



Comparative analysis of the Squamosa Promoter Binding-Like (SPL) gene family in Nicotiana benthamiana and Nicotiana tabacum

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SQUAMOSA promoter binding-like (SPL) proteins are transcription factors known to play key roles in growth and developmental processes, including leaf development, plant architecture and juvenile-to-adult and vegetative-to-reproductive phase transitions. In this study, we report the identification of 49 SPL genes in Nicotiana tabacum cv. K326 and 43 SPL genes in N. benthamiana lab strain. The SPL genes of the two species were classified into 8 phylogenetic groups according to the SPL classification in Arabidopsis. The exon-intron gene structure and SBP domains are highly conserved between homeologues and orthologues. 30 of the NbSPL genes and 33 of the NtSPL genes are targets of **miR156**. These results served as basis for candidate gene selection for a knockout experiment using CRISPR/Cas9 in N. benthamiana that revealed some clues about the biological function of NbSPL13 genes. The knockout of these genes brought to a significant delay of 4-5 days in flowering time, which could be the basis for the increase of available infiltration time for recombinant protein production. Additionally, the expression of SPL genes was analysed by RNA-seq at different growth stages for both tobacco and N. benthamiana, revealing that genes not under miR156 control were constitutively expressed throughout development and the other miR156-regulated genes varied their expression at different developmental stages.

Phylogenetic tree with SPL genes from N. tabacum, N. benthamiana and Arabidopsis thaliana



SPL gene subfamilies of *N. benthamiana* and tobacco exhibit similar exonintron structure and a conserved SBP domain



Expression profiles of NbSPL and NtSPL genes in leaf at juvenile (J), pre-flowering (P) and flowering (F) stages



NbSPI 1/12 1x		NtSPL	_1/12_1a
NbSPI 1/12_1/		NtSPL	_1/12_1b
		NtSPL	_1/12_2a
NbSPL1/12_2a	6	NtSPL	_1/12_2b
NbSPL1/12_2b		NtSPL	_1/12 [_] 3a
NbSPL1/12_3x		NtSPL	_1/12_3b
NbSPL1/12 3y	4	NtSPL	_1/12_4Ua
NbSPL13_1a (miRNA156)	-	NtSPL	_13_1a (miRNA156)
NbSPL13_1b (poSBP)(miRNA156)		NtSPL	_13_1b (miRNA156)
	100	NtSPL	_2/10/11_1a (miRNA1
NbSPL2/10/11_3a (miRNA156)	2	NtSPL	_2/10/11 1b (miRNA1
NbSPL2/10/11_3b (miRNA156)		NtSPL	_2/10/11 2a (miRNA1
NbSPL2/10/11_4Ub (miRNA156)		NtSPL	_2/10/11_2b (miRNA1
NbSPL3/4/5 2x (miRNA156)	0	NtSPL	_2/10/11_3a
NbSPL3/4/5_2v (miRNA156)		NtSPL	_3/4/5_1b (miRNA156
NbSPI 3/4/5 4a (miRNA156)		NtSPL	_3/4/5_2a (miRNA156



Gene editing of NbSPL13 gene subfamily results in delayed flowering in N. benthamiana



sgSPL1.5 sgSPL1.6



Flowering time

A) CRISPR/Cas9 construct for editing NbSPL13_1a gene

B) NbSPL13_1a structure

			NbSPL3/4/5_4b (miRNA156)	
			NbSPL3/4/5_5x (miRNA156)	
			NbSPL3/4/5_5y (miRNA156)	
			NbSPL3/4/5_6Ua (miRNA156)	
			NbSPL3/4/5_7a (miRNA156)	_
			NbSPL3/4/5_7b (miRNA156)	
			NbSPL6_1b (miRNA156)	
			NbSPL6_2a (miRNA156)	
			NbSPL6_2b (miRNA156)	
			NbSPL6_4a (miRNA156)	
			NbSPL6_4b (miRNA156)	-
			NbSPL6_5a (miRNA156)	
			NbSPL6_5b (miRNA156)	
			NbSPL7_1a	
			NbSPL7_1b	
			NbSPL8_1a	
			NbSPL8_1b	
			NbSPL9/15_1a (miRNA156)	-
			NbSPL9/15_1b (miRNA156)	
			NbSPL9/15_2a (miRNA156)	
			NbSPL9/15_2b (miRNA156)	1
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			NtSPL3/4/5 2b (miRNA156)
			NtSPL3/4/5 3Ub (miRNA15
			NtSPL3/4/5 4b (miRNA156)
			NtSPL3/4/5 6a (miRNA156)
			NtSPL3/4/5 6b (miRNA156)
			NtSPL3/4/5 7a (miRNA156)
			NtSPL3/4/5 7b (miRNA156
			NtSPL6 1a (miRNA156)
			NtSPL6_1b (miRNA156)
	-		NtSPL6_2a (miRNA156)
			NtSPL6_2b (miRNA156)
			NtSPL6_3Ua (miRNA156)
			NtSPI 6 4a (miRNA156)
			NtSPL6_4b (miRNA156)
			NtSPI 6 5a
			NtSPL6_5b (miRNA156)
			NtSPL7_1a
			NtSPL 7 1b
			NtSPL8_1a
			NtSPL8_1b
			NtSPL8 22
			NtSPL8_2b
			NtSPI 9/15 1a (miRNA156)
			NtSPI 9/15 1b (miRNA156)
			NtSPI 9/15 2b (miRNA156)
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C) Flowering time of NbSPL13 edited plants compared to WT. In total 4 groups of plants were analysed: NbSPL13_1a/1a/1b/1b (biallelic edition for both homeologous), NbSPL13_1a/1a/1b (heterozygous edition for 1b homeologous), NbSPL13_1a/1a (not edited in 1b). All of them showed a 4-5 days delay in flowering.

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A)

B)

C)

50-

F (100 DPS)

1a (miRNA156) b (miRNA156))/11 1a (miRNA156))/11 1b (miRNA156)

0/11_2a (miRNA156)

0/11_2b (miRNA156)