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MONITORING THIOREDOXIN GENE EXPRESSION BY REAL TIME QUANTITATIVE RT-PCR IN WHEAT GRAINS

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INTRODUCTION

Thioredoxins are small, heat-stable proteins that appear to be ubiquitous in all living cells (1). They catalyze dithiol/disulphide exchange reactions since their active site consists of a highly conserved sequence Trp-Cys-Gly-Pro-Cys which disrupts disulphide bridges of target proteins (2). Higher plants present two thioredoxin systems. One, the *f* and *m* system is located in the chloroplasts and regulate the redox state of enzymes involved in light-dependant CO_2 fixation (3). The second system is represented by the cytosolic thioredoxins *h*. Multiple *h*-type thioredoxins have been identified in plants (4,5,6). These proteins have been identified so in sieve tubes of several monocotyledonous and dicotyledonous plants where they are proposed to maintain sieve tube proteins and provide protection from oxidative damage. But most of the proposed thioredoxin proteins function are related to seed germination. In wheat seeds, it has been proposed thioredoxins *h* reduce gliadins and glutenins (7), activate thioalcalin, a calcium dependant protease (8) and reduce inhibitors of alpha-amylase and limit dextrinase (8). By these effects, reserves are mobilized and become available to the developing seedling. This work analyze the filling phase of wheat seed to evaluate the gene expression of two *h*-type thioredoxins.

MATERIEL AND METHOD

Gene expression. Soft wheat (*Triticum aestivum*, cv. Soissons) plants were grown in open field. Grains were harvested at different stages of development, and divided in two parts. The former was used to establish physiological data and the later was frozen until use. For RT-PCR purpose, cDNA was synthesized from total RNA extracted with the Rneasy plant minikit (Qiagen). Design of specific primers was done using the Primer express (Applied Biosystems) software. I (up: 5'-aggagcctgatagcgtg, down 5'-atcaccagcttgcggtct); II (5'-cacagcctggagcagtgga, down 5'-gcaaaattggagccataatg); 18S (up: 5'-agtaagcgcgagtcacagct, down: 5'-cattcaatcggtaggagcgac). Real-time PCR is a fluorescence-based method of quantifying PCR products during the exponential phase of amplification rather than after a fixed number of cycle. Relative quantification for a given gene, expressed as fold-variation over control is calculated after normalization and determination of difference cycle threshold (C_t) in target and control using the $2^{-\Delta C_t}$ formula (9) in a comparative method. Quantitative RT-PCR analyses were performed starting with 50 ng of reverse transcribed total RNA, with 50 nM of both sense and antisense primers in a final volume of 25 μ l using the Sybr Green PCR core reagents in an ABI PRISM 7700 Sequence Detection System instrument (Applied Biosystems). Expression profile for a given gene was monitored by relative quantification expressed over a the same gene as calibration control. The 18S RNA expression was used to normalize the reaction.

CDNA cloning and protein expression. mRNA was extracted with a mRNA isolation kit (Boehringer). Full-length sequences of both *h*-type thioredoxins were obtained by RT-PCR using their corresponding primers pair. I (up: 5'-atacatatggcgcgagagg; down: 5'-atctcagtgtagcagatgcagaacc), IIa (up: 5'-atacatatggcgcgctg; down: 5'-atctcagtgtagcagcgcgctgtag); IIb (up: 5'-atacatatggcgcgctg; down: 5'-atctcagtgtagcagcgcgctgtag). The orf was inserted in the pET16b expression vector (Novagen) after *Nde*I and *Xho*I digest. The insert was checked by sequencing. The resulting plasmid was then introduced in *E. coli* BL21(DE3)pLysS (Promega). Protein expression was induced by IPTG. Three hours after induction, cells were harvested by centrifugation and the pellet resuspended in a corresponding BugBuster lysis buffer (Novagen) with Benzonase Nuclease (Novagen). After incubation, soluble proteins were recovered in the supernatant by centrifugation. The expected molecular mass of over-expressed protein was purified by affinity chromatography in Ni-NTA His.Bind resin (Novagen) according to the manufacturer instructions.

RESULTS AND DISCUSSION

Two types of thioredoxin *h* were found in common wheat, cv Soissons (Fig.1). Type I (10), (118 AA, MW 12.69 kDa, pI 4.98) is homologous with a rice thioredoxin (11), while type II has two forms, a (AF 286593; 125 AA, MW 13.25Da, pI 4.79) and b (AF 420472; 130 AA, 13.79 kDa, pI 4.79). The rice-type (12 kDa) has, in addition to the two cysteines of the active site, an additional cysteine residue in position 11 located on a sheet of the N-terminal part of the model generated by homology (Fig.2). The program is available at www.cbs.dtu.dk/services/CPHmodels/ (12). If the structure corresponds to the model, this cysteine residue located on a flexible part of the molecule in a good orientation, could form a covalent bond allowing a dimerization of the molecule like it was observed with the human thioredoxin (13). The IIb form, initially found in durum wheat (14) is also present in common wheat. It differs from the IIa form by a longer and hydrophobic N-terminal part, characteristic whose one allotted a possible role in membrane anchorage (14). The genes corresponding with these thioredoxins were cloned and expressed as His-tag proteins. The molecules obtained show awaited characteristics (Fig.3).

The follow-up of the 18S RNA expression is used to standardize the reaction insofar as the grains undergo notable physiological modifications during the phase of filling, in particular, the variation of the water content. As expected, the expression of the 18S is relatively stable during the period observed (Fig. 6A). The point of intersection with the base line is grouped around cycle 19. One observes on the other hand in the follow up of the expression of the thioredoxins, a certain dispersion of C_t with a density around 30 (Fig.6B). This observation is translated after calculation by the following general profile (Fig 7). There is a decrease of the expression of the thioredoxin isoforms from anthesis up to 10 days, then this expression is stabilized. The major expression is thus associated with the period of cellular division up to the point of inflection, 10 days after anthesis, which characterizes the beginning of the water loss in the grain. The two types of thioredoxin followed show a parallel profile, although the expression of type I is higher. Tests by RT-PCR followed by sequencing of whole gene of the studied isoforms confirm their presence during the period observed.

The use of an antibody will permit to check the correspondence between the expression of the mRNA and the protein synthesis. In the same way the follow-up of each isoform over a longer period will make it possible to refine their respective profile. The next step of this work will investigate the targets of the thioredoxins before the phase of desiccation in the grain. Recently, it was shown that two thioredoxins *h* of the IIa type were located in the nucleus of aleurone and scutellum cells of germinating wheat seeds (15). This localization echoes what was underlined in the animal cell (16) and yeast (17). The confirmation of these data will make it possible a better understanding of the role of these metabolic proteins in the grain.

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IIa  MAASA-----ATATAAVGAGEVISVHSLEQWTMQIEEANAAKKLVVIDFTASWCGPCRI
IIb  MAAATATTTAAATAAAVGPGEVISVHSLEQWTMQIEEANAAKKLVVIDFTASWCGPCRI
I    MAEE-----GAVIACHTKQEFDTHMANGKETGKLVVIDFTASWCGPCR
IIa  MAPIFADLAKKFPAAVFLKVDVDELKSIAEQFSVEAMPFFLFMKEGDVKDRVVGAIKEEL
IIb  MAPIFADLAKKFPAAVFLKVDVDELKSIAEQFSVEAMPFFLFMKEGDVKDRVVGAIKEEL
I    IAPVFAEYAKKFPGALFLKVDVDELKDVAEAYNVEAMPFFLFKDGAKVDTVVGGRKDDI

IIa  TNK-VGLHAAQ--
IIb  TTK-VGLHAAA--
I    HTKIVALMGSASA
    
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Fig. 1 - Alignment of Soissons Thioredoxin type I and II).

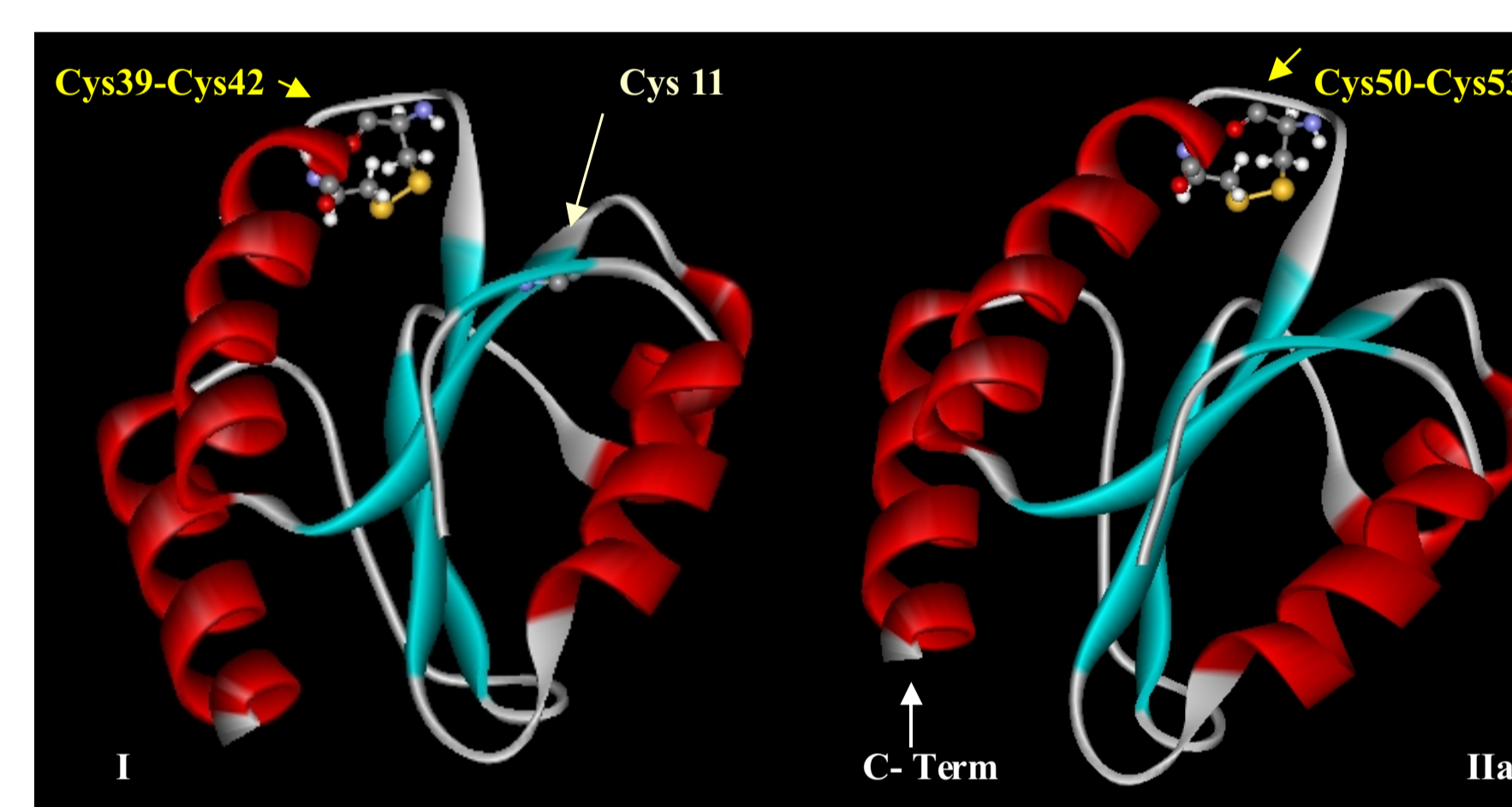


Fig. 2 -Predicted structures of the two thioredoxin types derived from the *C. reinhardtii* thioredoxin *h* (1 TOF).

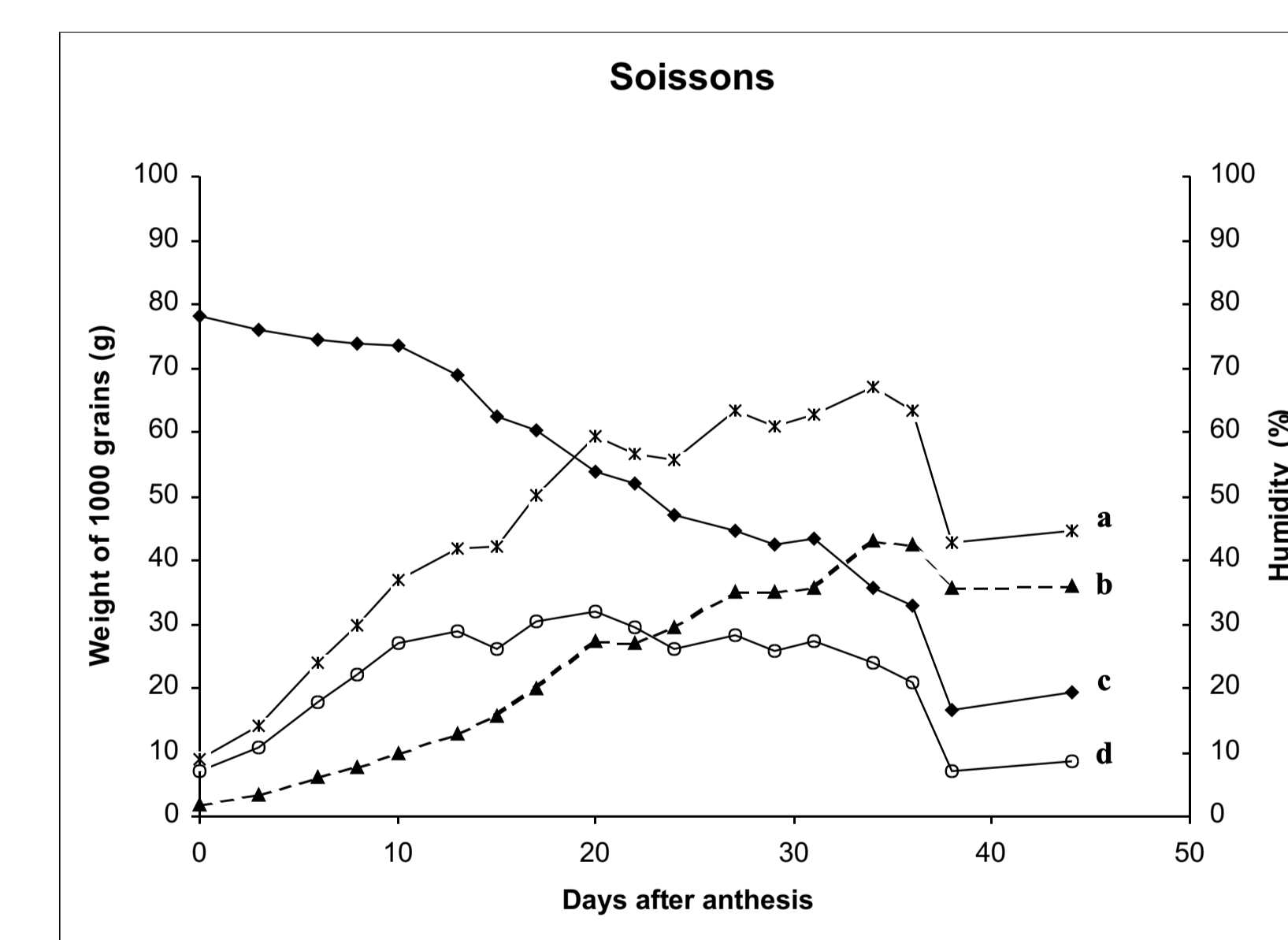


Fig.4 - Evolution of physiological data during grain filling. Fresh weight (a); dry weight (b); water content © and humidity (d).

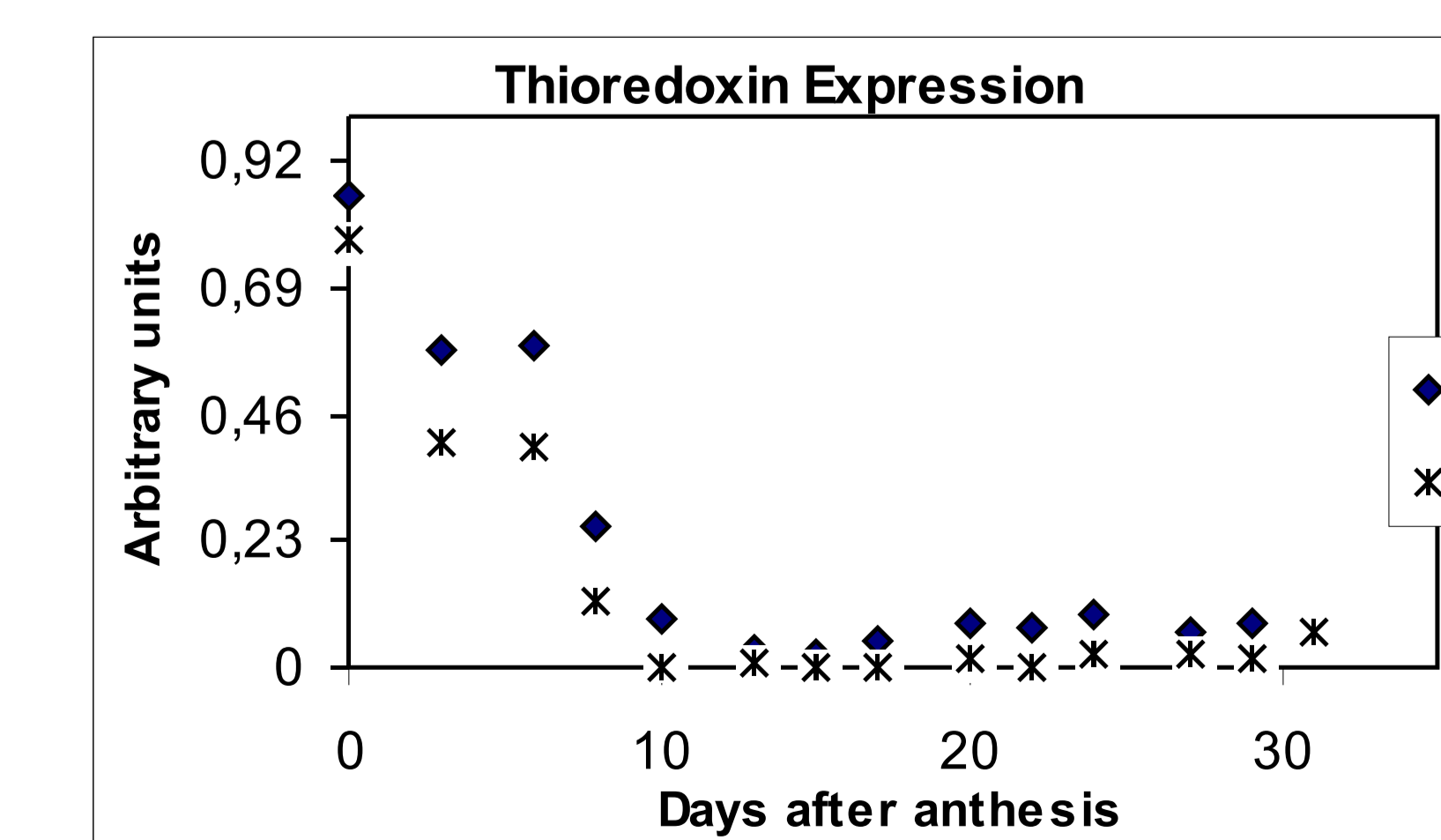


Fig.7 -General expression profile of two thioredoxin types by real-time PCR..

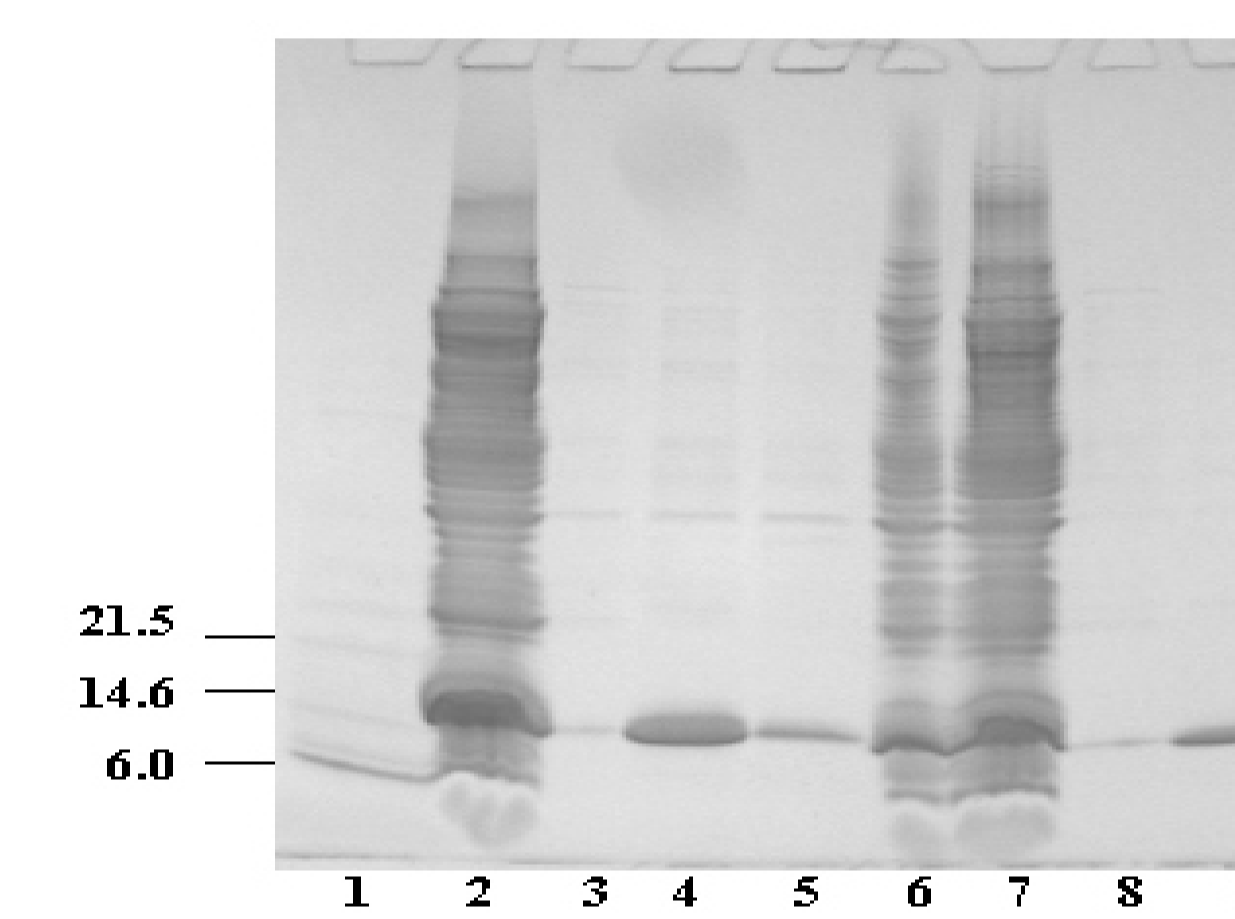


Fig. 3 - SDS-PAGE gel of purified Soissons thioredoxin proteins. Lane 1, protein markers (Novex, USA); lanes 2, 6,7 non-purified total protein after induction of IIa (lane 2) and II b (lanes 6,7); Ni-resin purified recombinant protein of IIa (4,5) and IIb (9).

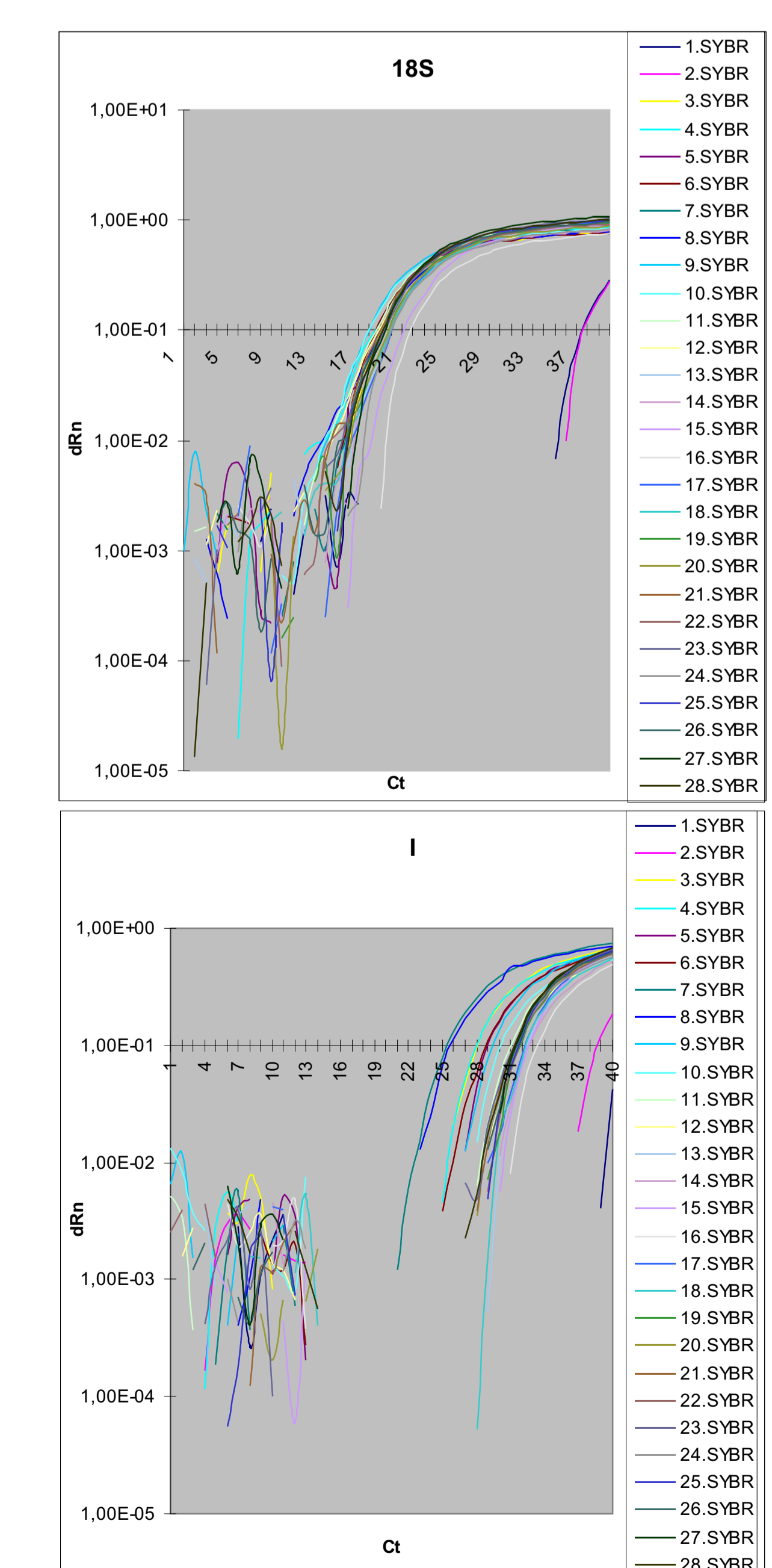


Fig.6-PCR amplification plots of cDNA. Cycle numbers plotted against change in normalized fluorescence (dRn) caused by binding of SYBR Green to dsDNA. Threshold cycle (C_t) is the point in time during cycling at which an amplification plot intersects with an arbitrary limit. The higher the initial amount of nucleic acid in the sample the smaller the C_t value.