Characterization and isolation of T-DNA tagged banana promoters active during *in vitro* regeneration and low temperature stress

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Abstract

A genome-wide T-DNA tagging strategy was pursued for the characterization and isolation of novel banana promoters. Embryogenic cell suspensions were transformed *via Agrobacterium tumefaciens* containing a promoterless, codon-optimized luciferase gene either without (*luc*$^{+}$) or with an intron (*luc*$^{+INT}$) next to the right T-DNA border. Approximately 89,000 transgenic cell colonies were first screened for baseline luciferase (LUC) activity at 26°C two to three months after transformation. A 1.6- to 4.3-fold higher tagging frequency was obtained with the *luc*$^{+INT}$ containing vector (pKCKUL1) than with the *luc*$^{+}$ vector (pETKUL2). Screening in real-time then continued under controlled temperature conditions in which LUC activity was monitored during a gradual decrease to different low temperature (LT) treatments including 18°C, 16°C, 12°C and 8°C. Luciferase activation frequency in cell colonies subjected to 26°C and different LT treatments ranged from 0.17% to 2.69%. Observed patterns included an enhanced, decreased or *status quo* LUC activity at LT relative to the 26°C LUC activity. Transgenic cell colonies responsive to 8°C were regenerated to *in vitro* plants with a real-time screening for LT regulated LUC activation at each developmental stage. The tagged lines ET2-17 and ET2-42 showed an enhanced LUC activity at 8°C at the cell colony stage. However, LUC activity of the latter line was not up-regulated by LT anymore at the shoot induction and *in vitro* plant stages. On the other hand, in the tagged line ET2-17 the up-regulation of LUC activity by LT at cell colony stage (10.7- fold) remained present at the shoot induction stage (2.5-fold up-regulation) but was absent at *in vitro* plant stage. In addition, other promoter-tagged lines with different levels of LUC activity at 26°C at cell colony stage showed a decrease of LUC activity at 8°C during the subsequent stages of the *in vitro* regeneration process (lines ET2-34, ET2-85 and ET2-156).

The number of T-DNA inserts in ten independent promoter-tagged lines tested averaged 3.6 with a range from 1 (line ET2-34) to 5 (lines ET2-17 and ET2-156). Isolation of T-DNA flanking sequences was accomplished *via* TAIL-PCR and I-PCR. Sequence analysis of these flanks revealed the presence of direct tandem repeats, vector backbone and/or T-DNA rearrangements in up to half of the lines analyzed. The lines ET2-49, ET2-89, ET2-111 and ET2-156 contained in one RB T-DNA flanking (5'-tagged) sequence the enhanced CaMV 35S promoter with part of the selectable marker gene *neo* from the tagging construct. Continuity of sequence between the corresponding right and left border sequences was revealed by linking PCR using flanking sequence specific primers. *In silico* analysis of the four 5'-tagged banana sequences in line ET2-17 suggested that two candidate promoters were tagged. The 5'-tagged sequence in line ET2-34 harbored a repetitive region, while in line ET2-85 three 5'-tagged sequences were retrieved with one most likely linked to a gene and containing a near-canonical TATA box.

An RT-PCR approach was followed to identify and confirm the sequence that activated LUC expression, which is of paramount importance in lines carrying multiple T-DNA copies. A

forward primer annealing to the 5'-tagged sequence near (within 1 to 70 bp) the RB T-DNA sequence was employed in combination with a reverse primer complementary to the *luc*$^{+}$ gene. Transcriptional fusion between (the) 5'-tagged sequence(s) and the *luc*$^{+}$ gene was detected in tagged lines ET2-17, ET2-34, ET2-85 and ET2-156.

Candidate promoter sequences were cloned upstream of the *uid*A^{INT} reporter gene and back-transformed to banana. One (17-1) of two transcriptionally active tagged sequences in the promoter-tagged line ET2-17 was found active in back-transformed banana lines throughout *in vitro* development confirming its promoter characteristics. The LT up-regulation of promoter sequence 17-1 in back-transformed lines at early undifferentiated cell colony stage resembled that of the original promoter-tagged line ET2-17. In contrast, the 5'-tagged sequence 34-1 did not show promoter activity in back-transformed cultures irrespective of the temperature treatment and developmental stage. Finally, the promoter activity of the 5'-tagged sequence 85-1 in back-transformed lines was similar to the relatively strong activity in the original promoter-tagged line ET2-85.

In conclusion, T-DNA tagging has proven a reliable and reproducible method to characterize and isolate novel promoters in banana. Despite their relatively low activity in *in vitro* cultures compared to the maize ubiquitin promoter, the discovered promoters might be useful for banana improvement which warrants further research.