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Silencing of antibody genes in plants with single-copy transgene inserts as a result of gene dosage effects

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Abstract The stability of Fab antibody fragment expression during plant development was studied using two homozygous Arabidopsis thaliana lines that contain single copies of the transgenes. These lines exhibited expression characteristics that are typical for homology-based post-transcriptional gene silencing. Their developmental silencing profiles differed markedly, presumably due to the influence of the genomic context on the T-DNAs. In both lines, a clear gene dosage effect could be observed: in contrast to the homozygous lines, derived hemizygous plants accumulated high levels of Fab fragments throughout development. Interestingly, silencing also occurred in double-hemizygous plants, which resulted from a cross between the two homozygous lines and had two copies of each T-DNA at non-allelic positions in their genome. In all cases, down-regulation of the Fab levels was strictly correlated with methylation of cytosine residues in the transcribed regions of the transgenes. Remarkably, this methylation was also found in regions in which the transgenes were non-homologous regions. Finally, the time point of down-regulation depended on the culture conditions and differed for leaves and roots of the same transgenic plant.

Keywords Gene dosage · Single-copy inserts · Transgene silencing · Threshold level

Introduction

Plants can synthesize several types of recombinant proteins with high fidelity. Although economically useful accumulation levels have been reported, the stability of accumulation levels is as important as the levels themselves. Indeed, introduced transgenes, especially when they are organized in repeat structures, are frequently silenced, resulting in dramatically decreased protein accumulation levels (for an overview, see De Wilde et al. 2000). Beyond any doubt, this gene silencing phenomenon hampers the general economic exploitation of plants as protein factories.

Previously, we have analyzed the stability of antibody and Fab production in various homozygous transgenic lines of Arabidopsis. Each line had a different and specific instability profile of antibody production due to silencing of the antibody transgenes (De Neve et al. 1999). Lines kd 12 and kd 27 are independent lines that each possess one copy of the K T-DNA and one copy of the H T-DNA at different, unlinked positions in their genome. Both lines have no vector backbone sequences linked to the T-DNAs and are homozygous for both T-DNAs. These lines exhibited expression characteristics that are typical for homology-based post-transcriptional gene silencing. In leaves of line kd 12, Fab production is developmentally down-regulated and down-regulation is meiotically reversible, whereas in leaves of line kd 27, accumulation levels seem to be low early on in development (De Neve et al. 1999). Given the observed protein and mRNA accumulation profiles in these lines and in their segregants, the single transgene inserts were proposed to trigger post-transcriptional gene silencing when expressed to a high level (De Neve et al. 1999). Here, we report a detailed developmental analysis of the accumulation levels and the corresponding DNA methylation patterns. We compared the accumulation levels in homozygous, hemizygous, and double-hemizygous plants to identify whether it is gene dosage and the associated high-level expression, or rather the presence...
of a second, allelic transgene copy that is the factor that triggers gene silencing. Furthermore, we analyzed whether gene silencing can be triggered at different time points in different organs of a given line. In addition, we show that culture conditions can influence the timing of down-regulation of the transgenes.

Materials and methods

Plant material

The homozygous transgenic plant lines kd 12 and kd 27 were obtained as described previously (De Neve et al. 1993). The K T-DNA contains a kanamycin resistance gene and an expression cassette for the light (α) chain of the MAK33 antibody or Fab (De Neve et al. 1993); the H T-DNA contains a hygromycin resistance gene and an expression cassette for the Fd chain of the MAK33 Fab. The immunoglobulin-encoding expression cassettes are homologous in different functional regions; the 35S promoter (approximately 950 bp) and the 3′ ends (approximately 300 bp) are common to both cassettes, and the α and Fd mRNAs share a 5′ terminal 116-nucleotide-long sequence and a 3′ terminal sequence of 204 nucleotides downstream of the stop codon. In line kd 12, an internal part of the K T-DNA of at least 400 bp was found to be duplicated and fused to the T-DNA left border. Seed stocks of hemizygous plants derived from line kd 27 and kd 12 were obtained by crossing the kd 27 line with a non-transformed Arabidopsis thaliana (L.) Heynh. C24 plant and by crossing two homozygous lines, k 12 and d 12, each possessing one of the two different T-DNAs, respectively. In addition, a seed stock of plants containing the K T-DNA derived from line kd 12, and the H T-DNA derived from line kd 27, both in a hemizygous state, was obtained by crossing the segregated homozygous lines k 12 and d 27. Finally, a line with two copies of each T-DNA at different chromosomal positions was obtained by crossing the homozygous lines kd 12 and kd 27.

Plant tissue culture

Seeds were germinated on selective solid standard medium containing MS salts and sucrose, and the plants were transferred to soil (greenhouse) 3 weeks after sowing. Alternatively, seeds were sown directly on soil in the greenhouse or were germinated in liquid medium, containing Gamborg’s B5 salts and 2% sucrose, in an Erlenmeyer flask, and grown under axenic conditions with continuous gentle shaking. Plants were grown at 22°C on a 16 h day/8 h night cycle.

Preparation of protein extracts and determination of total soluble protein levels

To construct a developmental Fab accumulation profile, plant material was isolated at fixed time points. For the analysis of Fab accumulation levels in leaves and roots, different plants from the same seed stock were extracted at each developmental point. At least five plants per seed stock were pooled. For the developmental analysis of Fab accumulation levels and the corresponding transgene methylation patterns, leaves of different sizes were isolated from six particular plants per seed stock at each time point. Alternatively, different plants from one homozygous seed stock were pooled and used for the analysis of Fab accumulation levels and the corresponding transgene methylation patterns 3 and 10 weeks after sowing.

Protein extracts were prepared from fresh plant material and the total soluble protein (TSP) content was determined using the Bio-Rad protein assay as described (De Neve et al. 1999).

MAK33 sandwich ELISA

Antiserum specific for the MAK33 antibody was produced in rabbit. IgGs were isolated and a sandwich ELISA was set up and performed as described by Bruyns et al. (1998). As a standard, a hybridoma-derived MAK33 Fab fragment (Roche Diagnostics, Brussels, Belgium) was used.

DNA extraction and Southern analysis

Simultaneously with the isolation of leaf material for the analysis of the Fab fragment accumulation, leaves at different developmental stages were collected from the six analyzed plants per seed stock, frozen in liquid nitrogen, and stored at −70°C until the day of DNA extraction. DNA was isolated and Southern analysis was performed on 0.75-μg aliquots of DNA as described by De Neve et al. (1997), after cleavage with EcoRV, SspI, Sau3AI, HpaI, or CiaI. Hybridization was performed with probes for the α- and the Fd-coding regions according to De Neve et al. (1997). The probes were labeled using the Gene Images random prime labeling kit (Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK) and hybridization and detection were carried out according to the protocol supplied with the Gene Images CDP-Star detection module (Amersham Pharmacia Biotech).

Results

Fab accumulation levels in leaves and roots of homozygous plants grown on solid medium

In leaves of plants of the homozygous line kd 12, high levels of Fab accumulation were detected 2 and 3 weeks after sowing. From week 5 on, Fab accumulation levels were up to 25-fold lower and remained low until 10 weeks after sowing (Fig. 1A). Root extracts of plants of this line showed low to intermediate accumulation levels at 2 weeks, high levels at 3 weeks, and more than 10-fold lower levels at 5–7 weeks after sowing. Ten weeks after sowing, accumulation levels were again relatively high (Fig. 1A).

In leaves of plants of the homozygous line kd 27, low Fab accumulation levels were detected at every time point analyzed (Fig. 1A). In contrast, root extracts of this line showed an intermediate, high, and approximately 10-fold lower Fab accumulation level 2, 3, and 5–7 weeks after sowing, respectively. Ten weeks after sowing, accumulation levels were again relatively high (Fig. 1A).

To monitor the potential effect on Fab accumulation levels of transfer of plants from axenic to greenhouse conditions, seeds of the homozygous line kd 12 were either sown on solid medium and transferred to soil 3 weeks later, or sown directly on soil; no essential differences could be detected (data not shown).

Fab accumulation levels in leaves and roots of plants grown in liquid medium

With a view to using plants grown in liquid culture for the secretion of recombinant proteins into the medium