

# Tolerance to, and Uptake and Degradation of 2,4,6-Trinitrotoluene (TNT) are Enhanced by the Expression of a Bacterial Nitroreductase Gene in *Arabidopsis thaliana*

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Z. Naturforsch. **60c**, 272–278 (2005)

*Arabidopsis thaliana* was transformed with a gene encoding a nitroreductase (NTR, E.C.1.6.99.7) with activity against a wide range of nitroaromatic compounds. The gene was transferred from *Escherichia coli* by an *Agrobacterium*-mediated *in planta* method. The obtained seeds were sowed to produce T1 plants, and they were assayed for the integration of the transgene in the plant genome. Transgenic plants that were positive with the PCR analysis were self-pollinated to produce T2 generation plants. Seven lines obtained were assayed for the NTR activity. While the non-transformed wild-type plants showed no detectable NTR activity, the enzyme activity of the transgenic plant lines was approx. 20 times higher. Using the line with the highest NTR activity, the phytoremediation characteristics of plants against 2,4,6-trinitrotoluene (TNT) was investigated. While the wild-type plants did not grow in the presence of 0.1 mM TNT, the transgenic plants grew almost normally in this condition. The uptake of TNT by seedlings of transgenic plants increased by 7 to 8 times when they were floated on TNT solution. HPLC analysis showed that the peak due to TNT taken up into plant body was much smaller in the transgenic plants as compared with that of the wild type, and that a number of peaks attributable to the degradation products of TNT, including 4-amino-2,6-dinitrotoluene, were detected in the extract from the transgenic plants. This indicates that the expression of bacterial NTR improved the capability of plants to degrade TNT.

**Key words:** Nitroreductase (TNT and NTR), Transgenic Plant, *Arabidopsis*, *nfsA*

## Introduction

Nitroaromatic compounds have been widely utilized in a number of industrial processes including the production of munitions, plastics, pharmaceuticals, textile dyes, and agricultural chemicals (Harter, 1985) and improper handling of them has led to the contamination of soil and groundwater in many countries (Spain, 2000; Esteve-Nunez *et al.*, 2001). The explosive 2,4,6-trinitrotoluene (TNT) is one of the most highly toxic and persistent nitroaromatic compounds. The toxic effects of TNT have been studied in several organisms; TNT reportedly causes liver damage and aplastic anemia in humans (Kaplan and Kaplan, 1982).

Nitroreductase (NTR, E.C.1.6.99.7) is an enzyme that utilizes NAD(P)H as an electron donor to catalyze a two-electron reduction of TNT to hydroxylaminodinitrotoluene (HADNT), which is subsequently reduced to aminodinitrotoluene (ADNT) derivatives. NTR genes have been reported from several microbes (Watanabe *et al.*, 1990; Bryant *et al.*, 1991; Zenno *et al.*, 1996; Goodwin *et al.*, 1998; Anlezark *et al.*, 2002). However, no NTR genes have so far been reported from plants.

Hannink *et al.* (2001) have reported that detoxification capability of tobacco against TNT is increased by the expression of NTR gene from *Enterobacter cloacae*. This study paved the way for the use of bacterial NTR genes for phytoremedia-

tion of nitroaromatic contaminants in the environment. We therefore investigated production and characterization of transgenic plants that bear the *nfsA* gene from *Escherichia coli*.

## Materials and Methods

### Chemicals

2,4,6-Trinitrotoluene (TNT) was purchased from ChemService (West Chester, PA, USA). External standards for TNT for HPLC analysis of degradation products of TNT such as 2-hydroxylamino-4,6-dinitrotoluene (2HA46DNT), 4-hydroxylamino-2,6-dinitrotoluene (4HA26DNT), 4-amino-2,6-dinitrotoluene (4A26DNT), 2-amino-4,6-dinitrotoluene (2A46DNT), 2,6-diamino-4-nitrotoluene (26DA4NT), and 2,4-diamino-6-nitrotoluene (24DA6NT) were obtained from AccuStandard (New Haven, CT, USA).

### Construction of plasmid

A plasmid pNAC in which a gene was encoding the major nitroreductase (*nfsA*) in *Escherichia coli* (Zenno *et al.*, 1996) was cloned between the *Bam*H I and *Sma* I sites of pUC19. The *nfsA* gene was excised from pNAC by *Sac* I and *Xba* I and inserted into the corresponding restriction sites of pChlCOD (Sakamoto *et al.*, 1998), a binary vector for *Agrobacterium*, bearing the kanamycin-resistant gene (*nptII*) and the hygromycin-resistant gene (*hph*). The resultant plasmid was designated pIGNFSA, in which each of the three genes *nfsA*, *nptII* and *hph* is under the control of cauliflower mosaic virus 35S promoter and nopaline synthase termination sequence.

### Transformation of *Arabidopsis*

pIGNFSA was introduced into *Agrobacterium tumefaciens* strain C58/pMP90 (Koncz *et al.*, 1984) by the freeze-thaw method (Chen *et al.*, 1994). *Arabidopsis thaliana* ecotype C24 was transfected with the *Agrobacterium tumefaciens* cells bearing pIGNFSA by the vacuum infiltration method (Araki and Kaya, 2000). Transfected plants were selected for kanamycin and hygromycin resistances, and these were allowed to set seeds. These seeds were sowed to produce the T1 generation. Putatively transformed T1 plants were analyzed for the integration of the transgene in the genome. Plant genomic DNA was isolated according to Ta-

kahashi *et al.* (2001), and subjected to PCR analysis. The following primer pair specific to the *nfsA* gene which gives a 824-bp amplified signal was used: forward, 5'-GCTGATGAACCGTCCACC-3'; reverse, 5'-AATTTTCACCCTGCACCTCTC-3'. PCR conditions comprised of 30 cycles of DNA denaturing at 94 °C for 1 min, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s. A number of PCR-positive T1 plants were self-pollinated, and T2 seeds were obtained. T2 plant line whose siblings were homozygous both in kanamycin and hygromycin resistances was regarded as a homozygous line, and seven such lines were used for further study.

### Determination of NTR activity

Fresh shoots of 7-week-old wild-type and transgenic plants were separately frozen with liquid nitrogen and homogenized in a mortar with a pestle in the presence of a buffer (pH 7.5) containing 100 mM Tris [tris(hydroxymethyl)amino-methane]-HCl, 20% (v/v) glycerol, 50 mM ascorbic acid, 2.5 mM EDTA, 5 mM DTT (dithiothreitol), and 1% (w/v) PVPP (200  $\mu$ l per 100 mg fresh shoots). The homogenate was centrifuged at 11,000  $\times$ g for 20 min, and the supernatant was used for enzyme activity assay. Protein in the supernatant or crude enzyme solution was assayed by Bradford (1976). A 7.5  $\mu$ l of the enzyme solution containing 2.25  $\mu$ g protein was added to 142.5  $\mu$ l of reaction mix containing 25 mM Tris-HCl, 10% (v/v) glycerol, 1 mM ascorbic acid, 2 mM DTT, 10 mM NADPH, and 0.2 mM nitrofurazone. The mix was incubated for 40 min at 30 °C. The absorbance at 400 nm ( $\epsilon = 12.96 \mu\text{M}^{-1} \text{cm}^{-1}$ ), corresponding to the amount of remaining nitrofurazone, was determined, from which the NTR activity was estimated.

### Test for TNT tolerance of transgenic plants

Ten seeds each of the wild-type and transgenic line were surface-sterilized with 2.5% (v/v) sodium hypochlorite, placed on square Petri dishes containing MS medium (Murashige and Skoog, 1962) with or without 0.1 mM TNT, allowed to germinate, and grown vertically under a 15-h light (70  $\mu\text{m photons m}^{-2} \text{s}^{-1}$ ) and 9-h darkness cycle at  $22 \pm 0.2$  °C for 21 d. TNT was dissolved in *N,N*-dimethylformamide (DMF) before being added to the culture medium.

### Uptake and degradation of TNT

Ten seeds each of the wild-type and transgenic line were surface-sterilized with 2.5% (v/v) sodium hypochlorite, placed in Erlenmeyer flasks containing 50 ml MS medium, and allowed to germinate and grow under the same conditions as described above for 10 d except that flasks were shaken at 120 r.p.m. using a rotary shaker (GYROTORY® shaker-model G2, New Brunswick Scientific, Edison, NJ, USA). Seedlings were then transferred (floated) aseptically onto a 50-ml MS medium in flasks with or without 0.25 mM TNT. They were incubated for 7 d more under shaking as described above.

TNT in the medium was quantified daily by using a 2690 HPLC system (Waters, Milford, MA, USA) equipped with a 4.6 mm × 250 mm C18 L-column (CERI, Tokyo, Japan) and 996 photodiode array detector (Waters, Milford, MA, USA). Medium samples were filtered through a 0.20- $\mu$ m Millex-LG filter (Millipore, Billerica, MA, USA), and 10  $\mu$ l of each sample were injected into the column. The sample was separated at room temperature with linear gradient mobile phases of 20% to 60% (v/v) methanol fed at a flow rate of 1 ml min<sup>-1</sup>. The absorbance at 230 nm was determined, and from this value the uptake of TNT by plants was quantified.

At the end of the culture in the presence of TNT, plants were harvested and separated into shoots and roots. They were then homogenized under liquid nitrogen in a mortar with a pestle, after which the homogenate was recovered with methanol (300  $\mu$ l per 100 mg fresh tissues) into a flask, and shaken for 24 h at 180 r.p.m. The extract was centrifuged and the resulting supernatant was analyzed for TNT and its degradation intermediates by HPLC under the conditions as described above.

## Results

### Transformation of *Arabidopsis*

A total of 13 independent T1 plants of *Arabidopsis thaliana* resistant to both kanamycin and hygromycin were obtained by the *in planta* method. Twelve of them showed the 842-bp band specific to the *nfsA*, confirming the integration of the transgene into the genome of *Arabidopsis tha-*

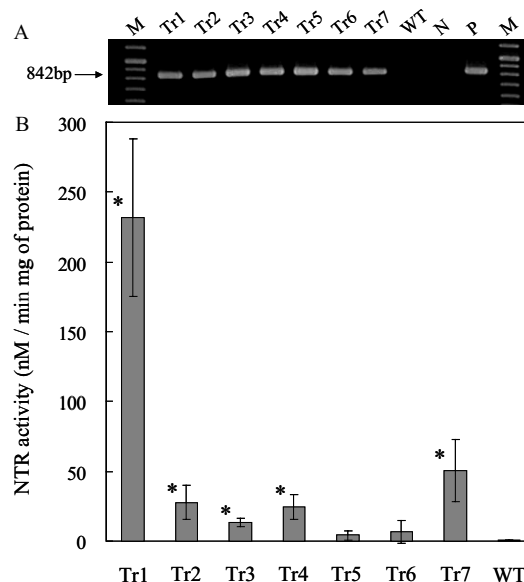


Fig. 1. PCR analysis (A) and NTR activity (B) results of the wild type (WT) and 7 transgenic lines (Tr1–Tr7). (A) The arrow indicates the 842-bp PCR band specific to *nfsA*; M, 100-bp ladder maker; N, negative control; P, positive control. (B) Columns and bars show, respectively, the NTR activity and standard deviation ( $n = 3–5$ ). \* $P < 0.05$ .

*liana* (Fig. 1A). Following the procedure described above a total of seven homozygous T2 lines were obtained.

### Determination of NTR activity

NTR activity was determined using NADPH as electron donor and nitrofurazone as an acceptor, respectively. Our results showed that the wild type did not exhibit any detectable NTR activity, while all 7 transgenic lines (Tr1 to Tr7) exhibited NTR activity. Five of the transgenic lines (Tr1 through Tr4 and Tr7) had NTR activity significantly greater than the wild type (Fig. 1B). Among the five transgenic lines, the enzyme activity varied from  $13 \pm 5$  to  $230 \pm 56$  nmol min<sup>-1</sup> mg<sup>-1</sup> of protein (mean of 3–5 replicates with SD). The transgenic line 1 (Tr1) that had the highest NTR activity was used in the following study.

### Comparison of TNT tolerance between wild-type and transgenic plants

Wild-type and transgenic plants were cultured (for 21 d) in the absence and presence of TNT to test the TNT tolerance. In the absence of TNT,

both parental and transgenic plants grew normally, an indication that the introduction of the bacterial gene in the plant genome did not result in any deleterious effect on the plant growth. In the presence of 0.1 mM TNT, wild-type plants germinated normally but the root formation and growth was severely inhibited, while the transgenic plants showed almost normal growth; the root biomass (fresh weight) was 0 and  $4.4 \pm 1.1$  mg (mean of 8–10 replicates with SD) for wild type and transgenic plants, respectively. Similar results were obtained with the shoot growth; the shoot biomass (fresh weight) was  $5.4 \pm 5.0$  and  $86.6 \pm 9.0$  mg (mean of 8–10 replicates with SD) for the wild type and transgenic plants, respectively. These results suggest that the expression of the bacterial NTR gene increase tolerance to TNT. This result keeps in line with those reported by others (Hannink *et al.*, 2001).

### Uptake of TNT

Fig. 2 shows the concentration of TNT in the medium plotted against the time of incubation in the presence of the wild type or transgenic Tr1 plants and in the absence of plants (NP). In the case of the wild type plants, the decrease in the

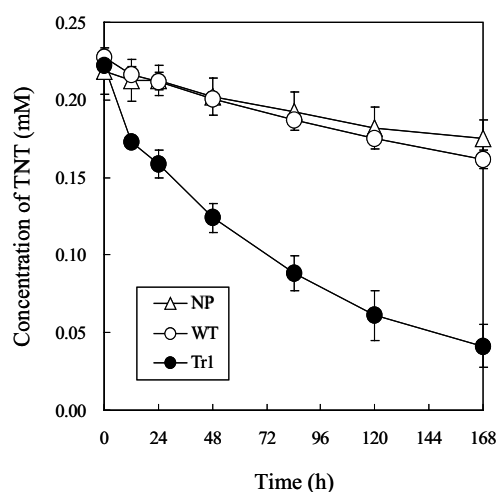


Fig. 2. The concentration of TNT in the medium plotted against the time of incubation in the presence of the wild-type (WT) or transgenic (Tr1) plants and in the absence of plants (NP). Ten-day-old seedlings grown aseptically were incubated in the medium containing 0.25 mM TNT for over 7 d. TNT concentration in the medium was determined daily by HPLC. Data represent the mean with standard deviation ( $n = 3$ ).

TNT concentration was very close to that of the no plant control. It seems likely that wild-type *Arabidopsis thaliana* has very limited capability to take up TNT (see also below).

On the other hand, the incubation with the transgenic plants resulted in a marked decrease in the TNT concentration in the medium. This indicates that the expression of the bacterial NTR much increases the capability of *Arabidopsis thaliana* to take up TNT from the medium. Interestingly, the value of uptake of TNT by the transgenic *Arabidopsis thaliana* depicted in Fig. 2 was estimated to be about  $20 \mu\text{mol d}^{-1} \text{g}^{-1}$  fresh weight from the initial slope. This value was 16 times greater than that reported in the transgenic tobacco ( $1.2 \mu\text{mol d}^{-1} \text{g}^{-1}$  fresh weight) expressing *Enterobacter cloacae nfsI* (Hannink *et al.*, 2001).

### Degradation of TNT by plant

Fig. 3 shows the typical HPLC chromatograms of the extracts from shoots (left panels) and roots (right panels) of the wild type and Tr1 transgenic plants. In the wild-type shoot extract, a distinct peak corresponding to TNT was detected. However, this peak was much smaller in the Tr1 shoot extract, implying that degradation of TNT is much pronounced in the Tr1 shoot. Consistent with this is the fact that there were a number of additional peaks, possibly due to the metabolism of TNT (compare Fig. 3B with Fig. 3A). HPLC analysis of three independent replicates showed that the amount of TNT in the extract of the wild type and Tr1 shoot was estimated to be  $6.8 \pm 0.6$  and  $1.2 \pm 1.1 \text{ ng mg}^{-1}$  fresh weight (mean of three replicates with SD), respectively.

Peaks due to 4A26DNT and 2A46DNT were identified in the extract of the wild type shoot (Fig. 3A). In the extract of Tr1 shoot, the 4A26DNT peak was even more intense but the 2A46DNT peak was not detected (Fig. 3B). The presence of an intense peak due to 4A26DNT in the Tr1 extract provides direct evidence that the expression of bacterial NTR enhances the degradation of TNT taken up into plant body. The amount of 4A26DNT in the extract of the wild-type and Tr1 shoot was estimated to be  $1.4 \pm 0.1$  and  $5.6 \pm 0.5 \text{ ng mg}^{-1}$  fresh weight (mean of three replicates with SD), respectively. The reason for the absence of 2A46DNT in the transgenic shoot is unclear.

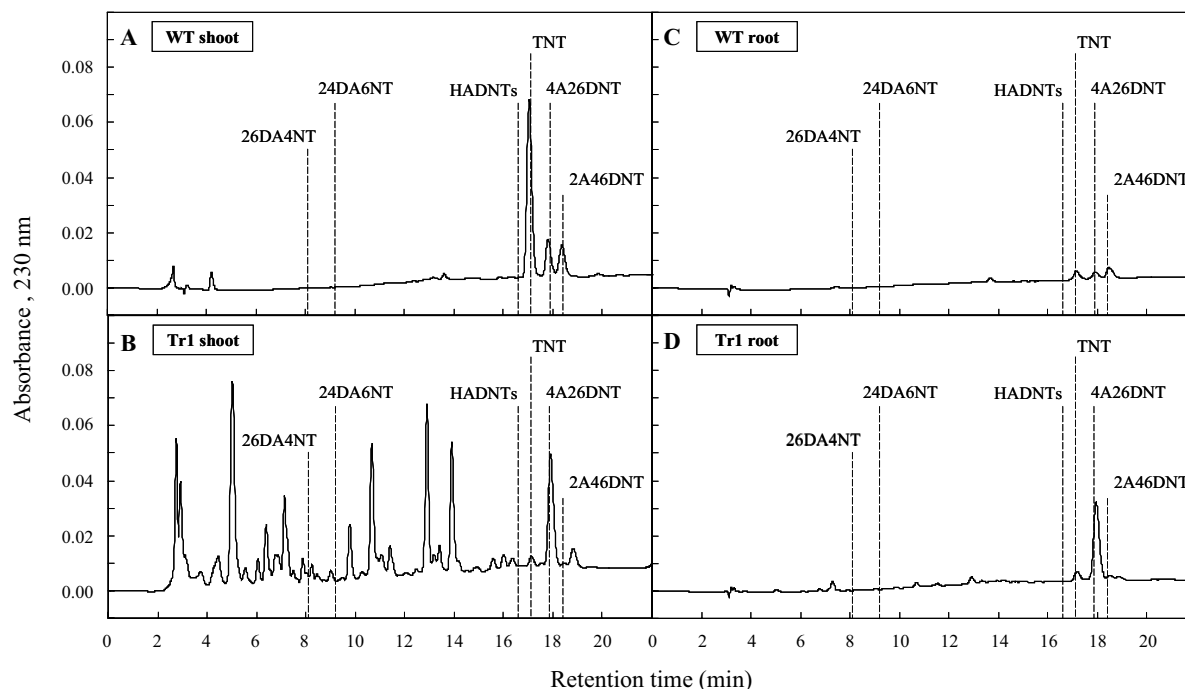


Fig. 3. Typical HPLC chromatograms of the extracts of shoots and roots of the wild-type (WT) and transgenic (Tr1) plants. The seedlings were incubated with 0.25 mM TNT for 7 d, after which they were separated into shoots and roots, and the extracts were prepared from each tissue. The extracts were analyzed by HPLC in comparison with various standard compounds of degradation intermediates of TNT. See text for details.

Thompson *et al.* (1998) have identified 4A26DNT as an intermediate degradation product in poplar trees fed with TNT. This agrees with our result shown in Fig. 3. The present study provides for the first time that the formation of 4A26DNT is enhanced by the expression of the bacterial NTR gene. In the previous study using transgenic tobacco expressing *Enterobacter cloacae nsfI* (Hanink *et al.*, 2001), 4A26DNT was not identified as a major degradation intermediate of TNT.

In contrast to the case of shoots, the HPLC signals were much sparse both in the extracts of the wild-type and Tr1 transgenic plants (see Figs. 3C and 3D). Detailed examination of the chromatograms, however, showed that the characteristics of the chromatograms of the extracts of both roots is somewhat different. In the Tr1 root extract, a strong peak of 4A26DNT and much less intense peak of TNT were detected, indicating uptake and metabolism of TNT in the roots. In fact, HPLC analysis of three independent replicates of Tr1 root extracts indicated that the amount of TNT and 4A26DNT was  $1.2 \pm 0.5$  and  $11 \pm 2.7$  ng mg<sup>-1</sup>

fresh weight (mean of three replicates with SD), respectively.

In the extract of wild type root, the intensity of all peaks including TNT and 4A26DNT was very weak. The amount of respective compound in this root was estimated to be  $1.4 \pm 0.5$  and  $1.2 \pm 0.3$  ng mg<sup>-1</sup> fresh weight (mean of three replicates with SD), respectively. It should be noted that in the present study uptake of TNT into shoots occurred directly through their surface, if not entirely, but not via roots since the uptake experiment was performed by the “floating method” where seedlings were floated on the TNT medium (see Materials and Methods).

## Discussion

In the present study, we have provided evidences that suggest that the rate of uptake of TNT by the transgenic Tr1 line of *Arabidopsis thaliana* ( $20 \mu\text{mol d}^{-1} \text{g}^{-1}$  fresh weight, see Fig. 2) is at least one order of magnitude greater than the respective value for the transgenic tobacco ( $1.2 \mu\text{mol d}^{-1}$



$\text{g}^{-1}$  fresh weight) expressing a nitroreductase from *Enterobacter cloacae* encoding by the *nfsI* (Han-nink *et al.*, 2001).

Interestingly, very similar values for the uptake of TNT were observed with the four other transgenic lines of *Arabidopsis thaliana* (data not shown), although the *in vitro* NTR activity varied by a factor of 20 among these five transgenic lines bearing chimeric *Escherichia coli nfsA*, from  $13 \pm 5$  to  $230 \pm 56 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$  (see Fig. 1A).

In the light of the fact that the amount of TNT in transgenic shoots ( $1.2 \pm 1.1 \text{ ng mg}^{-1}$  fresh weight) and roots ( $1.2 \pm 0.5 \text{ ng mg}^{-1}$  fresh weight) was approx. five to ten times less than that of 4A26DNT ( $5.6 \pm 0.5$  and  $10.6 \pm 2.7 \text{ ng mg}^{-1}$  fresh weight, respectively), a possible intermediate degradation product (see above), it is conceivable that the majority of TNT taken up into the plant body may have been rapidly reduced by the NTR enzyme.

Assuming that protein content of *Arabidopsis thaliana* is  $8 \text{ mg g}^{-1}$  fresh weight, and also assuming that *in vitro* NTR activity gives a direct measure for the uptake of TNT into plant body, we estimated a TNT uptake rate for the transgenic line. The lowest *in vitro* NTR activity was  $13 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$  on average among the five transgenic lines (see Fig. 1A). This line was estimated to take up TNT at a rate of  $8 \mu\text{mol d}^{-1} \text{ g}^{-1}$  fresh weight, which was approx. in the same order of magnitude to the observed uptake value ( $20 \mu\text{mol d}^{-1} \text{ g}^{-1}$  fresh weight). Therefore, the metabolism of TNT can influence the rate of uptake of TNT by the transgenic plants.

The uptake of TNT by non-transformed poplar has been shown by using radio-labeled TNT by

the previous authors (Thompson *et al.*, 1998). Our present finding of the uptake of TNT by the wild-type *Arabidopsis thaliana* (Fig. 3) agrees with this result. Whether or not a plant NTR gene exists and its involvement in the uptake process is to be addressed in future.

Our present study clearly demonstrates that the transgenic *Arabidopsis* expressing the bacterial NTR revealed enhancement of tolerance to TNT and capacity to uptake TNT, and increased degradation of TNT. However, detailed studies to identify degradation intermediate products by analyzing the unknown peaks in the chromatogram of the extract of NTR-expressing transgenic plants (Fig. 3) and to analyze whether or not the pathway of the degradation of TNT in NTR-expressors is the same with that of bacteria are vital to understand the metabolic process itself and to investigate phytoremediation of environmental contaminations by TNT. Production of transgenic woody species expressing this gene is an intriguing and important subject of a future study to mitigate nitroaromatic contaminants including TNT from the environment by plants.

#### Acknowledgements

This research was supported by grants from the Nippon Life Insurance Foundation to M. T., the European Commission (QLK3-CT-2001-00345) to J. L. R., the Grant Agency of the Czech Republic (no. 206/02/P065) and the Ministry of Education of Czech Republic (no. Z4 055 905) to A. N. and T. V., and the Japan Society for the Promotion of Science (JSPS-RFTF96L00604 and no.13GS0023) to H. M.

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