Biochemical and Biophysical Research Communications 397 (2010) 22–26

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of uricase as a potential target of plant thioredoxin: Implication in the regulation of nodule development

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ARTICLE INFO

Article history:
Received 28 April 2010
Available online 12 May 2010

Keywords:
Thioredoxin
Nodulin-35
Uricase
Nodule development

ABSTRACT

During symbiotic nodule development in legume roots, early signaling events between host and rhizobia serve critical determinants for the proper onset of nodule morphogenesis, nitrogen fixation, and assimilation. Previously we isolated thioredoxin from soybean nodules as one of differentially expressed genes during nodulation and noted its positive role in nitrogen fixation. To identify the target proteins of thioredoxin in nodules, we used thioredoxin affinity chromatography followed by mass spectrometry. Nodulin-35, a subunit of uricase, was found to be a target of thioredoxin. Their interaction was confirmed by pull-down assay and by bimolecular fluorescent complementation. With an increased uricase activity observed also in the presence of thioredoxin, these results appear to implicate a novel role of thioredoxin in the regulation of enzyme activities involved in nodule development and nitrogen fixation.

1. Introduction

Legume plants form root nodules, specialized organ for nitrogen fixation and assimilation through symbiosis with rhizobia. Signaling between two symbiotic partners is followed by a series of developmental responses such as root hair curling, cortical cell division, and infection thread formation [1,2]. This reprogramming of root cells enables plants to accommodate nitrogen-fixing bacteria. Changes in uninfected cells upon rhizobial infection are distinct enlargement of microbodies (peroxisomes) and proliferation of smooth ER [3]. Fixed nitrogens by bacteroids are transported from symbiosomes to the cytoplasm of plant cells and assimilated into glutamine via glutamine synthetase/glutamic acid synthase [4,5]. Tropical legumes such as soybean export nitrogen from nodules to the shoot in a form of ureides which are synthesized by uricase in uninfected cells [6,7]. Nodulin-35, one of the soybean nodulins with a molecular mass of 35,100, was localized to peroxisomes of the uninfected cells [8]. Expression of antisense nodulin-35 cDNA resulted in forming root nodules with severe nitrogen deficiency, suggesting an importance of ureide biosynthesis in tropical legumes [9].

Thioredoxins (Trxs) are protein disulfide oxidoreductases with a typical—WCG/PC—motif. By modulating redox potential of the targets, they regulate the enzymatic activities of various metabolic pathways [10]. A chloroplastic thioredoxin was found to activate several key enzymes of photosynthetic reaction after being reduced by the enzyme ferredoxin–thioredoxin reductase. Recently, with the identification of more new targets, the roles of thioredoxins have been extended to many other processes of the cell [11,12]. Different isoforms of thioredoxins are targeted into different subcellular compartments; Trxs *f* and *m* in plastids, Trx *h* in cytoplasm, and Trx *o* in mitochondria, to name a few. Several approaches have been employed to identify target proteins regulated by thioredoxins. Among those, affinity chromatography using a catalytically inactive thioredoxins with mutated active site followed by protein identification with mass spectrometry permitted isolation of targets from many different organisms including *Arabidopsis* [11,13,14].

From earlier studies, we isolated a thioredoxin gene (*GmTRX*) from soybean root nodules as one of differentially expressed genes during nodulation [15]. RNAi-mediated repression of the gene resulted in impaired development of nodules. In this study, an affinity chromatography with GmTRX mutated in the active site allowed identification of nodulin-35, a subunit of nodule-specific uricase, as an interacting partner. Specificity of the interaction between the thioredoxin and nodulin-35 was confirmed by subsequent *in vitro* pull-down assay using proteins expressed in *Escherichia coli*, and also by *in vivo* interaction study using the bimolecular fluorescence complementation (BiFC) assay in *Arabidopsis* protoplasts. We further tested a potential consequence of this interaction by *in vitro* assay and observed an enhanced uricase activity in the presence of thioredoxin. These suggest that thiore-

Abbreviations: TRX, thioredoxin; BiFC, bimolecular fluorescent complementation.

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doi:10.1016/j.bbrc.2010.05.040

doxins may also be involved in the regulation of nitrogen assimilation during nodule development of legume plants.

2. Materials and methods

2.1. Plant materials and bacterial strains

Soybean grown in a growth chamber at 28 °C with 16/8 h cycle was inoculated with rhizobia (*Bradyrhizobium japonicum* USDA110) and nodules from the plant roots were harvested 27 days after inoculation. Three-week-old tobacco (*Nicotiana benthamiana*) plants were used for infiltration using *Agrobacterium tumefacience* GV3101.

2.2. Affinity chromatography

Site-directed mutagenesis of *GmTRX* was performed by PCR. The mutated thioredoxin (*GmTRX$_m$*) was subsequently cloned into an Avi-tag containing vector pAC4 vector (Avidity, Aurora, CO) with NcoI and BamHI. The resulting construct was cloned into pET-28a(+) for protein expression. As a result, GmTRX$_m$ was fused to His-tag and Avi-tag at N-terminal and C-terminal ends, respectively. GFP was cloned in the same way as GmTRX$_m$ and used as negative control. The fusion construct of GmTRX$_m$ was expressed in *E. coli* strain BL21 (DE3) (EMD Chemicals, Gibbstown, NJ) containing *birA* encoding biotin protein ligase for biotinylation [16]. Proteins were purified by affinity chromatography using avidin-agarose resin (Thermo Fisher Scientific, Waltham, MA).

2.3. Identification of a GmTRX$_m$-target protein

Mature nodule proteins were used for the thioredoxin affinity chromatography. Eluted proteins were resolved on a 12% SDS–polyacrylamide gel electrophoresis and a few protein bands of interest were subjected to mass spectrometry (Applied Biosystems, Foster City, CA) as described previously [17].

2.4. Western blotting

cDNA of nodulin-35 was cloned into pET-28a(+) and expressed in *E. coli* strain BL21 (DE3). GmTRX$_m$- and GFP-immobilized resins were incubated with an *E. coli* cell lysate expressing nodulin-35 at room temperature for 2 h. Western blotting was performed after protein transfer to the Hybond-P membrane (GE Healthcare, Piscataway, NJ) with anti-His antibody.

2.5. Bimolecular fluorescence complementation (BiFC) analysis

BiFC assay was performed as described in Fang and Spector [18]. Full-length cDNAs of nodulin-35 and *AtTRX-o1* [19] were cloned into the binary BiFC vectors, p2YN and p2YC, respectively. The resulting fusion constructs were introduced into *Arabidopsis* protoplasts [20] or leaves of *N. benthamiana* [21]. Fluorescence signals were detected under the microscope using the GFP filter.

2.6. Uricase assay

Uricase activity was measured as described previously [7]. The reaction mixture contained 70 mM Tris–Cl (pH 8.0), 0.1 mM uric acid, and appropriate amount of enzyme samples.

3. Results

3.1. Generation and purification of recombinant GmTRX$_m$

Since repression of a thioredoxin in soybean nodules by RNAi approach resulted in an impairment of nodule development in our previous study [15], we wanted to identify the effectors through which the potential regulatory role of thioredoxin during nodule development is mediated. Thus, we set out to isolate thioredoxin-interacting proteins from soybean nodule tissues using affinity chromatography. Following the strategy of using a catalytically inactive mutant thioredoxin to increase stability of the interaction as successfully employed by other research groups previously [22], we generated a mutant version of GmTRX by substituting a conserved cysteine in the putative catalytic domain with serine (C62S) (Fig. S2). The mutant construct (GmTRX$_m$) was also designed to contain an *in vivo* biotinylation signal so that the protein can be readily purified and immobilized using the avidin-agarose resin (Fig. 1A).

Disulfide oxidoreductase activity of the GmTRX$_m$ was examined by the insulin reduction assay [23] to ensure it serves our intended purpose. As shown in Fig. 1B, GmTRX$_m$ displayed a substantial delay in the insulin reduction activity as compared with the wild type thioredoxin GmTRX. NADPH-dependent thioredoxin reductase that is needed to reduce thioredoxin *in vivo* to carry out the disulfide oxidoreduction reaction was substituted with DTT (0.33 mM). The slower enzyme kinetics of GmTRX$_m$ exhibited in the assay confirmed that it can be utilized as a viable affinity tool to bring down the interacting partners from cellular sources [22].

3.2. Identification of a potential target protein of thioredoxin in nodules

Our previous data suggested that GmTRX may play a role in a later stage of nodule development, rather than the early stage of nodule formation [15]. Thus, in order to isolate proteins functionally associated with GmTRX during soybean nodule development, total soluble proteins from mature, 27-day-old nodules were incubated with biotinylated GmTRX$_m$ proteins immobilized to avidin-agarose resins. Several GmTRX-specific protein bands were visible on Coomassie-stained SDS–PAGE gel after the affinity purification (Fig. S1). Among those, three bands that appeared repeatedly on multiple trials were selected for identification by Liquid Chromatography–Mass Spectrometry (LC–MS) analysis. One of these proteins, which was consistently observed as a prominent band of an apparent molecular mass of 35 kDa, turned out to be nodulin-35 (N-35), a soybean nodule-specific uricase (Fig. S1, band #3). The other was identified as a phosphoenolpyruvate carboxylase (PEPC) homolog (Fig. S1, band #1), whose interaction with thioredoxin is less surprising considering that the activities of many enzymes involved in carbon metabolism, including those of Calvin cycle, are regulated as the targets of thioredoxin [11]. On the other hand, the finding that uricase interacts with thioredoxin is rather intriguing because it could be the first example of thioredoxin in the control of nodulation through modulation of the key enzymes involved in the process. Therefore, we decided to further pursue to confirm and characterize the interaction between N-35 and thioredoxin.

3.3. Confirmation of the specific interaction between N-35 and thioredoxin

In order to confirm the specificity of the interaction and to determine if thioredoxin binds directly with N-35, *in vitro* protein pull-down assay was performed using recombinant N-35 and the GmTRX$_m$ expressed and purified from *E. coli*. Biotinylated GmTRX$_m$ immobilized to the avidin-agarose resin was used to test the specific pull-down of 6x-His-tagged N-35. As shown in Fig. 2, the GmTRX$_m$ could only co-precipitate N-35 (lane 4), as revealed by Western blot analysis with anti-His antibody, while 6x-His-tagged GFP included in the experiment as a negative control did not get pulled-down by GmTRX$_m$ (lane 5), demonstrating specific and di-

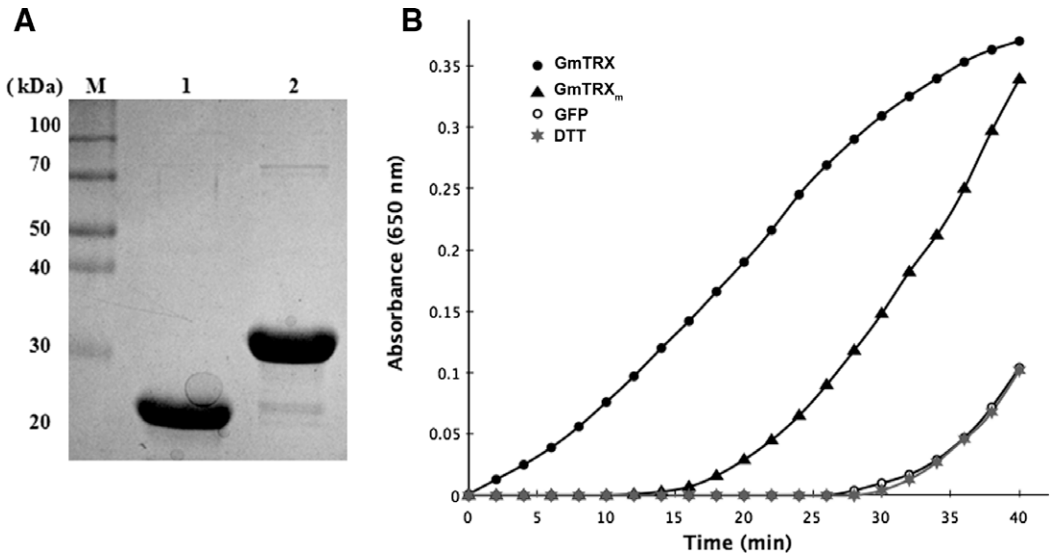

Fig. 1. Purification and activity of recombinant GmTRX$_m$. (A) Purification of GmTRX$_m$ and GFP from *E. coli* BL21 (DE3). GmTRX$_m$ was induced in *E. coli* BL21 (DE3) by 1 mM IPTG for 5 h and purified by using avidin-agarose resin (lane 1). GFP was purified in the same way (lane 2). (B) Insulin reduction activity of recombinant GmTRX$_m$ (▲) and GmTRX (●). The reduction of insulin was examined by measuring A_{650} for 40 min at 30 °C. The reaction was started by the addition of 0.33 mM DTT. GFP (○) or DTT (✱) alone was used as negative control.

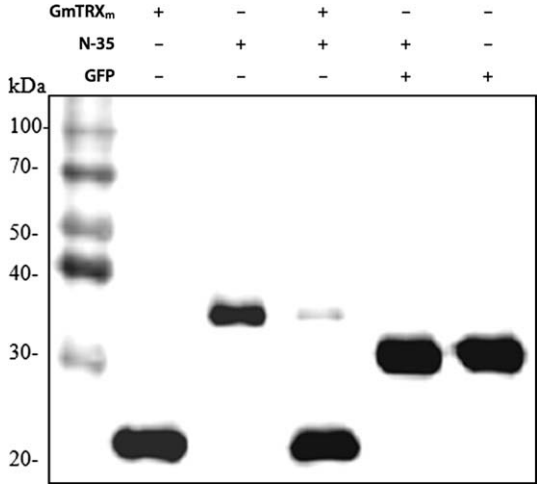

Fig. 2. Analysis of the interaction *in vitro* between GmTRX$_m$ and nodulin-35. GmTRX$_m$ or GFP bound to avidin-agarose resins was incubated with cell lysates of *E. coli* expressing nodulin-35 for 30 min at room temperature, respectively. Proteins were separated using SDS–PAGE, and detected by immunoblot analysis with anti-His antibody.

rect binding of the thioredoxin with N-35. To examine if this interaction is dependent upon disulfide bond formation, we also tried the same pull-down assays using the wild type GmTRX as well as the GmTRX$_m$ in the presence of an increasing amount of DTT added during the reaction. Contrary to the conventional thioredoxin–substrate interaction where the binding stability between the target and thioredoxin is negatively affected by DTT, our result showed no such DTT-dependent dissociation of N-35 from GmTRX (data not shown), suggesting that N-35 is not a substrate of the disulfide oxidoreductase activity of thioredoxin.

We further tested the specific interaction between thioredoxin and N-35 *in vivo*, by using the bimolecular fluorescence complementation (BiFC) approach [24]. N-35 is a subunit of nodule-specific uricase localized to peroxisome and GmTRX is a cytosolic form of thioredoxin. Therefore, although we were able to confirm the specificity of interaction between N-35 and the GmTRX by *in vitro* pull-down assay, the different subcellular localization of these proteins *in vivo* presented a concern for the authenticity of the interaction. We speculated that the actual interaction in the cell could probably occur between N-35 and a yet to be discovered peroxisomal form of thioredoxin. However, since no peroxisomal TRX has been identified to date, we opted to use a mitochondrial form of TRX in the BiFC assay as a best alternative to the peroxisomal TRX because mitochondrial proteins were often localized to peroxisome (See Section 4). The mitochondrial form of soybean TRX has not been well characterized notwithstanding the recent progress in the soybean genome project. On the other hand, the *Arabidopsis* genome contains two mitochondrial forms of TRXs (AtTRX-o1 and AtTRX-o2). Of these, we chose the AtTRX-o1 in our BiFC assay because it has a slightly higher homology with the GmTRX. It shares 37% sequence identity with the GmTRX at protein level (62% overall similarity), and excluding the N-terminal region where the mitochondrial targeting pre-sequence peptides are present, the two proteins display even much higher sequence similarity (Fig. S2). In this experiment, we fused *nodulin-35* cDNA and *AtTRX-o1* to p2YN and p2YC vectors, respectively, each of which contains the N-terminal 158 amino acids (YN) and the C-terminal 80 amino acids of YFP (YC) under the 35S promoter of cauliflower mosaic virus. Strong YFP fluorescence was observed when AtTRX-o1 and N-35 were co-expressed in *Arabidopsis* protoplasts (Fig. 3). Meanwhile, the co-expression of a ribosomal protein S6 and AtTRX-o1 did not produce any YFP fluorescence, serving as a correct negative control. *Agrobacterium*-mediated infiltration of the fusion constructs into tobacco (*N. benthamiana*) produced the same results of fluorescence as above (data not shown). These imply that N-35 indeed interacts with a thioredoxin in plant cells, even though it still remains unresolved which thioredoxin in soybean interacts with N-35. Interestingly, the co-localization pattern in our data showed the GFP fluorescence in a specific subcellular compartment(s), rather than being distributed evenly across the cytoplasm. Whether this observed location was peroxisome, or mitochondria, or both, was not determined in this experiment.

3.4. Uricase activity of N-35 in the presence of thioredoxin

In order to obtain an insight into a biological significance of the interaction between thioredoxin and N-35, we examined whether

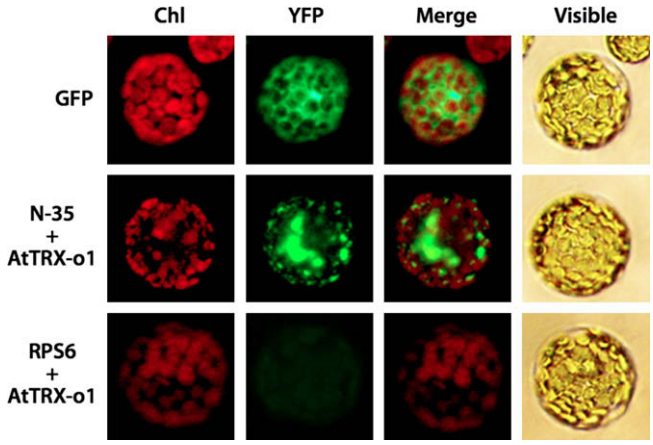

Fig. 3. Bimolecular fluorescence complementation (BiFC) analysis of thioredoxin and nodulin-35 molecular interaction in *Arabidopsis* protoplasts. Shown are representative fluorescent images of YFP obtained from *Arabidopsis* protoplasts expressing the indicated proteins. Where indicated, fusions of nodulin-35 to an N-terminal fragment of YFP (YN) and AtTRX-o1 to a C-terminal fragment of YFP (YC), or fusions of a ribosomal protein S6 to an YN and AtTRX-o1 to an YC were introduced into protoplasts of *Arabidopsis* leaves or infiltrated into tobacco leaves (*N. benthamiana*). Fluorescence and bright field images were obtained after incubation of transfected or infiltrated samples in plant growth chamber for appropriate times. GFP (326-GFP) was used as positive control.

the uricase activity of N-35 would be changed by the interaction with thioredoxin. N-35 containing *E. coli* cell lysates were incubated with purified GmTRX and then uricase activities of the mixture were measured according to the method of Suzuki and Verma [7]. The reaction rate of N-35 was, indeed, increased in the presence of thioredoxin whereas GFP protein included as a negative control in place of thioredoxin showed no effect on the uricase activity (Fig. 4). Taken together, these data indicate that through the interaction with thioredoxin, the uricase activity may be positively regulated, which is consistent with our previous observation of the stimulatory effect of GmTRX on nodule development.

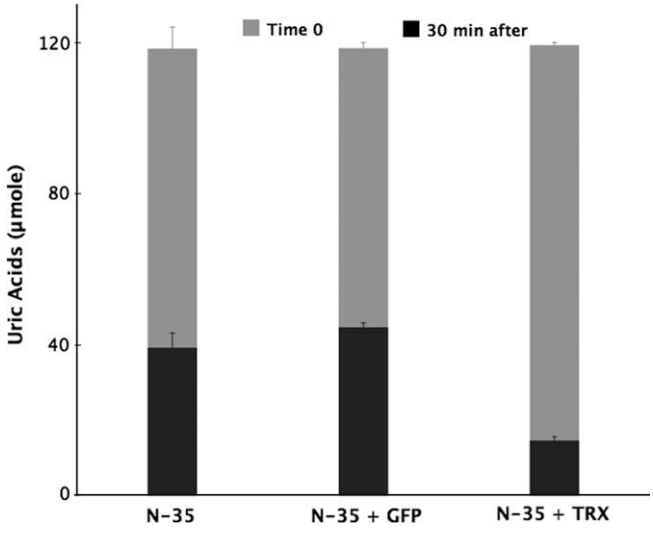

Fig. 4. Uricase activity in *E. coli* lysates expressing nodulin-35 in the presence of GmTRX. Enzyme reaction was performed with the lysates of *E. coli* expressing nodulin-35 after addition of 1 mM uric acid. In order to examine the effect of thioredoxin on the uricase activity, purified GmTRX or GFP was incubated with the lysates of *E. coli* expressing nodulin-35 at room temperature for 30 min and then uric acid was added to the mixture. For each sample, the amount of uric acids at the end of the reaction (black bar) was compared with that of the beginning of the reaction (gray bar).

4. Discussion

Our *in vitro* and *in vivo* interaction data showed that N-35 and thioredoxin can form a specific interacting complex. Taken together with the *in vitro* uricase assay result which demonstrated a stimulatory effect of thioredoxin on the uricase activity, it may be possible that there is a peroxisomal form of thioredoxin functioning in nitrogen-fixing legume roots to regulate activities of the enzymes in nitrogen assimilation.

Although most thioredoxins regulate protein activity by thiol–disulfide exchange reactions, Kumar et al. [25] reported the isolation of twenty thioredoxin-targeted proteins in *E. coli* which do not have cysteine residues. These proteins were suggested to act independently of thiol redox activity as there have been examples to support that thioredoxins also function as structural components in regulating target proteins [26,27]. N-35 and GmTRX were still associated when they were mixed together with DTT (data not shown). Thus, it appears that N-35 interacts with thioredoxin independently of thiol redox activity. If this was the case, mutating the active sites for isolation of thioredoxin targets in soybean nodules was not necessary to stabilize the putative heterodimeric complex [25]. But we could not exclude a possibility that other treatments to affect a disulfide bond may result in dissociation of N-35 from thioredoxin.

Peroxisomes are organelles with active oxidative metabolism such as the formation of hydrogen peroxide and superoxide radicals. Many antioxidant enzymes and stress-related proteins are present in peroxisomes to prevent damages from the reactive oxygen species. Recent findings suggest that mitochondria and peroxisomes share some morphological and functional components [28]. These two organelles have close metabolic link to each other. They also seem to have a biogenetic link, suggesting coordination in biogenesis, dynamics and turnovers of each organelle. Recently, Neuspiel et al. [29] provided evidence for vesicular transport from the mitochondria to peroxisomes, revealing the close relationship between these two organelles [30]. Both organelles also contain diverse antioxidant molecules. For example, PMP20, a protein localized to peroxisomes and mitochondria, exhibited the activity of thioredoxin peroxidase although thioredoxin was not found in peroxisome yet [31,32]. In our study, a mitochondrial type of thioredoxin (AtTRX-o1) was used to examine its *in vivo* interaction with the soybean nodule-specific uricase, nodulin-35 (N-35), in *Arabidopsis* protoplasts because while there is no peroxisomal type of thioredoxin reported to date, mitochondria and peroxisome share some components with each other [33]. The BiFC signal (YFP fluorescence) could be detected only in the protoplasts cotransfected with AtTRX-o1 and N-35, indicating a specific interaction of the N-35 and the mitochondrial type of thioredoxin in these cells. Moreover, rather than being evenly distributed throughout the cytoplasm, the YFP fluorescence in these cells was observed as discrete spots within the cytoplasm suggesting that peroxisome and/or mitochondria might be the site for the interaction. It is probable that the actual interaction occurring in soybean nodules may also involve a mitochondrial form of TRX that was indirectly translocated to peroxisome.

As demonstrated in this study that addition of TRX can increase the uricase activity of N-35 *in vitro*, TRX may have a similar positive role in regulating the activity of N-35 in nodule. Elucidating the existence of functional thioredoxin in peroxisomes would further validate their interaction and the potential significance observed by our present study, and therefore, future direction of the studies will focus on the efforts to demonstrate the biological significance of the interaction between nodulin-35 and thioredoxin on nodule development.

Acknowledgments

This research was supported by the SRC Research Center for Women's Diseases of Sookmyung Women's University (2008) and by the Seoul R&BD (Research and Business Development) Program (Grant No.: 10582).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.05.040.

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