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PATHWAYS FOR SYNTHESIS, AND POSSIBILITIES FOR GENETIC MODIFICATION OF SUGAR ESTER ACYL GROUPS PRODUCED BY TRICHOMES OF SOLANACEOUS SPECIES

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Summary. Glandular trichomes of many plant species synthesize and secrete a variety of phytochemicals that play an important protective role against pathogens and pests. Acyl sugars, in particular, are powerful natural insecticides. We demonstrated the existence of the α-ketoacid elongation (αKAE) pathway, an alternative to the fatty acid synthase (FAS) pathway, for the extension of carbon chains of acyl groups in sugar esters (SE). The α-KAE pathway utilizes one carbon elongation per cycle in contrast to two carbons per cycle in FAS, and it was found to function in a variety of tobaccos and petunia. FAS-mediated elongation was confirmed to participate in the synthesis of SE acyl groups in *Solanum pennellii* and *Datura metel*, and also in the synthesis of odd-carbon-length acids of epicuticular waxes from in tobacco and *Brassica oleraceae*. We attempted to knock down (RNAi-mediated) the central for the α-KAE pathway condensing enzyme isopropyl malate synthase (IPMS) using two IPMS-related genes from *S. pennellii*. The RNAi constructs were introduced via *Agrobacterium*-mediated transformation of leaf discs of *N. tabacum* T.I. 1068, and the resulting plants displayed significant changes in 2-methylbutyric and 3-methylvaleric groups produced by glandular trichomes.

Key words: αKAE, IPMS, Solanaceae, sugar esters, trichomes.

Abbreviations: αKAE – alpha keto acid elongation, EWFA – epi-cuticular wax derived long-chain fatty acids, FAS – fatty acid synthase, IPMS – isopropyl malate synthase, RNAi – RNA interference, SE – sugar esters.

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INTRODUCTION

Plants posses epidermal protrusions on their surfaces, called trichomes. They differ in shape, size, ocurrence of glands and give certain odor and appearance to the plants. Glandular trichomes synthesize various kinds of specialized secondary metabolites, including terpenoids, flavonoids, phenylpropanoid derivatives and SE (for details see Wagner, 1991; Schilmiller et al., 2008). Many trichome-secreted biochemicals are of commercial importance as natural pesticides, flavorants, adhesives, emulsifiers, fragrances, food additives (Wagner et al. 2004). Solanaceae species, including tobaccos, tomatos, petunias and Datura synthesize a variety of diterpenes and sugar esters, and as recently discovered – anti-fungal proteins (Shepherd et al., 2005). Noteworthy is the amount of SE, deposited on the leaf surface, reaching in some cases 2-10% of leaf dry weight (Kroumova et al., 1994). Because of the commercial value, abundance, and accessibility of the compounds, they are candidates for plant metabolic engineering with a potential to convert Solanaceous species into factories for producing natural products, and to improve their natural resistance to insects. This could be possible only after the pathways for synthesis of these metabolites are clarified and the enzymes involved are characterized. Here we review of the progress achieved in delineating the biosynthetic pathways leading to SE. Also, we discuss some attempts (by us and others) to change SE acyl group content using a reverse genetics approach to knock down isopropyl malate synthase (this paper) or 2-oxo isovaleryl dehydrogenase (Scolombe et al, 2008).

Review of the biosynthetic pathways Stable isotope-labeling studies

Sugar esters are common trichome products in the Solanaceous family, and their abundance can vary from 23% of trichome exudate weight in *N. tabacum* T.I. 1068 to 100% in *N. obtusifolia* (unpublished). Chemically, they consist of glucose or sucrose esterified with a variety of short and medium chain, strait or branched (iso and anteiso) acids/fatty acids from acetate (C2) to 8-MeNonanoate (C10) (in *S. pennellii*) (Arrendale et al, 1990; Cutler et al., 1992). The precursor role of branched-chain amino acids in the biosynthesis of iso and anteiso fatty acids had been elucidated in bacteria and animal tissues in the early 1990s (Mori and Kaneda, 1982; Oku and Kaneda, 1988). Using radiolabeling studies, Kandra et al. (1990) and Kandra and Wagner (1990) showed that some branched short-chain, as well as strait-chain tobacco SE acyl groups are derived from amino acid precursors and suggested that they are synthesized via reactions common to Leu synthesis/ degradation. Leucine (Leu) is synthesized from the deaminated Valine (Val) derivative 2-oxo-3 Methyl Butyrate via 3 reactions, leading to its elongation by one carbon, forming 2-oxo-4 Methyl Valerate. The 3-step reaction is called "α-keto elongation". The names of the enzymes in order of operation are: 2-isopropyl malate synthase (EC 4.1.3.12), isopropyl malate dehydratase (EC4.2.1.33), isopropyl malate dehydrogenase (EC 1.1.1.85) (and 2-oxoisovalerate dehydrogenase (EC 1.2.1.25)). The α-keto elongation reaction is incorporated in many anabolic biochemical pathways, including bio-synthesis of coenzyme B (Howell et al.,

1998), Leucine (Strassman and Ceci, 1963), Lysine via a-aminoadipate pathway in fungi (Nishida and Nishiyama, 2000), glucosinolates in Brassicaceae species (Graser et al., 2000), N-butyl and N-amyl alcohols in yeasts (Stoddard, 1980), the TCA cycle (Melendez-Hevia et al. 1996), and certain acyl groups of sugar esters of Solanaceous species (Kroumova et al., 1994, Kroumova and Wagner, 2003). Most of the above pathways utilize a single cycle of α-keto elongation, while later we discovered a repeating cycling mechanism leading to formation of SE acyl groups, elongated by one carbon atom. The cycling was also confirmed for synthesis of aliphatic chain of glucosinolates (Graser et al., 2000). Keeping in mind "the broad specificity of the reactions with the alkyl portion of α-keto acid substrates", the following more trivial names were suggested: alkyl malate synthase, alkyl malate dehydratase and alkyl malate dehydrogenase (Kandra and Wagner, 1990). In the classical Val and Leu pathways the two α-keto acids are precursor for amino acids, or undergo oxidative decarboxylation to form acyl-CoAs en route to their degradation. The enzyme complex, generally called alkyl, 2-keto (isovaleryl) dehydrogenase, involved in this reaction is important in removing the ketoacids from the cycling en route to formation of sugar esters (Scolombe et al., 2008). Acyl groups of sugar esters in Solanaceae family (Severson et al., 1985) present iso-branched (coming from Val and Leu), anteiso- branched (coming from Ileu) or straight-chain (coming from Thr) homologs, differing only by one carbon in the backbone of the molecules. Also it is known that epicuticular wax-derived long-chain fatty acids (EWFA) are odd- and even-numbered, iso- and anteiso-branched. It was proposed earlier, that cycling of ketoacid products through α-ketoacid elongation (αKAE) leads to lengthening of strait,medium-chain acyl groups of tobacco SE (Kandra and Wagner, 1990). We made a conjecture that this is also the mechanism for elongation of iso- and anteiso-branched medium-chain fatty acyl groups of SE, but could not exclude the alternative mechanism for there formation by 2-carbon elongation via FAS pathway where the primer molecules could be propyonyl-CoA, 2-MePropyonyl-CoA, 2-MeButyryl-CoA or 3-MeButyryl-CoA, instead of acetyl-CoA. Experiments were conducted to test both hypotheses by using labeled precursor acetate and by predicting the labeling pattern of the carboxyl carbons of the product, depending on the pathway utilized. Figure 1 (Kroumova and Wagner, 2003) shows the predicted labeling pattern of the carboxyl carbon for both paths of elongation – two carbon elongation (FAS) and one carbon elongation (αKAE). More detailed pattern labeling is given in Kroumova et al., 1994. According to the αKAE hypothesis when [1-^{14}C]acetate is used, the FAS pathway will lead to a labeled carboxyl group, but [2-^{14}C]acetate will not. The opposite is expected for the αKAE pathway, where only [2-^{14}C]acetate will result in labeled carboxyl carbon. Percentage labeling was predicted from the labeling in the starter molecules and the number of elongation cycles. The same prediction was done for the epi-cuticular waxes fatty acids (EWFA) (Table 2, Kroumova and Wagner, 1999).

We radioactively labeled epidermal peels or isolated trichomes (in which SE are synthesized) with [1-^{14}C]- and [2-^{14}C]-acetate.

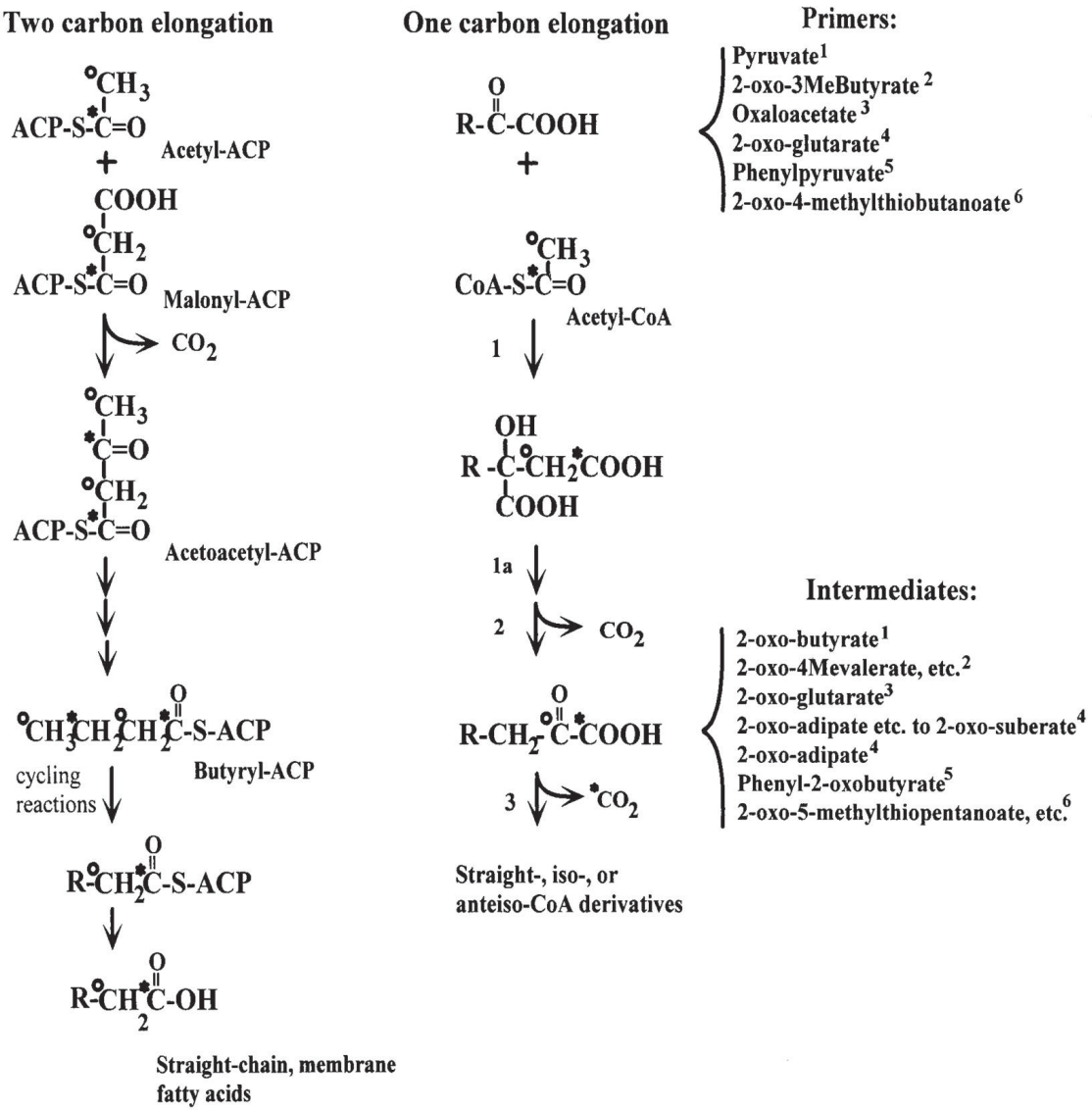

Fig. 1*. Diagram contrasting two carbon elongation (FAS mediated) and one carbon elongation (αKAE) pathways. Carbon 1 of acetyl-CoA is identified as (*) and carbon 2 as (o). Enzymes catalyzing reactions 1, 1A, 2 and 3 are assumed to be: 2-isopropylmalate synthase, EC 4.1.3.12; isopropylmalate dehydratase, EC 4.2.1.33; 3-isopropylmalate dehydrogenase, EC 1.1.1.85; and 2-oxovalerate dehydrogenase, EC1.2.1.25. The primers for various αKAE reactions are identified with corresponding intermediates using the same superscript.

*Planta, v. 216, pp:1013-1021 (2003), Kroumova A. B., G. J. Wagner. Different elongation pathways in the biosynthesis of acyl groups of trichome exudate sugar esters from various solanaceous plants, Fig.1. With kind permission of Springer Science+Business Media.

Table 1. Percentage of total molecule label in the carboxyl carbons of SE acyl groups and membrane fatty acids after labeling petunia (*Petunia* x *hybrida*), *N. bentamia*, *N. gossei*, *N. glutinosa*, *D. metel*, and *S. pennelii* trichomes with [2-^{14}C]- and [1-^{14}C]acetate. *Ac* acetate. *2MeBut* 2-methylbutyric,*4MeVal* 4 methylvaleric ,*Val* valeric, *Hex* hexanoic, *Hept* heptanoic, *Oct* octanoic, *Dec* decanoic, *4(*or *5)MeHex* 5(or 6)-methylhexanoic, *5(*or *6)MeHept* 5(or 6)-methylheptanoic, *8MeNon* 8-methylnonanoic, *NP* non predicted, *N* molecules not labeled. These are combined data from Kroumova and Wagner (2003).

Acyl group or Membrane fatty acid	Precursor		Predicted labeling in carboxyl carbon			
			αKAE		FAS	
	[2-^{14}C]Ac	[1-^{14}C]Ac	[2-^{14}C]Ac	[1-^{14}C]Ac	[2-^{14}C]Ac	[1^{14}C]Ac
Petunia						
4MeVal	40.4 ± 8.7[a]	N	33.0	0.0	0.0	50.0
Hept	19.2 ± 1.3	N	20.0	N	0.0	33.3
N. bentamiana						
5MeHept	19.4 ± 0.8	N	20.0	0.0	0.0	33.3
6MeHept	18.4 ± 2.1	N	20.0	0.0	0.0	33.3
N. gossei						
5MeHex	26.5 ± 1.1	N	25.0	0.0	0.0	50.0
5MeHept	20.1 ± 1.2	N	20.0	0.0	0.0	33.3
N. glutinosa						
4MeHex	26.4 ± 44.4	N	25.0	0.0	0.0	50.0
5MeHex	22.9 ± 4.4	N	25.0	0.0	0.0	50.0
D. metel[b]						
Hex	4.8 ± 1.5	35.5	33.3	N	0.0	33.3
Oct	4.6	24.7	16.6	N	0.0	25.0
L. pennellii[c]						
3MeBut	57.9 ± 6.0	N	50.0	0.0	NP	NP
8MeNon	2.8	32.6 ± 2.1	14.3	0.0	0.0	25.0
Dec	2.8	28.9 ± 3.9	12.5	N	0.0	20.0
Membrane FA						
Palmitate	4.1 ± 1.5	17.2 ± 0.7	NP	NP	0.0	12.5
Oleate	3.2 ± 0.8	15.8 ± 2.9	NP	NP	0.0	11.1

[a]Means from three Schmidt degradations with standard deviation;
[b]40 h labeling;
[c]6 h labeling

Table 2*. Radioactivity in carboxyl carbons of epi-cuticular wax FA, labeled with [2-^{14}C] or [1-^{14}C] acetate (% of total)

FA	NC2326	Kohlrabi		Brussels sprouts	Expected radioactivity	
	[2-^{14}C]Acetate	[1-^{14}C]Acetate	[2-^{14}C]Acetate	[2-^{14}C]Acetate	[1-^{14}C]Acetate	[2-^{14}C]Acetate
14:0	5.0	14.1, 11.7	1.9, 2.6	l	14.3	0.0
15:0	4.3	8.9, 8.3	6.3	l	11.6 to 16.6 or 0[a]	0 or 12.5[a]
16:0	2.6, 1.2	12.3, 17.2	2.2, 5.8	l	12.5	0.0
18:0	1.2	n.e.	n.l.	l	11.1	0.0
i15:0	2.3	n.l.	n.l.	n.l.		0 or 11.1 to 16.6[a]
a15:0	3.3	n.f.	n.f.	l		0.0
a17:0	n.f.	n.l.	n.l.	1.8		0.0

n.f. – not formed; n.e. – not examined; n.l. – not labeled; l – low label

[a]The first value or range is that expected without α-oxidation, and the second is that with α-oxidation involved. Ranges consider variations due to extent of label in primers that are elongated to form these fatty acids.

*Reprint from Phytochemistry, 50, A.B.Kroumova , G. J. Wagner, "Mechanisms for elongation in the biosynthesis of fatty acid components of epi-cuticular waxes", pp .1341-1345, Copyright (1999), with permission from Elsevier.

The acyl groups were recovered via extraction with solvent, methanolysis and than HPLC. Individual acids were collected and subjected to Schmidt degradation, where the carboxyl carbon was removed. Its radioactivity was compared with the radioactivity of the reminder of the molecule (Kroumova and Wagner, 1995). For the analysis of EWFA, leaves from selected plants species were labeled through the petiole, or alternatively lower leaf epidermis was peeled and incubated in a radiolabeling solution. After a series of purifications and a methanolysis, FAs were subjected to Schmidt degradation in the same way as for acyl groups from SE (Kroumova and Wagner, 1999). Table 1 shows our radiolabeling data of the most prominent SE acyl groups from Petunia, *N. bentamiana*, *N. gossei*, *N. glutinosa*, *Datura metel* and *S. pennellii*, and from membrane fatty acids (FA). The data were compared with the labeling predicted by FAS and αKAE. The results are consistent with the conclusion that SE acyl groups from Petunia and tobaccos are synthesized via αKAE pathway, while SE acyl groups from *D. metel* and *S. pennellii* are formed from the FAS pathway. 3MeBut from S. pennelii showed 50% labeling in the carboxyl group from [2-^{14}C] acetate. Its synthesis does not involve αKAE elongation, but starts via Leu-related reactions. The membrane FAs showed the expected classical FAS pathway of elongation.

Table 2 (Kroumova and Wagner, 1999) presents similar comparative data, related to carboxyl radiolabeling of EWFA from *N. tabacum* NC2326, Kohlrabi, and Brussels sprouts. When [2-^{14}C] acetate was used, their labeling was low within or close to the method error (3%). In contrast, labeling with [1-^{14}C] acetate resulted in ample carboxyl labeling. The information from15:0, i-15:0, a-15:0 and a-17:0 EWFA carboxyl labeling via [2-^{14}C] acetate (background labeling level) supports the conclusion that αKAE is not involved in chain elongation of odd-length, straight or branched chain EWFA. On the basis of the data we could also exclude the participation of α-oxidation (removal of the carboxyl group from the final elongation products). Both reactions would have given substantial carboxyl labeling. The results imply that for EWFA, odd-length primers (2- or 3-MeButyryl-CoA or propyonyl-CoA) are elongated via FAS reaction to form the above products. Palmitic acid (16:0) serves as a control and shows labeling, as expected, entirely consistent with FAS elongation of malonyl-CoA. αKAE reactions were postulated also to participate in the synthesis of glucosinolates in of Brasicaceae species (*Brassica napus*, *Eruca sativa*, *Arabidopsis thaliana*) (Chrisholm and Wetter, 1964)). Glucosinolates are secondary products, containing nitrogen and sulfur, and the anabolic pathway of some starts with chain-elongation of methionine (met GSL). By using stable isotope-labeled tracers, followed by MS and NMR, Graser et al. (2000) confirmed participation of αKAE in chain elongation reactions. The biosynthesis was suggested to involve up to 11 cycles of αKAE elongation (Halkier, 1999). The operation of the αKAE pathway in *N. bentamiana* trichomes was supported also by gene expression studies. Microarray analysis (Slocombe et al., 2008) showed up-regulation of IPMS genes only in tobacco, but not in *S. pennellii*, where FAS functions.

MATERIALS AND METHODS

RNAi constructs for knock down of IPMS genes from *S. pennellii* and *Nicotiana tabacum* T.I. 1068

IPMS genes from several plant species have been retrieved from the GenBank. We compared the amino acid sequences of two *S. pennellii* (former *Licopersicon pennellii*) and two Arabidopsis IPMS genes (At1g18500, At1g74040) and found three conserved motifs of 10 and 12 aa. In order to recover fragments of the *S. pennellii* IPMSa (AF004165) and IPMSb (AF004166) genes , primers were selected in such a way as to be specific for each gene, and so that the PCR amplified products contain the motifs. The oligos had the following sequences: from IPMSa: forward primer GGGAAGCAGTGAAATATGCTA, and reverse primer CAATAAACATTAG-GTTGGAGA; for IPMSb: forward primer GGTGTTGTATAATTGAGGCTG, and reverse primer ACGGTTGTGTAGA-AAGGCCA. Total RNA was isolated from leaves of *S. pennellii* with a small scale phenol-free total RNA isolation kit (Ambion Inc. Austin, TX). cDNA was synthesized from mRNA using Advantage RT-for-PCR kit (Clontech,CA, USA). PCR was performed using the above primers and PCR Master mix (Promega, Madison, WI). The amplified 900 bp fragments were inserted into the cloning vector pEW9495 (pUC19 with introduced into the multiple cloning site partial, 1,025-bp, β-D-glucuronidase (GUS) gene, Wang and Wagner., 2003) on both sides of the GUS linker in sense and antisense orientation, in order to create a dsRNA with a single-stranded GUS loop after the RNA transcription. The RNAi cassettes were sequenced to ensure their authenticity and orientations. Created cassettes were transferred to the shuttle vector pKYLX71 behind 35S promoter (Schardl et al., 1987) via restriction-digestion from the cloning vector and ligation with the shuttle vectors. The new plasmids were named pL1 (for Licopersicon IPMSa) and pL2 (for Licopersicon IPMSb. Plasmids were introduced to *Agrobacterium tumefaciens* strain LBA4404 containing pGV3850 by triparental mating.

Transformation and regeneration of *S. pennellii*

S. pennellii seeds were obtained trough the Germplasm Resources Information Network (GRIN) system (Accession Lot PI 246502 5901, Peru, order 202116). Plants were grown in the greenhouse or *in vitro*, and used as a stock of explants for transformation. *A. tumefaciens* transformation was done essentially as described in Horsch et al, 1988 with some modifications. Agrobacterium was grown on LB medium, containing 50 mg/L Km, 50 mg/L Rif and 35 mg/L Gm. Leaf or petiole explants from sterile-grown plants were inoculated with the bacteria for 2 days in the dark, than blotted dry and transferred to MS medium supplemented with Gamborg B-5 vitamins (Sigma-Aldrich Co. LTD, Irvine, UK), 3% Sucrose, 3 mg/l BAP and 0.15 mg/L IAA ((Ruf et al., 2001) supplemented with 25 mg L^{-1} Km and 500 mg L^{-1} Mefoxine. In about a month small meristemoid calli were formed, mainly at leaf bases and the cut edges of the petioles. These calli were transferred to shoot-formation medium containing MS salts, Gamborg B-5 vitamins, 3% sugar,

2.0 mg L^{-1} Kin and 0.1 mg L^{-1} IAA, 25 mg L^{-1} Km and 500 mg L^{-1} Mefoxine. After 10 days, well-formed shoots were cut and transferred to a rooting medium (the same medium without growth regulators) containing 80mg/L Km and 400 mg/l Mefoxine, in PLANTCON® containers (MP Biomedicals, LLC). Rooted plants were transferred to fertilized Pro-Mix (Premier Horticulture Inc., Canada) in the growth room.

Transformation and regeneration of *N. glutinosa*, cv 24A

Axenic tissue sources were prepared and transformation was done according to Horsch et al. (1988). Important step before transformation was to preculture explants (cut cotyledons and first leaves) on regeneration medium (MS salts, Gamborg B-5 vitamins, 3% sucrose, 1 mg L^{-1} BA, 0.05 mg L^{-1} NAA and 0.7% agar) for 2 days. Further they were co-cultivated with *A. tumefaciens* for 2 days and then transferred to a regeneration medium containing 15 mg L^{-1} Km and 250 mg L^{-1}, Ticarcillin (GlaxoSmithKline). Small calli and shoots formed in 30 days. They were transferred to the above medium without NAA to stimulate stem growth and formation of more leaves. In another month, shoots were transferred to a rooting medium (the same, without growth regulators), containing 10 mg L^{-1} Km and 200 mg L^{-1} Ticarcillin.

Transformation and regeneration of *N. tabacum* T.I. 1068

Axenic tissue sources were prepared and transformation was done using the leaf disc method (Horsch et al, 1988). Kanamycin-resistant plantlets were transferred to soil and further analyzed.

GC analysis of acyl groups of sugar esters

Fatty acid butyl esters were prepared according to Severson et al. (1985) and were analyzed with HP 6890 GC System/ HP 5973 Mass Selective Detector. The peaks were identified by using standards and by comparing the retention times.

RESULTS

Reverse genetics approach

In order to be able to exploit the existence of αKAE to over-produce and modify synthesis of branched and straight, short and medium-chain FA, the enzymes involved in the pathway should be confirmed and characterized. Although we suggested participation of four enzymes in αKAE, related to Leu synthesis/degradation (Figure 2, reaction 1, 1a, 2 and 3), direct evidence for their involvement is still not available. It is not clear if the same alkyl-malate synthase uses different α-keto acids as substrates, or if there are different isoforms; how alkyl-malate synthase(s) is (are) related to the Leu IMPS; which enzyme determines the specificity of acyl groups in different species - alkyl-malate synthase or alkyl, 2-keto dehyrogenase? We attempted to determine if IPMS participates in the cycling reactions and if there is preference to certain substrate keto acids in this reaction. We applied the reverse genetic approach to knock down IPMS gene expression using double stranded RNAi (dsRNAi).

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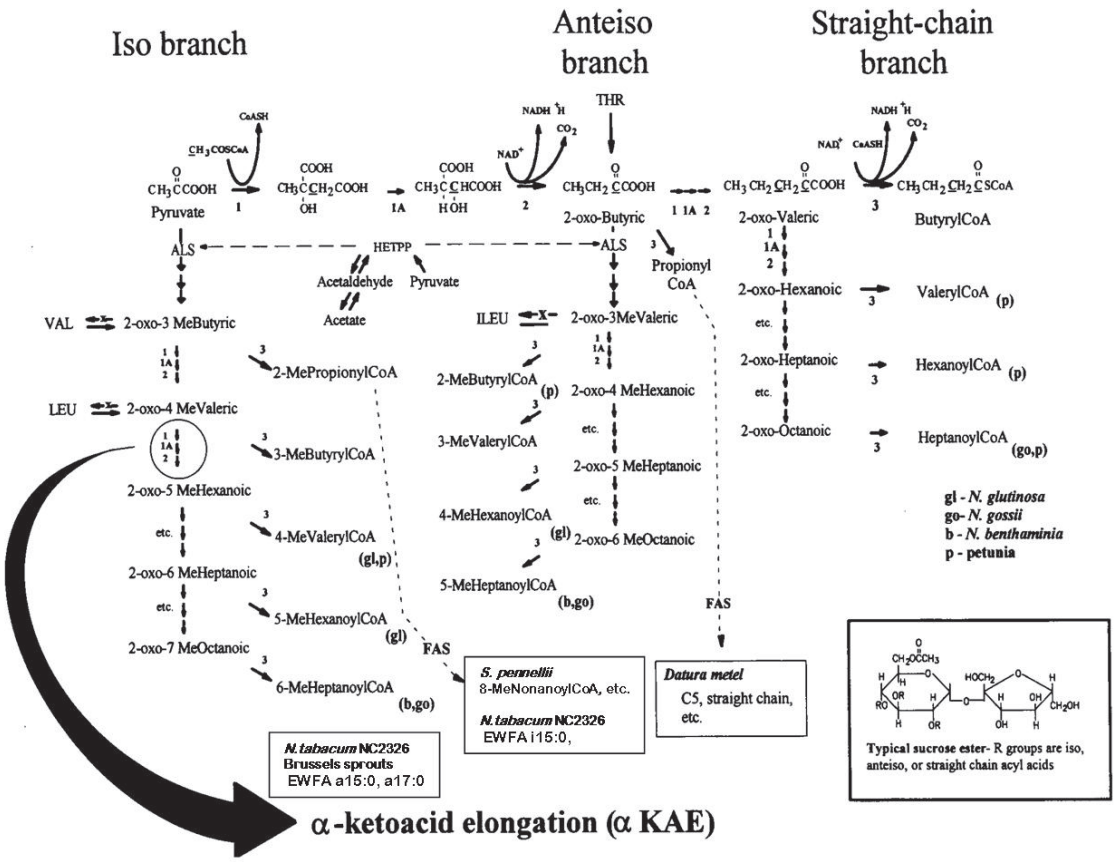

Figure 2*. Pathways for synthesis of Val (VAL), Leu (LEU) and Ileu (ILEU), one carbon elongation of 2-oxo-acid intermediates via αKAE (in Petunia x hybrida and Nicotiana species) and two carbon elongation via FAS in *S. pennellii*, *D. metel*, Brussels sprouts and *N. tabacum* cv. NC2326. Carbon 2 of acetate is underlined to show its fate in the elongation of pyruvate to form butyrylCoA via αKAE. Enzyme reactions catalyzing 1, 1A, 2 and 3 are defined in the legend of Fig.1. Letters in parentheses following various CoA products denote principal acyl groups found in SE of different tobaccos and petunia (e.g., gl for *N. glutinosa*, etc.). ALS acetolactate synthase, HETPP hydroxyethylthiamine phyrophosphate, Me methyl, THR threonine.

*Planta, v. 216, pp:1013-1021 (2003), Kroumova, A. B., G. J. Wagner. Different elongation pathways in the biosynthesis of acyl groups of trichome exudate sugar esters from various solanaceous plants, Fig.2. With kind permission of Springer Science+Business Media.

We synthesized primers on the basis of database sequences for two IPMS (a and b) genes from *S. pennelii* (AF004165 and AF004166) and used these to amplify part of the genes from cDNA. We then introduced them in sense and antisense orientation, separated by a GUS spacer into an dsRNAi construct. We performed leaf disc transformation with *Agrobacterium*, carrying the constructs in binary plasmid, and transformed *N. tabacum* T.I. 1068, *N. glutinosa* cv. 24a and *S. pennellii* with the constructs.

Transformation of *N. tabaccum* T.I. 1068 with *L. pennellii* IPMSa and IPMSb constructs.

Twenty one plants transformed with IPMSa dsRNAi and thirty seven plants, transformed with IPMSb dsRNAi were regenerated. Their SE were collected, saponified and the acyl groups were esterified with butanol. To obtain acyl group profiles of individual plants, butyl esters were analyzed by gas chromatography (GC). Qualitative composition of acyl groups was not changed, i.e. they consisted of MePropionate, 2- and 3MeButyrate, and 3- and 4 MeValerate. Knockdown with IPMSa and IPMSb resulted in similar changes in the abundance of certain acyl groups. Fig. 3 shows relative acyl group abundance in SE of To plants transformed with IPMSa or IPMSb constructs. Changes were observed among anteiso branched acyl groups. All of the shown clones had reduced 3-MeValerate and increased 2-MeButyrate compared to non-transformed control. These plants were chlorotic, some of them with curly leaves but fertility was normal. Some of the other plants had normal GC composition (like the control shown in Fig. 3), but were also phenotipically impaired. In contrast, wild-type control and controls, transformed with empty plasmid had normal appearance. The chlorotic phenotype could be a consequence of disturbed synthesis of photosynthetic proteins or non-specific knock down of photosynthetic genes with some nucleotide similarity to IPMS gene.

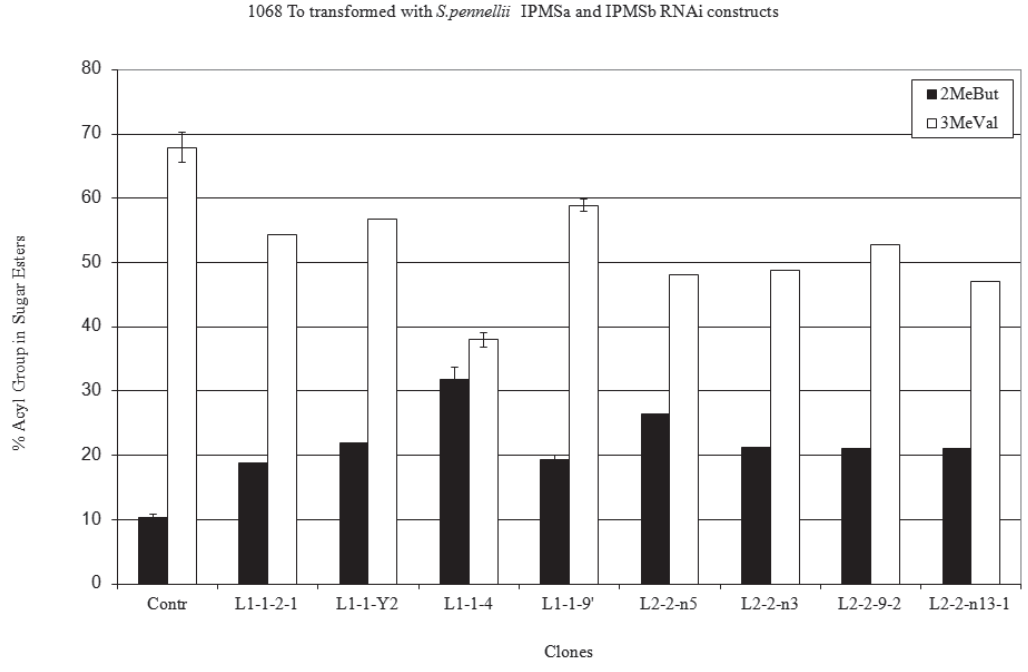

Figure 3. Relative acyl group abundance in sugar esters of *N. tabacum*, T.I. 1068, transformed with IPMSa or IPMSb genes from *S. pennellii*. L1-clones contain IPMSa dsRNAi construct, and L2 clones – IPMSb dsRNAi construct. *2MeBut* 2-Methyl Butyric, *3MeVal* 3-Methyl Valeric.

Transformation of *N. glutinosa* cv. 24a with *S. pennellii* IPMSa and IPMSb constructs.

Totally 20 To plants (transformed with IPMSa dsRNAi or IPMSb dsRNAi) were analyzed as the above. SE acyl groups contained MePropionate, 2-and 3 MeButyrate, 3- and 4 MeValerate, and 4- and 5 MeHexanoate. Three plants showed substantially reduced 5 MeHexanoate. Twenty percent of regenerants showed abnormal leaf morphology (curly leaves), which was not directly correlated with the changed GC profile.

Transformation of *S. pennellii* with *S. pennellii* IPMSa and IPMSb constructs.

Totaly 39 plants, transformed with IPMSa dsRNAi or IPMSb dsRNAi, were regenerated and their SE acyl groups were analyzed with GC. There was no phenotype or GC profile change from control plants and those transformed with empty vector. They contained MePropionate, 2 and 3 MeButyrate, 8 MeNonanoate, Decanoate and Laurate. Four transformants showed increased abundance of 8-MeNonanoate. Some of regenerants were chlorotic. The branched -chain amino acids, Leu, IsoLeu and Val are essential aminoacids, synthesized only in plants. Enzymes, involved in Leu synthesis/degradation were studied in detail in Arabidopsis. There are at least 2 potential genes for IPMS, 4 potential genes for IPMDHT, 3 potential genes for IPMDH, and 7 identified branched-chain aminotransferases (Binder et al, 2007). Alkyl, 2-keto (isovaleryl) dehydrogenase, the enzyme that converts keto acids from αKAE to acyl-CoAs, exists as a multienzyme complex of units E1 (with two subunits), E2 and E3 (Mooney et al, 2002; Taylor et al., 2004). IPMS is a key enzyme, involved in the metabolism of Leucine. A gene unequivocally encoding IPMS activity in plants has not been characterized thus far. In Arabidopsis two IPMS-like genes were identified on the basis of similarity of their sequences to IPMS in other organisms (including high aa identity with *S. pennellii* IPMSa and IPMSb), and on the basis of substrate specificity of the purified proteins. They showed differences only in their Km's and quaternary structures. IPMS1 knockdown mutants had no change in the soluble Leu content, and only small (but statistically higher then the control) increase in Val content (de Kraker et al., 2007). Interestingly, regenerants had curly leaves and were slightly chlorotic. We observed similar phenotypical changes in *N. tabacum* 1068 and *S. pennellii*, knockdowns for the *L. pennellii* genes. Arabidopsis IPMS2 knockdown plants had no change in soluble amino acid content and were normal. In another case, Brassica IPMS was over expressed in Arabidopsis, but most of the recovered lines (7 of 8) showed silencing. Plants were dwarfed, pale with crinkled rosette leaves. They showed reduced soluble Leu and increased Val, Ser,Gly, Thr, Met, and His (Field et al., 2006). The reasons for these changes could not be found. It is not clear if they are direct effect of IPMS knockdown or represent pleiotropic effects. The above examples imply that IPMS is a critical enzyme and its impairment probably would lead to an unhealthy plant. For this reason its synthesis is highly regulated. In the case of Arabidopsis, it is likely that each

IPMS enzyme efficiently compensates for the lack of another that is knocked down. Regarding the chain elongation, probably more than one enzyme is involved in elongation of different α-keto acid intermediates. At least this is the case in α-keto acid elongation of ω-methylthio-2-oxoalkanoic acids derved from methionine. MAM1 and MAM2 (methylthioalkylmalate synthase) genes participate in formation of short carbon chains (Kroymann et al, 2001; Textor et al, 2004 from Benderoth 2009), but MAM3 is involved in synthesis of aliphatic glucosinolates with long carbon chains via αKAE (Field et al, 2004;Textor et al, 2007). MAM synthases determine variability in glucosinolate alkyl chain length. MAM genes, together with IPMS genes belong to enzyme class EC2.3.3.They share a high degree of similarity. Phylogenetic analysis shows that MAMs have originated from IPMS (Benderoth et al. 2009). We found some changes in acyl group abundances from *N. tobacum*, *N. glutinosa* and *S. pennellii*. We speculate that there is similarity between the IPMS and IPMS-like genes responsible for condensation of 2-oxo4 MeHexanoate from anteiso path and 2-oxo-6 MeHeptanoate from iso path. According to the RNAi mechanism, if short 21nt nucleotides are identical with some RNA areas, they will lead to the destruction of the entire RNA. Knockdown of enzymes, not involved directly in amino acid anabolism, but majorly in secondary metabolite pathways (as in our case putative IPMS-like ones), could lead to changes in the synthesis of the final products. Slocombe et al. (2008) demonstrated, via down regulation of branched-chain keto-acid dehydrogenase complex (enzyme 3 in Fig. 2), that this enzyme is required for BCFA and acyl sugar synthesis in *N. benthamiana* and S. *pennelii*. Knockdown of E1-β unit of the complex in *N. bentamiana* resulted in significant reduction in amount of SE, 3-fold reduction of SE acyl groups and 8-fold reduction of E1-β transcript levels (determined by RT-PCR). 8-MeNonanoic acid was reduced 40% in *S. pennellii*. Also *iso*- and *anteiso*- wax alkanes in both species were reduced significantly. The metabolic changes were not coupled with any phenotypic disturbances (Slocombe et al. 2008). Notably, in *N. bentamiana*, all SE acyl group were reduced without any preferences to certain chain length. This implies that at least the E1-β unit of BCKD complex does not determine acyl diversity and SE composition. The Arabidopsis enzymes methylthioalkylmalate isomerase (functional analog of enzyme 1a, Fig.1) and methylthioalkylmalate dehydrogenase (functional analog of enzyme 2, Fig. 1) were targeted for metabolic profiling by reverse genetics (Sawada et al., 2009). The knockdowns had substantially reduced levels of methionine-derived glucosinolates (met-GSLs) with C4-C8 chains.

Conclusion

Among species of Solanaceae family, there are two pathways for elongation of fatty acids: two-carbon elongation via FAS synthase and one-carbon elongation via αKAE.

Epi-cuticular wax long chain fatty acids are synthesized via FAS mediated elongation from even- or odd-numbered straight- or branched-chain precursors. Specific modification of SE acyl composition will only be possible after the specific enzymes

involved in the synthesis of acyl groups are better characterized.

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