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Research article

Small RNAs were involved in homozygous state-associated silencing of a marker gene (*Neomycin phosphotransferase II*: *nptII*) in transgenic tomato plants

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ABSTRACT

Homozygous state-associated co-suppression is not a very common phenomenon. In our experiments, two transgenic plants 3A29 and 1195A were constructed by being transformed with the constructs pBIN-353A and pBIN119A containing *nptII* gene as a marker respectively. The homozygous progeny from these two independent transgenic lines 3A29 and 1195A, displayed kanamycin-sensitivity and produced a short main root without any lateral roots as untransformed control (wild-type) seedlings when germinated on kanamycin media. For the seedlings derived from putative hemizygous plants, the percentage of the seedlings showing normal growth on kanamycin media was about 50% and lower than the expected percentage (75%). Southern analysis of the genomic DNA confirmed that the homozygous and hemizygous plants derived from the same lines contained the same multiple *nptII* transgenes, which were located on the same site of chromosome. Northern analysis suggested that the marker *nptII* gene was expressed in the primary and the hemizygous transformants, but it was silenced in the homozygous transgenic plants. Further Northern analysis indicated that antisense and sense small *nptII*-derived RNAs were present in the transgenic plants and the blotting signal of *nptII*-derived small RNA was much higher in the homozygous transgenic plants than that of hemizygous transgenic plants. Additionally, read-through transcripts from the *TRAMP* gene to the *nptII* gene were detected. These results suggest that the read-through transcripts may be involved in homozygous state-associated silencing of the *nptII* transgene in transgenic tomato plants and a certain threshold level of the *nptII*-derived small RNAs is required for the homozygous state-associated co-suppression of the *nptII* transgene.

1. Introduction

In genetically modified plants, the reliable and stable expression of transgenes is a prerequisite for the successful application of gene technology to agriculture. Previous studies have demonstrated that introduction of transgene containing sequences homologous to an endogenous gene into plants usually result in co-suppression of transgene and endogenous genes [1,2]. Co-suppression was first described for the chalcone synthase (CHS) gene in *petunia* [3], which was not unique to *CHS* but appeared to be a general phenomenon affecting many endogenous genes in sense transgenic tomato plants [4,5]. Gene co-suppression has been studied extensively in dicot plants [6] and is important to consider in transgene experiments [7,8]. The phenomenon was found in a considerable proportion of the transgenic plants transformed with sense constructs.

Co-suppression of homologous endogenous gene normally occurs in plants with high-level expression and/or multiple copies of the transgenes [2,9]. The complex nature of the experiments concerning co-suppression makes it difficult to define the molecular mechanisms involved. The mechanisms regarding gene co-suppression have been intensively investigated in plants [10]. Several mechanisms have been proposed to explain this phenomenon [6,11,12]. Endogenous mRNA levels contributed significantly to co-suppression [13,14]. Co-suppression has been shown to be involved in transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) [15–17], which both can be explained by RNA interference (RNAi) [18–20]. TGS was linked to methylation of the promoter sequence [21], while PTGS was

Abbreviations: CaMV, cauliflower mosaic virus; CHS, chalcone synthase; dsRNAs, double stranded RNAs; hpRNA, hairpin RNA; IR, inverted repeats; PTGS, post-transcriptional gene silencing; RdDM, RNA-directed DNA methylation; RNAi, RNA interference; siRNAs, small interfering RNAs; TGS, transcriptional gene silencing.

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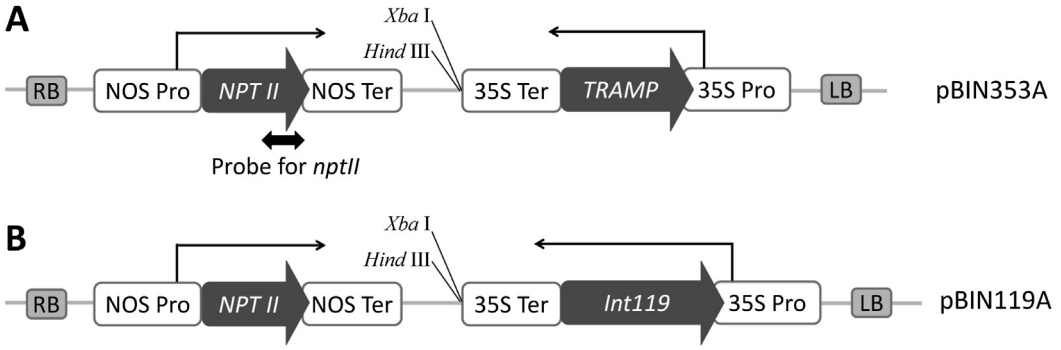

Fig. 1. Structures of T-DNA region of the pBIN353A (A) and pBIN119A (B) binary vector. The probe for Southern and Northern blot of *nptII* was indicated in T-DNA maps. LB, left T-DNA border region; RB, right T-DNA border region; NOS Pro, nopaline synthase promoter; *NPTII*, Neomycin phosphotransferase gene; NOS Ter, nopaline synthase terminator; 35S Ter, Cauliflower Mosaic Virus (CaMV) 35S terminator; *TRAMP*, tomato ripening associated membrane protein gene (GenBank accession No. X73848); 35S Pro, Cauliflower Mosaic Virus (CaMV) 35S promoter.

thought to be caused by sequence-specific RNA degradation [11]. PTGS, beginning with the expression and recognition of double stranded RNAs (dsRNAs) [22–24], was the manifestation of a mechanism that suppresses RNA accumulation in a sequence-specific manner.

Homozygous state-associated co-suppression is not a very common phenomenon and was reported in the transgenic tobacco plants [25,26]. Specific feedback regulation depending on gene product levels has been postulated [26]. The primary transgene was transcribed at a relatively high rate, and by duplication of these rates, transcript levels in homozygous plants reached a critical threshold which caused the suppression of both the transgene and the endogenous gene(s). The suppression of the marker gene *nptII* was found in doubly transformed tobacco plants, which contained one copy of the *nptII* transgene and was retransformed with the *HYG* gene encoding hygromycin resistance [21]. In the progeny of doubly transformed plants, the expression of *nptII* gene was often suppressed. This suppression could be correlated with methylation of the promoter of the *nptII* gene. They suggested that the homologous sequences in both T-DNAs were in some way responsible for the methylation in the doubly transformed plants. To date, not much attention has been paid to uncover the mechanisms regarding homozygous state-associated co-suppression.

In this study, we constructed two transgenic plants 3A29 and 1195A, containing *nptII* gene as a selective marker respectively. The homozygous progeny exhibited kanamycin-sensitivity and produced a short main root without any lateral roots as untransformed control (wild-type) seedlings when germinated on kanamycin media. Our experimental results suggest that the small RNAs may be involved in homozygous state-associated silencing of the *nptII* transgene in transgenic tomato plants and a certain threshold level of the small *nptII*-derived RNAs is required for the homozygous state-associated co-suppression of the *nptII* transgene.

2. Results

2.1. Southern analysis of T_1 transgenic plants from line 3A29 and line 1195A

In order to obtain homozygous progeny and to study the inheritance of the *nptII* transgene in the progeny from two independent transgenic lines 3A29 and 1195A (T_0 progeny), Southern analysis of genomic DNA was carried out on the T_1 progeny which were obtained by self-pollination of transformants 3A29 and 1195A. Genomic DNA was extracted from young leaf tissue of 12 T_1 plants in line 3A29, 10 T_1 plants in line 1195A and wild-type plants. Equal amounts (30 μg) of genomic DNA samples were digested with *Hind* III which have no cut sites in the *nptII* sequence, electrophoresed on a 0.8% (w/v) 1 × TAE agarose gel and analysed by Southern blot.

In line 3A29, when probed with the *nptII* coding sequence, there were three bands visible in 11 T_1 plants (3A29-2 to 3A29-12), but not any bands visible in one T_1 plant (3A29-1) and in the wild-type plant (Fig. 2A). These results indicated that the plant 3A29-1 might have lost the insert and be an azygous plant. The relative intensity of the bands was increased in plants 3A29-3 and 3A29-12, which suggests that these two progeny may be homozygous plants and that the other 9 T_1 plants may be hemizygous plants. In the line 1195A, when probed with the *nptII* coding sequence, there were also three bands visible in all of 10 T_1 plants, but not any bands visible in the wild-type plant (Fig. 2B). The relative intensity of the bands was increased in plants 1195A-1, 1195A-6 and 1195A-10, which suggests that they may be homozygous plants and that the other 7 T_1 plants may be hemizygous plants.

The same prominent bands were present in all T_1 transgenic plants from the same lines and this suggested that these copies of the transgene may be located on the same site of chromosome. It is possible that several segments of the T-DNA were co-integrated

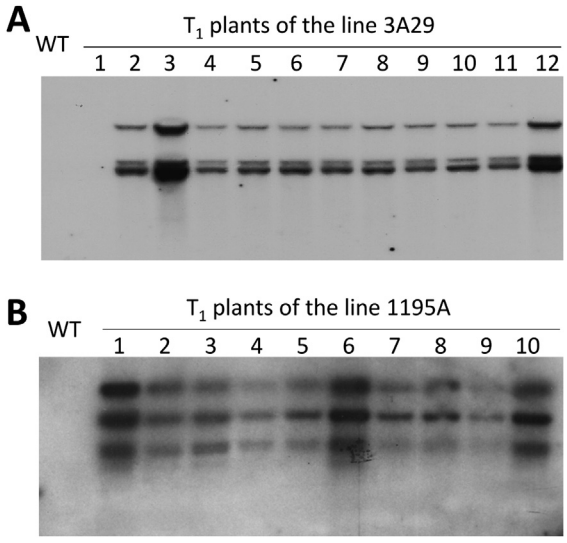

Fig. 2. The copy number of the T_1 progeny was analysed by Southern blot. The genomic DNA samples of 12 T_1 plants in line 3A29 (A), 10 T_1 plants in line 1195A (B) and wild-type plants extracted from young leaf tissue were digested with *Hind* III and probed with the *nptII* coding sequence.