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Intron splicing in 5′ untranslated region of the *rolA* transcript in transgenic apple

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Summary

The *rolA* gene encoded on the Ri plasmid of *Agrobacterium rhizogenes* causes developmental alterations, including dwarfing characteristics in the transgenic plants. In an attempt to introduce dwarfing characteristics into apple rootstocks for breeding purposes, the *rolA* gene was incorporated into the apple rootstock M26 and obtained four transgenic clones. All the clones exhibited reduced growth compared to untransformed control plants but different degree of dwarfing and wrinkled leaves. In the present study, expression of the *rolA* gene was further investigated by analysing the structure of the *rolA* transcript and the levels of the *rolA* mRNAs from these clones. The nucleotide (nt) sequence of the *rolA* transcript showed two forms of the transcript: one, the unspliced form, was co-linear with the *rolA* sequence in the genomic DNA; the other was spliced mRNA in which an 85-base pair (bp) intron sequence in the 5′ untranslated region (5′UTR) was spliced out. The position of splicing is different from that in *Arabidopsis thaliana* but similar to the splicing site found in tobacco. The transcription start region of the *rolA* gene in apple was 206 bp upstream of that in Arabidopsis and 277 bp upstream to *Nicotiana tabacum* transcription start. A hairpin-like secondary structure and an upstream open reading frame (uORF) were revealed in the *rolA* 5′UTR. The levels of the *rolA* mRNA in the apple transgenic clones were analysed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). The results showed slight variation in the shoot tissues of the transgenic clones.

Abbreviations: bp, base pair; DIG, digoxigenin; dNTP, deoxyribonucleoside triphosphate; GUS, *ß*-glucuronidase; nt, nucleotide; RT-PCR, reverse transcription-polymerase chain reaction; uORF, upstream open reading frame; UTR, untranslated region

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Introduction

The plant pathogen *Agrobacterium rhizogenes* causes the hairy root disease of plants (Riker et al., 1930). During infection, the bacteria insert a part of their DNA (T-DNA) from the extrachromosomal Ri plasmid into the chromosomal DNA of infected plant cells (Zambryski et al., 1989). The *rolA* gene is one of the T-DNA genes encoded on the Ri plasmid of *A. rhizogenes* (White et al., 1985; Slightom et al., 1986). In order to understand the function of the *rolA* gene, Schmulling et al. (1988) and Sinkar et al. (1988) transformed the single *rolA* gene under its own promoter into tobacco (*Nicotiana tabacum*) and found that the transgenic tobacco plants displayed the phenotypic characteristics of wrinkled leaves and stunted growth with shortened internodes. These phenotypes were even more enhanced when the *rolA* gene was driven by the CaMV 35S promoter (Carneiro and Vilaine, 1993). Regulation of the *rolA* gene expression has been studied. Spatial and temporal regulations of the gene expression in transgenic tobacco have been investigated by promoter analysis (Guivarc'h et al., 1996). The results showed that the entire *rolA* promoter was composed of three domains, A, B and C. Domains B and C were sufficient to induce wrinkled leaves and short internodes. The domain C was responsible for the dwarfing phenotype with normal leaves. The regulation of *rolA* expression at the transcriptional or post-transcriptional levels has been investigated in *Arabidopsis thaliana* and tobacco (Magrelli et al., 1994; Spena and Langenkemper, 1997). It has been shown that the *rolA* gene contains an intron in its 5′ untranslated region (5′UTR) and intron splicing occurred in the plant cell. Structural difference between spliced and unspliced transcripts influenced, not the steady-state concentrations of *rolA* mRNA, but the *rolA* phenotype. The dwarfism correlates with the intron splicing. However, the differential expression of initiation of the *rolA* gene has been shown (Carneiro and Vilaine, 1993; Magrelli et al., 1994; Spena and Langenkemper, 1997), with different splicing donor sites (5′GT) in Arabidopsis and in tobacco. In order to introduce dwarfing characteristics into apple rootstocks for breeding purposes, the *rolA* gene has been transferred into the apple rootstock M26; four transgenic clones were obtained, all of them exhibiting reduced growth compared to the untransformed control plants. However, the degree of dwarfing and wrinkled leaves varied among the four clones (Holefors et al., 1998). The present study investigates the structure, intron splicing in 5′UTR and the expression level of the *rolA* transcript in the apple transgenic clones.

Materials and methods

DNA and RNA isolation

For DNA and RNA isolation, 5-week-old *in vitro* propagated transformed and untransformed shoots of the apple rootstock M26 were used. Plant genomic DNA was isolated according to Aldrich and Cullis (1993).

For RNA isolation in the present study, a modified method based on the hot phenol method (Pawlowski et al., 1994) and CsTFA isopycnic centrifugation was established. After LiCl precipitation and centrifugation, the pellet was dissolved in 500 μL of TE buffer in an Eppendorf tube. An equal volume of 5.7 M CsCl in 50 mM TE buffer, instead of CsTFA, was added to precipitate RNA. The tubes were stored on ice for 4 h. After centrifugation the pellet was dissolved in 200 μL of DMPC-H_2O.

Reverse transcription-polymerase chain reaction (RT-PCR)

Before reverse transcription-polymerase chain reaction (RT-PCR), the residual chromosomal DNA in the total RNA preparation was removed by an RNase-free DNase treatment. One μg of total RNA was reverse transcribed with AMV reverse transcriptase using the First Strand cDNA Synthesis Kit (Boehringer Mannheim, Germany). Oligo $p(dT)_{15}$ was used as primer for first strand cDNA synthesis. The resulting cDNA was diluted in sterile water to the concentration 12.5 ng/μL for PCR reaction. The reactions in which reverse transcriptase were omitted were used as the negative control for each clone. For determination of the transcription start site and sequencing of the *rolA* transcript, the following upstream primers were used: pr1: 5′-ACACAGCTTGAAAGGAAATCCG-3′(−306/−285 nt), pr2: 5′-GCCTAGCGTTCGGACAGCCA-3′(−180/−161 nt), pr3: 5′-AACGCTTCAA TACGGTGAG-3′(−100/−82 nt) and pr4: 5′-ATGGAATTAGCCGGACTAAACG-3′ (+1/+22 nt). The downstream primers were pr5: 5′-TTCAGGTCCACACAGCAACAAC-3′ (+524/+503 nt), pr6: 5′-CTGAAACAT-GAAATAGGTGC-3′ (−2/−21 nt), pr7: 5′-AGCTTATTTTAACTTAACAG-3′ (+383/+364 nt) and pr8: 5′-TCATATGACTATCTAATCT-3′ (+567/549 nt). The RT-PCR reaction mixture contained 1 μL of the cDNA, 2.5 μL of 10 × reaction buffer, 2.5 μL of 25 mM $MgCl_2$, 0.5 μL of 10 mM deoxyribonucleoside triphosphates (dNTPs), 0.3 μL of 20 μM of both 5′ and 3′ gene-specific primers described above and 0.2 μL Taq DNA polymerase (5 U/μL) in a total reaction volume of 25 μL. Amplification was performed in a thermal cycle (Perkin Elmer Gene AmpR 2400) with the following program: initial denaturation at 95 °C for 5 min followed by 30 cycles at 95 °C for 15 s, 59.2 °C for 15 s and 72 °C for 30 s. The program was ended with a final elongation step, at 72 °C for 5 min. A 15-μL sample of the PCR products was