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Nuclear and cytoplasmic sites for anti-sense control

Marc Cornelissen

Plant Genetic Systems NV, J. Plateaustraat 22, B9000 Gent, Belgium

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ABSTRACT

In transgenic tobacco, anti-sense control of the chimaeric *bar* (bialophos resistance) gene is manifested by a reduced *bar* transcript level and a reduced synthesis of *bar* translational product (PAT, phosphinothricin acetyl transferase) per transcript (1). This study shows that the anti-*bar* gene controls the transcript level and the translation efficiency of the *bar* mRNA through independent events and at different cellular sites. On the basis of these results a model for the mechanism of anti-sense control is proposed.

INTRODUCTION

Recently, a number of reports confirmed the potential of artificial anti-sense control in plants (reviewed by 2). The expression of anti-sense genes caused in all cases a significant reduction of the target RNA and protein levels. However, little is known about the nature of gene regulation by anti-sense RNA in eucaryotes. By analogy with procaryotic natural anti-sense control, it is believed that transcripts with a distinct sequence complementarity base-pair. RNA duplex formation would interfere with the expression pathway and could result in either nuclear transcript degradation (*Dyctiostelium*, 3), block of transcript transport to the cytoplasm (mouse L cells, 4) or translation inhibition (*Xenopus* oocytes, 5).

We have shown previously (1) that in transgenic tobacco the anti-*bar* gene controls *bar* expression by reduction of the *bar* mRNA levels and the synthesis of PAT per *bar* mRNA. This study focuses on the question whether different cellular sites are involved in this regulatory process. It is shown that the reduced translation efficiency and the reduced abundance of the *bar* mRNA are the result of two independently occurring interactions between sense and anti-sense mRNA.

MATERIALS AND METHODS

Tissue culture

Isolation of SR1(T-GSFR166) and SR1(T-GSFR166, T-GSC1) was described previously (1). SR1(T-GSFR166,T-GSC1/3) was obtained by introduction of the T-DNA of pGSC3 in SR1(T-GSFR166, T-GSC1) via *Agrobacterium* mediated T-DNA transfer. Transformants were identified by their ability to grow on medium containing 20 mg/l chloramphenicol. Protoplast preparation was carried out essentially as described (6).

DNA manipulations

pGSC3 is a pGSC1 derivative (1). The filled-in Asp718-HindIII fragment of pDE118 (a gift of J.Denecke) containing the $P_{TR2'}$*cat* (chloramphenicol acetyltransferase) gene was ligated into the StuI-HindIII fragment of pGSC1 carrying the anti-*bar* gene, to replace the P_{nos}*nptII* gene as plant selectable marker.

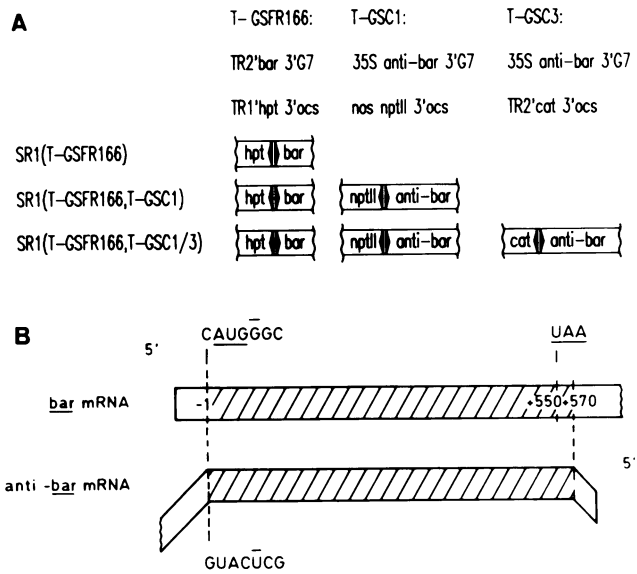

Figure 1. Schematic presentation of the anti-sense model system. (a) SR1(T-GSFR166) which harbours two copies of a T-DNA carrying the *bar* and *hpt* genes, was supertransformed with two copies of a T-DNA containing an anti-*bar* gene, generating SR1(T-GSFR166,T-GSC1) (1). SR1(T-GSFR166,T-GSC1/3) was obtained by introduction of two copies of the T-DNA of pGSC3 in SR1(T-GSFR166,T-GSC1) via *Agrobacterium* mediated T-DNA transfer. Southern blot analysis indicated that none of the T-DNAs integrated during the previous two transformations, had undergone rearrangements during the third transformation cycle (not shown). Construction of pGSC3 is described in Materials and Methods. (b) Sequence complementarity between *bar* and anti-*bar* RNA is indicated by hatched boxes. Mismatching nucleotides are overlined, translation start and stop codons are underlined. Numbering is relative to the first nucleotide of the translation initiation codon.

RNA manipulations

Total RNA was extracted from protoplast samples frozen in liquid nitrogen as described (1). Cytoplasmic RNA was extracted from protoplasts which were washed in 250 mM NaCl. Subsequently 2 ml extraction buffer I (10 mM Tris-HCl pH 9.0, 150 mM NaCl, 1.5 mM $MgCl_2$, 12.5 mM EGTA, 5 mM Dithiotreitol, 0.65% NP-40) and 1000 units RNAguard (Pharmacia) were added to 10^7 protoplasts. The cells were gently vortexed for 10 seconds and centrifuged for 1 minute at 10000 g (HB4), 4°C. The supernatant was collected and 2 ml extraction buffer II (1% SDS, 350 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl pH 7.5, 7 M urea), 2 ml phenol and 2 ml chloroform were added. After extraction, cytoplasmic RNA was precipitated with ethanol and dissolved in H_2O.

Filters and hybridisations with ^{32}P labeled riboprobes complementary to *bar* and *hpt* mRNA (1) were done according to Amersham protocols.

Transcription inhibition

A mixture of 0.4 ml actinomycin D (5 mg/ml; Boehringer) and 1 ml chloroquine (20 mg/ml; Sigma) was added to 50.10^6 protoplasts in 15 ml of medA (6). At 30 minutes intervals, samples of 3 ml were taken and frozen in liquid nitrogen.

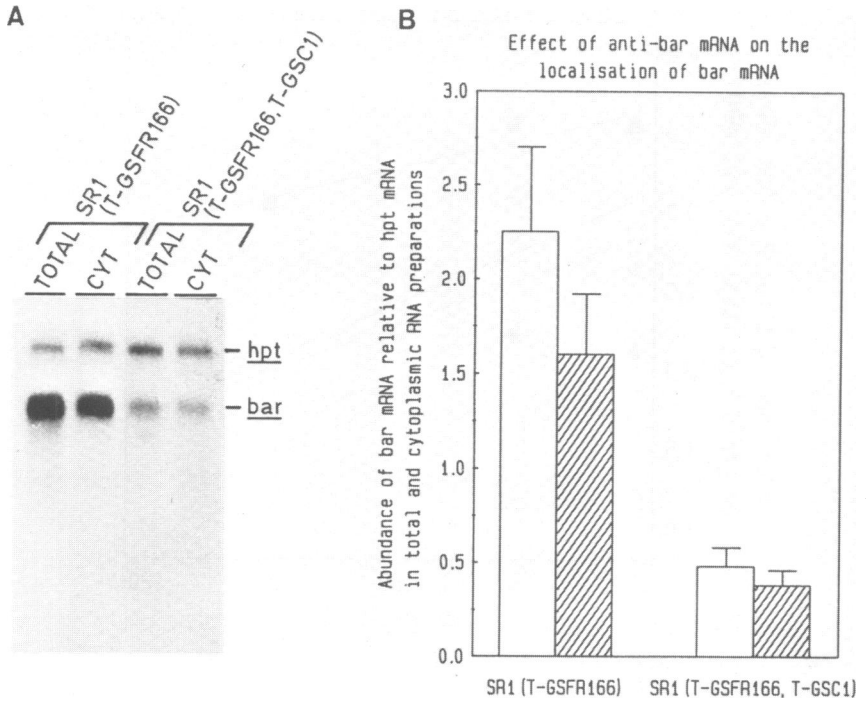

Figure 2. Localisation of *bar* and *hpt* transcripts in total and cytoplasmic RNA preparations of leaf protoplasts of SR1(T-GSFR166) and SR1(T-GSFR166,T-GSC1) by northern (a) and slot blot analysis (b). (a): Equal amounts of RNA were applied to each lane. (b): Open bars: total RNA, hatched bars: cytoplasmic RNA. Abundances were determined by slot-blot analysis with the aid of calibration curves obtained with dilution series of cold *bar* and *hpt* transcripts as described (1). The error in the abundances is ± 10%.

RESULTS

Anti-bar mRNA interferes with translation of bar mRNA

In leaf protoplasts, expression of the anti-*bar* gene results in a respectively four and three fold reduction of the abundance and the translation efficiency of the *bar* mRNA (1). Although approximately equimolar amounts of *bar* and anti-*bar* mRNA are present in the protoplasts, accumulation of duplex RNA was not detected.

The reduced synthesis of PAT per *bar* mRNA can be explained by either a reduced translation efficiency of the target mRNA in the cytoplasm or by a block in the transport of the target mRNA to the cytoplasm. To distinguish between these two possibilities, the abundance of *bar* mRNA in cytoplasmic and total RNA preparations from leaf protoplasts of SR1(T-GSFR166) and SR1(T-GSFR166,T-GSC1) (Fig. 1) was determined. The abundance of *hpt* (hygromycin phosphotransferase) mRNA present in each preparation was measured to provide an internal standard. Figure 2a clearly shows that the *hpt* mRNA level is comparable in the single and double transformant whereas the *bar* mRNA level is significantly reduced in the double transformant. Both *hpt* and *bar* mRNA are localised mainly in the cytoplasm, independent of the presence of anti-*bar* mRNA. Thus the ratios between the *bar* and *hpt* mRNA levels in the total and cytoplasmic RNA fractions of both

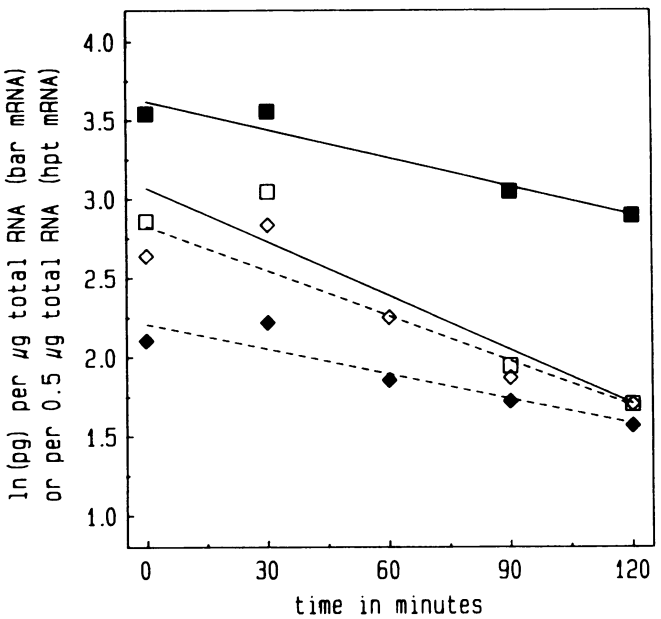

Figure 3. Degradation profile of *bar* and *hpt* mRNAs in the presence and absence of anti-*bar* mRNA after addition of transcriptional inhibitors. SR1(T-GSFR166): solid lines, filled squares: *bar* mRNA, open squares: *hpt* mRNA; SR1(T-GSFR166,T-GSC1): dashed lines, filled diamonds: *bar* mRNA, open diamonds: *hpt* mRNA. Linear regression indicates a half-life for *bar* mRNA of 135 and 119 minutes in the presence and absence of anti-*bar* mRNA, respectively. For *hpt* mRNA, these values are 75 and 62 minutes indicating that the stability of *bar* mRNA relative to that of *hpt* mRNA (135/75 = 1.8 and 119/62 = 1.9) is identical in both cell types. Abundances were determined by slot-blot analysis with the aid of calibration curves obtained with dilution series of cold *bar* and *hpt* transcripts as described (1). Inhibition of transcription was achieved by adding a mixture of 0.4 ml actinomycin D (5 mg/ml) and 1 ml chloroquine (20 mg/ml) to 50.10^6 protoplasts in 15 ml of medA (6). At 30 minutes intervals, samples of 3 ml were taken and frozen in liquid nitrogen. As a negative control, RNA was extracted from protoplasts incubated in the absence of transcriptional inhibitors at t = 0 and t = 90 minutes (not shown).

the single and double transformant are comparable (Fig. 2b). This implies that *bar* mRNA accumulates in the cytoplasm irrespective of the presence of anti-*bar* mRNA. Mechanistically it implies that in the cytoplasm translation of *bar* mRNA is hindered by anti-*bar* mRNA. Since no accumulation of duplex mRNA is detected (1), the interference must result from unstable interactions.

Abundance and translation efficiency of the bar mRNA are controlled by independent events

A next question is whether the reduced translation efficiency of the *bar* mRNA is somehow related to its decreased cytoplasmic abundance. To this end, the cytoplasmic stability of *bar* mRNA in SR1(T-GSFR166) and SR1(T-GSFR166,T-GSC1) protoplasts was analysed. Leaf protoplasts of both types were incubated in the presence of a mixture of the transcriptional inhibitors actinomycin D and chloroquine. Samples were taken at 30 minutes intervals and analysed for their mRNA contents. The amount of *hpt* mRNA diminished in time at similar rates in both the single and double transformant (Fig. 3) whereas non-treated samples did not display such decrease (not shown). The half-life of *bar* mRNA

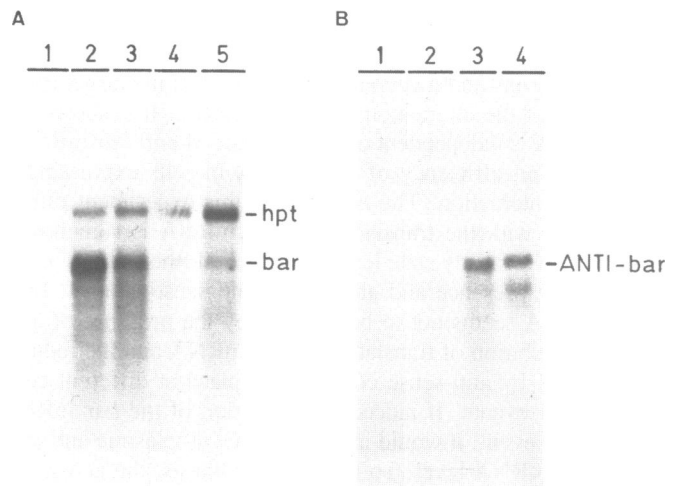

Figure 4. The effect of the anti-*bar* gene dosage on the *bar* mRNA level. RNA was extracted from leaf protoplasts incubated for one day at low light intensity at 24°C. Under these conditions the *bar*, anti-*bar* and *hpt* genes direct rather constant mRNA levels. (a) Northern blot analysis of total RNA of SR1 (lane 1), SR1(T-GSFR166) (lane 2), SR1(T-GSFR166,T-GSC1) (lane 3) and SR1(T-GSFR166,T-GSC1/3) (lanes 4 and 5). Each lane contains 2.5 μg total RNA with the exception of lane 5 which carries 5 μg. Hybridisations to *bar* and *hpt* mRNA are indicated. (b) Northern blot analysis of 2.5 μg total RNA extracted from SR1 (lane 1), SR1(T-GSFR166) (lane 2), SR1(T-GSFR166,T-GSC1) (lane 3) and SR1(T-GSFR166,T-GSC1/3) (lane 4). Hybridisation to anti-*bar* mRNA is indicated.

in these samples was significantly longer than that of *hpt* mRNA and was independent of the presence of anti-*bar* mRNA. The measurements probably do not yield the actual half-lives as inhibition of transcript formation places the cells under stress. However, the different half-life values for *bar* and *hpt* mRNA indicate that the decreases in transcript abundances are due to inhibition of transcription. The identical half-life of *bar* mRNA in the presence and absence of anti-*bar* mRNA is, therefore, biologically significant. It strongly suggests that the reduction of the *bar* mRNA level by anti-sense control is not caused by a cytoplasmic event, but results from an interaction at an earlier stage in the expression pathway.

The bar mRNA level is a function of the anti-bar gene dosage

This latter observation leads to the hypothesis that the level of anti-*bar* mRNA in the cytoplasm does not reflect the extent of reduction of the *bar* mRNA level. To test this idea, the effect of a further increase of the anti-*bar* gene dosage on the *bar* mRNA level was analysed. Two additional copies of the anti-*bar* gene were introduced into SR1(T-GSFR166,T-GSC1), yielding SR1(T-GSFR166,T-GSC1/3) (Fig. 1). Northern blot analysis (Fig. 4) and slot blot analysis (not shown) of total RNA extracted from leaf protoplasts of SR1(T-GSFR166,T-GSC1/3) revealed that the increase in the number of anti-*bar* genes resulted in a four fold reduction of the *bar* mRNA level relative to that of SR1(T-GSFR166,T-GSC1). However, the anti-*bar* mRNA level had only slightly increased. This shows that indeed the reduction of the *bar* mRNA level is not a function of the steady state level of the anti-*bar* mRNA but may be rather controlled by the number of anti-*bar* transcripts synthesized.

DISCUSSION

In a previous study, we demonstrated that anti-sense control of the *bar* gene is manifested by reduced target RNA levels and a decreased translation of the target mRNA (1). In the present work the nature of the interaction is studied further. It is shown that the cellular distribution of *bar* mRNA is independent of the presence of anti-*bar* mRNA. This implies that the reduced translation efficiency of *bar* mRNA in cells expressing anti-*bar* RNA is due to a cytoplasmic interaction. The outcome of this experiment raised the question whether the interference with the translation of *bar* mRNA is somehow related to the reduction of the *bar* mRNA steady state level. To this end, the stability of the *bar* mRNA was analysed both in the presence and absence of anti-sense control. Interestingly, the half-life of the *bar* mRNA seems not to be affected by the presence of anti-*bar* mRNA. This suggests that the inhibition of translation of *bar* mRNA and the reduction of the *bar* mRNA steady state level by anti-sense control take place at different cellular locations and thus are independent events. If indeed the reduction of the *bar* mRNA steady state level is not a cytoplasmic event, it would imply that the cytoplasmic anti-*bar* mRNA level is not related to the *bar* mRNA level. To test this hypothesis, the anti-sense gene dosage was increased by an additional transformation cycle. Analysis of total RNA revealed that the increase of anti-sense genes resulted specifically in a further decrease of the target mRNA level, whereas the anti-*bar* mRNA level remained approximately constant. Mechanistically this may imply that the target mRNA level is controlled by the amount of anti-sense RNA synthesized and that the final levels of sense and anti-sense mRNA present in the cytoplasm represent mRNAs which escaped a destructive interaction.

On the basis of the present results and our previous data (1) a model for anti-sense gene regulation can be proposed. Anti-sense control of a target gene results in a reduced cytoplasmic target mRNA level. This is not due to an increased turnover of the target mRNA in the cytoplasm, but is more likely the result of a reduced transport from the nucleus to the cytoplasm. It can be envisaged that in the nucleus a complementary transcript either interferes with synthesis and/or processing of a nascent target transcript, or triggers degradation by base-pairing to free or nascent transcripts. At this level 'sense and anti-sense transcripts' are indistinguishable as the interaction will apply to transcripts of both types. Transcripts which appear in the cytoplasm are probably those that escaped the inhibitory interaction. If both sense and anti-sense transcripts accumulate in the cytoplasm, translation of the target transcripts becomes less efficient. The underlying mechanism seems not to be based on stable duplex formation of the complementary transcripts and remains to be elucidated.

The impact of each of the two inhibitory mechanisms may differ for each set of complementary genes. The results indicate that the different mechanisms of inhibition previously observed (3–5) do not exclude each other, but are typical for the experimental conditions and the genes under study.

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