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METABOLIC ENGINEERING OF PLANT BIOMASS PRODUCTION OF BIOFUEL

Uncertainties of fossil oil supplies and growing demand for energy provoked a surge of interest in renewable energy resources and greatly stimulated intense academic and applied research on biofuel, plant-derived ethanol and oil [1, 2]. Ethanol has been traditionally produced by fermentation plant produced sugars (sugarcane, sugar beet) and starch (corn, potato). A lot of efforts and resources are currently directed to develop second generation technologies for ethanol production from the lignocellulosic materials which compose the majority of plant biomass [3]. Another challenge lies in the efficient production of biodiesel fuels based on plant oils [4].

Biodiesel production is usually associated with seeds of selected plant species, such as sunflower, soybean, rapeseed or jatropha, which accumulate oil in the form of triacylglycerols (TAGs) as storage reserves. While the oil content can reach 40–50% of seed dry weight, the yields of oil-rich seeds are rather limited. Despite accumulation in seeds, primary lipid synthesis occurs in green photosynthetic tissues, leaves and stems [5], which constitute a significantly larger portion of plant biomass than seeds. A number of recent studies indicated that gene manipulations enable the relocation or elevation of oil storage in alternative plant organs such as roots, stems or leaves making the green biomass a plausible system for manufacturing biodiesel [6, 7]. It is well documented that enhanced expression of some enzymes involved in lipid metabolism can lead to increased oil accumulation in different plant organs [5, 6]. The second set of published data relates to channeling oil accumulation to leaf tissues by leaf-specific expression of genes coding for transcription factors that regulate seed development and maturation. When constitutively expressed in leaves, some of transcription factors, e.g. LEC1, LEC2 or WRI1, induced the formation of seed-like structures and stimulated oil synthesis in the vegetative green tissues [8].

In an attempt to investigate the potential of plant biomass as a new source of biodiesel oil, we chose tobacco, a plant that has been widely used in genetic engineering experiments. Tobacco, a well-established industrial crop used primarily for non-food consumption, is widely cultivated in more than 100 countries worldwide, yielding up to 170 tons per hectare of green tissues when grown for biomass production instead of smocking [9]. Analysis of leaves from 56 tobacco species revealed that the average content of extractable fatty acids (FA), which is the major component of biodiesel fuel, is about 3% per dry weight [10]. While

the representation of oil in green biomass is much lower than in the oil-crop seeds, the sheer volume of biomass and the possibilities of metabolic engineering makes tobacco an attractive and promising "energy plant" platform. It could also serve as a model for the utilization of developed engineering strategies in other high-biomass plants for biofuel production.

Here, we present our data on enhanced accumulation of fatty acids in tobacco biomass following the constitutive expression of an Arabidopsis gene DGAT, coding for diacylglycerol acyltransferase, a key enzyme in TAG biosynthesis, and inducible expression of transcription factor LEC2, a master regulator of seed maturation and oil storage.

Additionally, as a feedstock for cellulosic ethanol fermentation, tobacco has two main advantages over existing feedstocks: 1) it contains relatively high amount of easily fermentable sugars and, 2) the content of lignin, which significantly hampers cellulose degradation and therefore contributes to high processing expenses, is much lower in tobacco compared to other feedstocks considered for ethanol production such as switch grass or corn stover.

Materials and Methods

Generation of expression vectors and plant transformation. Full length *Arabidopsis thaliana* cDNAs coding for diacylglycerol acyltransferase (DGAT, Ac.# BT008883), and Leafy Cotyledons-2 transcription factor (LEC2 Ac.# DQ446296) were used for construction plant expression cassettes. DGAT under the control of RbcS promoter and LEC2 under the control of ethanol-inducible AlcA promoter, were put into plasmid pBIN-Plus and the resulting vectors were used for *Agrobacterium*-mediated transformation of tobacco.

DNA and protein analysis. The presence of DGAT and LEC2 expression cassettes in transformed tobacco lines was confirmed by PCR analysis. The expression of DGAT and of LEC2 polypeptides has been confirmed by protein Western blot analysis.

Stimulation of LEC2 expression. The expression of LEC2 gene was induced by stimulating AlcR-promoter in 6-8 week old tobacco with 0.1% or 1% water solution of acetaldehyde. Induction was repeated twice with an interval of 24h and samples were taken for lipid analysis and mRNA expression analysis at 24h, 48h and 120h after the initial treatment.

Lipid extraction and analysis. For Liquid Chromatography (LC-MS) analysis total lipids were isolated by hexane extraction from lyophilized plant tissues. Extracted lipids were separated and analyzed using a Dionex UltiMate 3000 LC system partnered with an Applied Biosystems (Foster City, CA) 4000 Q Trap mass spectrometer with an electrospray ionization source. TAG amounts, adjusted for the internal standard, were calculated from a standard curve of triolein. For Gas Chromatography (GC) analysis of fatty acids, total lipids were extracted into chloroform using the methanol-chloroform procedure of Bligh and Dyer [10]. Extracted lipids were methylated and the fatty acids esters were analyzed by GC using Shimatzu Model GC-8APF gas chromatographer. To identify individual fatty acids, retention times of sample peaks were compared to peaks of

certified methylated fatty acid standards of rape seed oil. Fatty acid content was quantified using heptadecanoic acid as an internal standard added to samples prior to extraction.

Results and Discussion

Overexpression of DGAT results in increased TAG synthesis and accumulation of fatty acids. Tobacco, *Nicotiana tabacum*, cv. Wisconsin and a variety with elevated sugar content, NC-55, were transformed with *Arabidopsis* gene DGAT under the control of strong RbcS promoter with the aim of enhancing the synthesis of triacylglycerols (TAGs) in tobacco leaves. Prior to chromatographic lipid analysis, selected kanamycin resistant tobacco plantlets were grown for 3 months in a greenhouse and visually pre-selected by microscopic analysis of Sudan IV stained oil bodies in leaf samples. The plants with an increased number of oil bodies were subjected to analysis of TAGs by liquid chromatography mass spectroscopy (LC-MS) and/or to the analysis of total extracted fatty acid esters by Gas-chromatography (GC).

LC-MS analysis revealed up to a 20-fold increase in TAG accumulation in the tobacco plants overexpressing DGAT as compared to wild type. This increase in TAGs, which represent only 15-20% of total fatty acids in leaves, translated into an overall 2-fold increase of extractable fatty acids, up to 5.6% of tobacco dry biomass as determined by quantitative GC analysis, compared to 2.8% fatty acid content in untransformed tobacco, cv. Wisconsin. The reasoning for using high-sugar tobacco variety NC-55 in this study was the assumption that it could contain a higher background lipid levels because sugars are the primary precursors in fatty acid biosynthesis [5]. Indeed, the wild type cv. NC-55 demonstrated almost 30% higher total FAs compared to cv. Wisconsin, totaling 3.7% of dry weight. Overexpression of Arabidopsis DGAT enzyme in NC-55 tobacco led to persistently high accumulation of fatty acids above 5% of dry weight, with the highest FA level of 6%.

Enhanced TAG synthesis causes change in oil fatty acid composition. The observed increase in total FA accumulation in tobacco leaves was accompanied by a drastic shift in the fatty acid composition. Three fatty acids, linolenate, linoleate and palmitate, are usually predominant in the green parts of plants including tobacco [9]. In transgenic tobacco lines of cv. Wisconsin, the proportion of linolenate was reduced to 30-40% as compared to 61% in wild-type plants, while oleate increased from 1.5% to 20-25% in total extracted fatty acids. Changes in the TAG fraction of tobacco oil in DGAT-overexpressing tobacco were even more profound, with a decrease in linolenate from 60% to about 20%, and accumulation of 50-60% of oleate. Although the exact fatty acid profiles differ among different transgenic lines due to variations in linolenate (18:3), linoleate (18:2), palmitate (16:0) and oleate (18:1) proportions, the trend toward an increased proportion of more saturated oleate and decrease of unsaturated linoleate was obvious. In the context of developing tobacco biomass oil into a diesel fuel, such a shift is definitely desirable for making biodiesel with better fuel qualities.

Stimulation of LEC2 expression leads to both enhanced oil accumulation and a shift in oil fatty acid pattern. In order to confirm the concept of regulated accumulation of oil in tobacco biomass, we generated and tested tobacco plants expressing the LEC2 gene under the control of ethanol-inducible AlcA promoter of *Aspergillus nidulans* [12]. We opted for an inducible promoter in this case to avoid potential problems that constitutive expression of *LEC2* could cause by interfering with the plant growth/development program [13]. To stimulate the expression of LEC2, roots of three month old plants were treated by soil drenching with 0.1% and 1% acetaldehyde, which is a product of ethanol metabolism and a physiological inductor of AlcA. While the level of oil accumulation varies between individual plants 0.1% acetaldehyde treatment increased extracted fatty acids up to 5.5%, and 1% acetaldehyde treatment resulted in a more than double increase of total extracted FA, from 2.9% to 6.8% of dry weight in selected lines. Similar to tobacco plants over-expressing DGAT, we observed a shift in fatty acid composition following the stimulated expression of LEC2.

Additional means of increasing oil accumulation include using other strong enhancers/promoters in combination with DGAT or other key enzymes influencing oil biosynthesis such as acetyl-CoA carboxylase or thioesterase [5, 6], gene amplification technology [14], or inhibition of the pathways of lipid breakdown [15].

Conclusion

By generating both oil and ethanol, tobacco has the potential to produce substantial amount of renewable biofuel to be considered as one of the promising "energy crop" platform; the obtained data could be also used to develop efficient strategies for metabolic engineering of other plants with enhanced accumulation of fatty acids to be used as biodiesel.

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Summary

Tobacco plants engineered to constitutively overexpress DGAT, a key enzyme in plant lipid biosynthesis, or to inducibly express a transcription factor LEC2 that govern seed maturation, have about two-fold increase in total fatty acid content in their green biomass. The increased fatty acid content was accompanied by changes in their composition that favor development of tobacco as a renewable source of biofuel.

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О РЕГИОНАЛЬНОМ РЕГИСТРЕ МАРКЁРОВ САХАРНОГО ДИАБЕТА 2 ТИПА

В настоящее время в Украине более миллиона людей больны сахарным диабетом (СД). Примерно 86% случаев заболевания СД приходится на 2 тип. Считается, что реальное количество больных в 2–3 раза больше за счёт не выявленных и скрытых форм заболевания. Эти люди с высокой генетической предрасположенностью к заболеванию должны стать объектом целенаправленной профилактики СД. Выявление таких людей проводится по специальным признакам – маркёрам наследственной предрасположенности. К настоящему времени накоплена обширная информация о ДНК-маркёрах и генах-кандидатах СД [1–5]. Это создаёт хорошие возможности для выявления группы повышенного риска, однако практическое использование результатов молекулярно-генетических исследований ещё отстаёт от научных разработок.

Эффективность использования маркёров зависит, как известно, от разницы их частот в сравниваемых группах, поэтому диагностическая ценность каждого маркёра имеет локально-популяционное значение. СД 2 типа — заболевание этноспецифичное. У современных народов, предки которых в течение тысячелетий занимались земледелием, заболеваемость сахарным диабетом значительно ниже, чем у тех, чьи предшественники в недавнем прошлом вели образ жизни охотников, собирателей или скотоводов. В европейских странах сахарным диабетом 2 типа больны 3–6% населения, в Полинезии и Микронезии — 25–30%. Среди жителей США европейского происхождения больные СД 2 типа составляют 5%, среди афроамериканцев — 10%, выходцев из Мексики — 24%. У индейцев Пима СД 2 типа поражены 35%, а в возрасте 55–64 лет — 70% населения. В ходе предшествующей генно-культурной коэволюции у народов-земледельцев сформировался генофонд, адаптированный к пище с высоким гликемическим индексом. Генофонд народов, которые вследствие глобализации приобщились к западной цивилизации, но не прошли адаптации в процессе биологической эволюции, оказался не приспособленным к этим условиям. Эпидемию СД, которая поразила эти народы, генетики рассматривают как реакцию определённых генотипов на новые факторы среды. Понимание этого приводит к мысли о необходимости разрабатывать систему мер по профилактике СД 2 типа в