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BRIEF COMMUNICATION

Ectopic expression of the *Osmyb4* rice gene enhances synthesis of hydroxycinnamic acid derivatives in tobacco and clary sage

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Abstract

In this work, we report the ectopic expression of the *Osmyb4* rice gene, encoding the Myb4 transcription factor, in *Nicotiana tabacum* and *Salvia sclarea*. Transcriptional analysis of T_2 homozygous tobacco plants overexpressing *Osmyb4* revealed that Myb4 activated the transcription of several genes of the phenylpropanoid pathway such as *PAL, C4H, 4CL1, 4CL2* (encoding phenylalanine ammonia-lyase, cinnamic acid 4-hydroxylase, 4-coumarate: Co A ligase1, and 4-coumarate: Co A ligase2). Moreover, the Myb4 increased expression of *HQT* encoding hydroxycinnamoyl-CoA: quinate transferase which specifically triggers the accumulation of chlorogenic acid (CGA). In addition, increased acccumulation of rosmarinic acid (RA) was found in transgenic plants of both species. These results open the possibility of using the *Osmyb4* gene to increase the production of specific bioactive hydroxycinnamates.

Additional key words: chlorogenic acid, Myb transcription factor*, Nicotiana tabacum*, phenylpropanoid pathway, rosmarinic acid, *Salvia sclarea.*

In plants, the phenylpropanoid pathway produces the majority of phenolic compounds that play key biological roles during growth and development. They are also important for plant defence against pathogens, plant-microbe/animal interactions as well as in reparative mechanism against environmental stresses. In addition, these secondary metabolites have significant economic value for human health or modern industries (Treutter 2010). Thus, it is of interest to deepen the knowledge about the biosynthesis of this class of bioactive compounds and to enhance their accumulation in plants.

Among phenylpropanoids, chlorogenic acid (CGA) and rosmarinic acid (RA), two hydroxycinnamic acid derivatives, are the most abundant antioxidants synthesized in tobacco and clary sage plants, respectively (Niggeweg *et al*. 2004, Petersen *et al*. 2009). The increased content of CGA, which is also present in different plant products such as apples, pears, tomatoes, potatoes, eggplants and coffee (Farah *et al*. 2008), is associated with an increase in tolerance to oxidative stress and resistance to fungal pathogens and RA participates in plant defence system against fungal and bacterial infections or predators (Niggeweg *et al*. 2004, Petersen *et al.* 2009). Besides, these two compounds have well documented multiple pharmacological properties (Korkina 2007, Leone *et al.* 2007). Both compounds are esters of caffeic acid. In *Solanaceae,* the hydroxy-cinnamoyl-CoA: quinate hydroxycinnamoyl transferase (HQT) catalyses the final limiting step of CGA biosynthesis whereas in *Lamiaceae* a similar acyl-transferase, the rosmarinic acid synthase (RAS), is critical for RA accumulation (Petersen *et al*. 2009). The phenyl-propanoid pathway is well-characterized (Fig. 1) and genes encoding several enzymes, acting in different

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Abbreviations: 4CL - 4-coumarate:CoA ligase; CGA - chlorogenic acid; C4H - cinnamic acid 4-hydroxylase; EPSPS - 5-enol-pyruvylshikimate 3-phosphate synthase; EV - empty vector; HPLC-DAD - high performance liquid chromatography - with diode array detection; HQT - hydroxycinnamoyl CoA quinate transferase; PAL - phenylalanine ammonia lyase; RA - rosmarinic acid; RAS - RA synthase; RT-PCR - reverse transcription - polymerase chain reaction.
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branches of this pathway, have been cloned from many plants (Naoumkina *et al*. 2010). It has been reported that the coordinated action of several transcription factors regulates the expression of phenylpropanoid biosynthetic genes (Hartmann *et al*. 2005). Namely, MYB factors acting as activators or repressors have been well characterized (Pattanaik *et al*. 2010, Vogt 2010).

It has been previously reported that the rice *Osmyb4* gene, when ectopically expressed in *Arabidopsis thaliana* and in other plant species, activates the transcription of stress-related genes and improves tolerance to abiotic and biotic stresses (Vannini *et al*. 2004, 2006, Pasquali *et al.* 2008, Laura *et al.* 2010, Park *et al*. 2010). Furthermore, ectopic *Osmyb4* expression in the *A. thaliana* enhanced the synthesis of sinapoyl malate (SM), the major phenylpropanoid in this plant family (Mattana *et al.* 2005, Vannini *et al.* 2006, Milkowski and Strack 2010).

This last feature prompted us to investigate the potential of Myb4 in enhancing the synthesis of bioactive phenylpropanoids in *Nicotiana tabacum* and *Salvia sclarea,* two species characterized by a high content of hydroxycinnamic acid derivatives.

Seeds of *Salvia sclarea* L. and *Nicotiana tabacum* L. (cv. Samsun NN) were germinated on Murashige and Skoog (1962; MS) medium at 25 °C. *Osmyb4* over-expressing tobacco lines were generated by 2 d of co-cultivation with a recombinant *Agrobacterium tumefaciens* LBA4404 strain containing the binary vector pGA470 (EV) and pGAMyb4 (Vannini *et al*. 2004). Among several independent homozygous T_2 lines (100 % germination on selective kanamycin medium) with single insertion site (χ^2 = 3:1; $P \leq 0.001$), three lines showing different *Osmyb4* expression level were selected for molecular and metabolic analysis. T_2 tobacco plants transformed with the empty vector (EV) were generated as control plants. *S. sclarea* transgenic hairy roots were obtained by co-cultivation of leaf disks from 15-d-old seedlings with *Agrobacterium rhizogenes ATTC*-15834 strain carrying the pGA470 (EV) or pGAMyb4 vector according to Gangopadhyay *et al* (2008). Stable transformants were identified by absence of *virC* gene and presence of *rolB/rolC* genes in genomic DNA using PCR amplification (data not shown). Genomic DNA and RNA were extracted by using the *DNeasy* and *RNeasy* plant mini kits (*Qiagen*, Valencia, USA), respectively. Gene expression in sage hairy roots and tobacco plants was assessed by RT-PCR. First strand cDNA was synthesized with a cDNA synthesis kit (*Superscript II RT*, *Invitrogen*, Carlsbad, USA). PCR amplification was performed using initial denaturation step at 94 °C for 1 min, 30 cycles of denaturation at 94 °C 1 min, annealing 1 min according to the different primers T_m, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. The primer pairs used were described in Table 1.

Osmyb 4 expression in tobacco plants was quantified by qPCR according to Pfaffl (2001). The transgene expression was normalized to EF 1α as tobacco house-keeping gene. For primer pairs see Table 1.

Phenotype of tobacco plants was analysed by measuring plant height and the internode length in 12-week-old plants (n = 10). Growth (dry mass) of clary sage hairy roots was measured for 5 weeks until the stationary phase starting from 0.5 g inoculation fresh mass. The number of secondary roots was measured every week for one month. Total proteins from fine powder of young tobacco leaves grounded in liquid nitrogen were extracted in phosphate buffer saline (PBS 1×), 0.5 M NaCl, 10 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM EDTA (pH 8). Phenylalanine ammonia-lyase (PAL) and 4-coumarate: Co A ligase (4CL) activities were assayed according to Saunders and McClure (1974) and Knobloch and Hahlbrock (1977), respectively. All the enzymatic assays were run in triplicate and enzymatic activities expressed in μmol mg^{-1}(protein) min^{-1}. Lyophilized *N. tabacum* leaves and *S. sclarea* hairy roots (0.5 g) were extracted with MeOH containing 0.1 % trifluoracetic acid (TFA). Phenolics were quantified by HPLC combined with UV diode array (UV DAD; *Agilent Zorbax* eclipse *XDB* C_8 column 250 × 4.6 mm) with the following elution gradient combining solvent A (0.1 % TFA in water) and B (0.1 % TFA in CH_3CN): 0 - 20 min 15 % B, 20 - 35 min 95 % B, and 35 min 100 % B. Content of chlorogenic acid (CGA and rosmarinic acid (RA) was

Table 1. Primer pairs used for RT-PCR and qPCR.

	Genes	Forward	Reverse	Acc. No.
RT-PCR	*OsMyb4*	5'-TCGGCTTCTTGTGCTTCTTGC-3'	5'-AGGGAAGGAGCAAGCACAAT-3'	Y11414
	rolB	5'-TGAGCATGTGCTGTTTTTGG-3'	5'-GATTCAACCATATCGGAGCG-3'	X64255
	rolC	5'-TTAAAAGCAGGCAGGTGTCC-3'	5'-GAGCTGTCTTTCTCCGTTGTTT-3'	X64255
	Nt18S	5'-CCCAAAGTCCAACTACGAGC-3'	5'-TAGATAAAAGGTCGACGCGG-3'	AJ236016
	NtEPSP	5'-AACTGTTGCACCCAACTTCC-3'	5'-AAGTTGATGGAGCGATTTGG-3'	AB049816
	NtPAL	5'-CTCGAAGCAAGTCCTTTTCG-3'	5'-ATTCTCCGAGCTTGTCAACG-3'	D17467
	Nt4CL1	5'-TGGGATGGTTGAGAAGAAGG-3'	5'-ATTTGTGCCGCCTATTGTTC-3'	U50845
	Nt4CL2	5'-GAAGGATTTTGCCAGATGGA-3'	5'-TGACAGAAGCTGGACCAGTG-3'	U50846
	Nt C4H	5'-AAAAATGGATCTTCTCTTAC-3'	5'-ACTGGGATTGGTCCT-3'	AJ937847
	NtHQT	5'-TCCCCCTCATCATCTTTCAG-3'	5'-GGGCAAAGCCTAGACCTACC-3'	AJ582651
qPCR	*EF 1α F*	5'-TACCACCCCCAAGTATTCCA-3'	5'-TGTTGTCACCTTCCAAACCA-3'	AF120093
	Osmyb4	5'-CCGGAGGGACATAGCTAACA-3'	5'-CTACTGGGTGCATCTCATCG-3'	

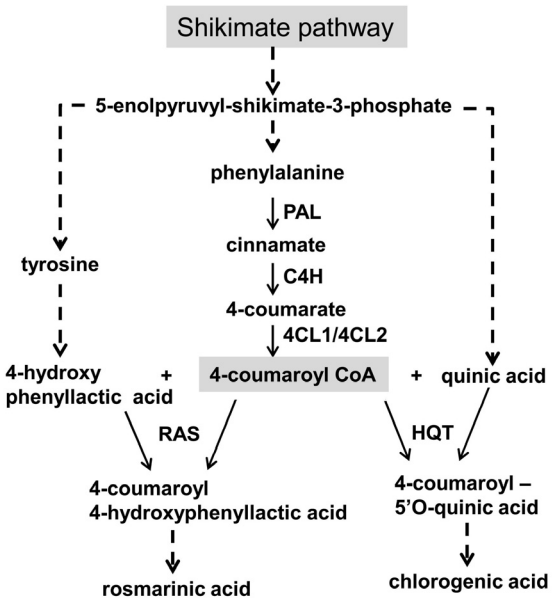

Fig. 1. Schematic overview of phenylpropanoid pathway in plants leading to the synthesis of chlorogenic acid and rosmarinic acid.

determined by measuring the absorbance at 254 and 280 nm, respectively and quantified using calibration curves of commercial standards.

The *Osmyb4* overexpression affected the phenotypes of transgenic tobacco plants and *S. sclarea* hairy roots (Fig. 2*A*,*D*) similarly as previously reported for *Arabidopsis* and apple (Vannini *et al*. 2004, Pasquali *et al.* 2008). Namely, three transgenic tobacco plant lines had shorter internodes (1.46 ± 0.040, 1.50 ± 0.037, and 1.90 ± 0.05 cm) and a reduced plant height (107.5 ± 0.57, 107.2 ± 0.50, and 106.5 ± 0.57 cm) compared to empty vector (EV) control plants (3.92 ± 0.09 and 118.7 ± 0.92 cm, respectively). As well, transgenic clary sage hairy roots were more densely packed showing a higher number of secondary roots (2.5 ± 0.2 *versus* 1.5 ± 0.3 in EV) but a higher growth rate reduction 5 weeks after inoculation (0.69 ± 0.02 *versus* 0.61 ± 0.03 in EV). Indeed, it has been extensively reported that the constitutive expression of TFs might affect plant growth and morphology (Sun *et al.* 2011).

Based on previously reported data on *Osmyb4* transgenic *Arabidopsis* plants (Mattana *et al* 2005, Docimo *et al*. 2008), we selected three *Osmyb4* over-expressing tobacco lines according to their transgene expression level (Fig. 2*B*) and tested the ability of Myb4 to activate the expression of phenylpropanoid pathway genes in tobacco plants. As shown in Fig. 2*C*, the expression of *EPSPS* (encoding 5-enolpyruvylshikimate 3-phosphate synthase) and genes encoding enzymes belonging to the phenylpropanoid pathway (*PAL*, *C4H*, *4CL1* and *4CL2*) were increased in transgenic plants. Interestingly, Myb4 also up-regulated the expression of the *HQT* gene encoding hydroxycinnamoyl-CoA: quinate transferase, the enzyme involved in the last committed step in CGA biosynthesis (Choi *et al*. 2004). Consistent with the enhanced gene up-regulation, *Osmyb4* overexpressing plants had a higher enzymatic PAL and 4CL activities, almost two-fold higher for PAL and one-fold increased for 4CL compared to EV plants (Table 2).

The final effect of this coordinated activation of genes belonging to the phenylpropanoid pathway was a six- to ten-fold increase in the leaf content of CGA, varying from 94.3 ± 17.9 (line 3) to 179.1 ± 56.6 mg g^{-1}(d.m.) (line 1) over the CGA content of 15 mg g^{-1}(d.m.) in control plants (Table 2). Previous attempts to improve CGA production by overexpressing *PAL*, *HQT*, or both genes triggered only a two/five-fold increase in CGA content (Howles *et al*. 1996, Chang *et al*. 2009). The higher CGA amount reached by *Osmyb4* overexpression may be associated to the coordinated up-regulation of several phenylpropanoid genes including the *HQT* gene.

In *S. sclarea*, the expression of *rolB/C* and *Osmyb4* genes was confirmed by RT-PCR (Fig. 2*E*) and the potential effect of *Osmyb4* was evaluated directly on the accumulation of phenylpropanoid compounds in the hairy roots. HPLC-DAD analysis revealed that the major difference was in RA amount whereas no differences were found for other phenolic compounds (data not shown). Quantitative analysis by HPLC established a two-fold increase of RA in transgenic compared to control hairy roots (Table 1).

Although PAL activity inhibition in *Salvia miltiorrhiza* blocks RA synthesis, it has been suggested

Table 2. Activities of PAL and 4CL (using cinnamic acid or *p*-coumaric acid as substrates) [mmol g^{-1}(protein) min^{-1}] and CGA content [mg g^{-1}(d.m.)] in the three overexpressing lines of transgenic tobacco plants compared to EV (pGA 470 empty vector). RA content [mg g^{-1}(d.m.)] in two independent *S. sclarea Myb4* overexpressing hairy roots, compared to EV hairy roots. Mean ± SE, $n = 5$; different letters indicate significant differences according to the Duncan's test ($P < 0.05$ for enzymatic activities and $P < 0.001$ for contents).

Plants	Parameters	EV	Line 1	Line 2	Line 3
Tobacco	PAL	4.6 ± 0.30^{a}	8.6 ± 0.20^{b}	8.2 ± 0.10^{b}	8.2 ± 0.10^{b}
	4CL (cinnamic acid)	2.1 ± 0.01^{a}	3.3 ± 0.10^{b}	3.2 ± 0.04^{b}	3.0 ± 0.03^{b}
	4CL (*p*-coumaric acid)	4.1 ± 0.03^{a}	4.6 ± 0.04^{ab}	4.5 ± 0.05^{ab}	4.6 ± 0.02^{ab}
	CGA	15.0 ± 2.80^{a}	179.1 ± 56.6^{b}	128.1 ± 16.2^{b}	94.3 ± 17.9^{c}
Salvia sclarea	RA	14.2 ± 0.30^{a}	38.8 ± 0.36^{b}	38.9 ± 1.05^{b}	

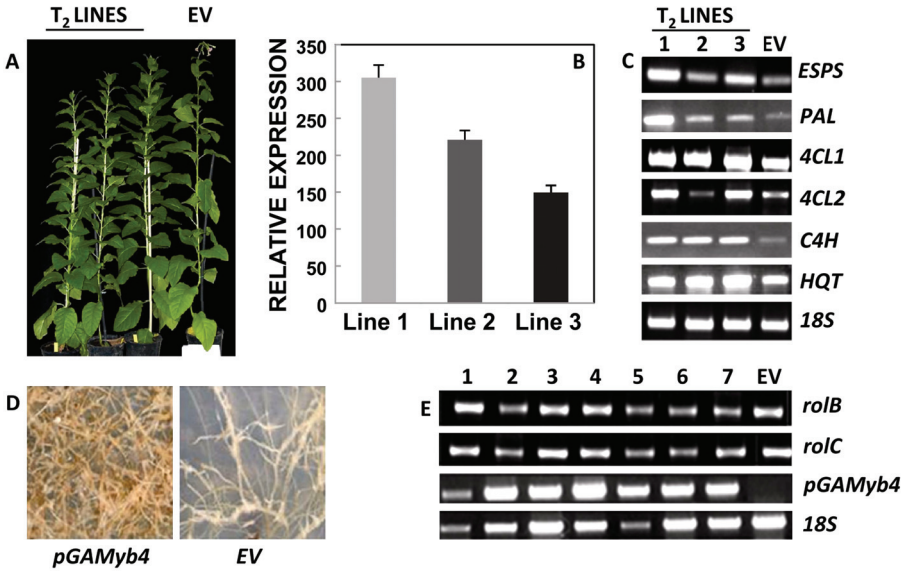

Fig. 2. Analysis of *Osmyb4* overexpression in tobacco plants and clary sage hairy roots. *A* - Plant phenotype of *Osmyb4* overexpressing (T_2 lines) of *N. tabacum*; *B* - quantitative expression level of the *Osmyb4* in three T_2 independent transgenic tobacco plants; *C* - expression analysis of the phenylpropanoids pathway genes (*EPSPS*, *PAL*, *4CL1, 4CL2*, *C4H*, and *HQT*) in *Osmyb4* and control T_2 tobacco plants (EV) by semi-quantitative RT-PCR; *D* - phenotype of clary sage hairy roots compared to the EV roots; *E* - expression of *rolB/C* and *Osmyb4* genes in independent *Osmyb4* hairy root line and a control EV line. Gene expression was assessed by semi-quantitative RT-PCR and using 18S rRNA as an internal control.

that its accumulation involves other enzymes (Chen and Chen 2000, Yan *et al.* 2006). Therefore, as demonstrated for CGA accumulation in transgenic tobacco, the increase of RA, here reported, may depend on the coordinated up regulation of several genes belonging to the phenyl-propanoid pathway as well as genes involved in the synthesis of 3-(4-hydroxyphenyl) lactate.

Altogether our and previous data suggest that *Osmyb4* overexpression specifically increases the synthesis of the most naturally abundant end-products of phenylpropanoid pathway (Vannini *et al*. 2006, Docimo *et al*. 2008). This accumulation may depend on the specific coordinated activation of several genes (*i.e*. *PAL*, *C4H*, and *4CL*) and of specific key genes, such as *HQT* and *SMT* (encoding sinapoyl-glucose:malate sinapoyl transferase) in tobacco and *Arabidopsis*, respectively (Vannini *et al*. 2006, Docimo *et al*. 2008). Actually, we did not observe any change in the content of other secondary metabolites such as nicotine in tobacco or salvianolic acid in clary sage. Accordingly, in tobacco transgenic plants, the expression of alkaloid biosynthetic genes (encoding, *e.g*., ornithine decarboxylase and putrescine *N*-methyl-transferase) was unaffected (data not shown).

In conclusion, our data and recent microarray analysis of transgenic rice plants (Park *et al.* 2010) indicate that *Osmyb4* is a general and evolutionary conserved regulator of the phenylpropanoid pathway. Therefore, beside its primary role in plant stress response, it represents a tool to enhance the synthesis of specific bioactive phenyl-propanoid end-products in other crops and medicinal plants.

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