^{32}P dCTP-labeled Tac 9 probe. The hybridization was essentially the same as described in Southern blot analysis section.

2.6. Fatty acid analysis

Lipids were extracted from different tissues of tobacco according to the method of Bligh and Dyer [18]. The individual lipids separation and the fatty acid analyses were carried out according to the method of Xu et al. [19]. Relative fatty acid compositions were calculated as the percentage that each fatty acid represented of the total measured fatty acids. An additional indirect method of assessing the cumulative effects of *FAD*2 activity during leaf fatty acid synthesis is through an oleic desaturation proportion (ODP) parameter as described [13].

2.7. SiRNA analysis

Small interference RNA (*SiRNA*) purification, separation and hybridization were performed essentially as described [20]. Twenty- and 23-mer oligonucleotides from *FAD*2 452 bp fragment were synthesized and used as molecular size markers and positive controls in hybridizations performed with DNA probe. Probes were the same as used in Southern blot. Prehybridization and hybridization were at 38 °C in the same solution to that used in southern blot.