Comparative Analysis of Latex Transcriptome Reveals Putative Molecular Mechanisms Underlying Super Productivity of *Hevea brasiliensis*

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**Abstract**

Increasing demand for natural rubber prompts studies into the mechanisms governing the productivity of rubber tree (*Hevea brasiliensis*). It is very interesting to notice that a rubber tree of clone PR107 in Yunnan, China is reported to yield more than 20 times higher than the average rubber tree. This super-high-yielding (SHY) rubber tree (designated as SY107), produced 4.12 kg of latex (cytoplasm of rubber producing laticifers, containing about 30% of rubber) per tapping, more than 7-fold higher than that of the control. This rubber tree is therefore a good material to study how the rubber production is regulated at a molecular aspect. A comprehensive cDNA-AFLP transcript profiling was performed on the latex of SY107 and its average counterparts by using the 384 selective primer pairs for two restriction enzyme combinations (Apol/Msel and TaqI/Msel). A total of 746 differentially expressed (DE) transcript-derived fragments (TDFs) were identified, of which the expression patterns of 453 TDFs were further confirmed by RT-PCR. These RT-PCR confirmed TDFs represented 352 non-redundant genes, of which 215 had known or partially known functions and were grouped into 10 functional categories. The top three largest categories were transcription and protein synthesis (representing 24.7% of the total genes), defense and stress (15.3%), and primary and secondary metabolism (14.0%). Detailed analysis of the DE-genes suggests notable characteristics of SHY phenotype in improved sucrose loading capability, rubber biosynthesis-preferred sugar utilization, enhanced general metabolism and timely stress alleviation. However, the SHY phenotype has little correlation with rubber-biosynthesis pathway genes.

**Introduction**

Natural rubber (cis-1, 4-polyisoprene) is an important industrial raw material with wide applications. It has significant advantages, making it difficult to be replaced by synthetic alternatives in most applications [1]. *Hevea brasiliensis* (para rubber tree), a native tree of Brazil, is the sole commercial species cultivated for rubber production due to the high quality, high yield, easy harvesting and processing. Over 90% of natural rubber is produced in the Southeast Asia, particularly in Thailand, Indonesia and Malaysia. Increasing demand for rubber has driven a six-fold price increase since 2002, prompting studies into the mechanisms of high productivity, and a search for alternative sources of natural rubber [2]. Rubber biosynthesis occurs on the surface of a special type of organelle (rubber particle) in the cytoplasm (latex) of the rubber-producing laticifers or latex vessels [3].

Since the 1920s, great progress has been made in rubber breeding, mainly based on 33 seedlings of Wickham trees. In Malaysia, a series of rubber clones (varieties) recommended for plantation displayed a gradual increase in the mean annual yield, 1175 kg/ha for PR1084 (selected in the 1920s), 1425 kg/ha for RRIM501 (1928-1931), 2000 kg/ha for RRIM600 (1937-1941), and 2125 kg/ha for RRIM712 (1947-1958).

However, the yields of the most productive clones currently available are still much inferior to the theoretical yield of rubber trees, which predicted 7,000-12,000 kg/ha/yr [4]. Conventional hand-pollination based rubber breeding is now confronted with a number of challenges for developing more productive rubber clones. These challenges include the long time needed for
accurate selection (~30 yrs), the low female fertility that limits recombination, and the difficulty in efficiently utilizing new germplasms. Genetic transformation is a promising technique to overcome these barriers. Current rubber clones can be intentionally improved by transgenic engineering if molecular mechanisms underlying rubber productivity are well understood and the key genes involved are identified. The recent release of a draft Hevea genome [5] and other ongoing Hevea genome sequencing programs will be greatly beneficial to this purpose.

In *H. brasiliensis*, the general metabolic pathway leading to rubber biosynthesis (RB) is now clear [3], and all the genes involved have been identified, especially owing to the application of Illumina second generation sequencing technology in the latex and bark of Hevea tree [6-8]. Some of the RB genes have been cloned and further characterized, notably the cis-prenyltransferase (CPT) [2,10], rubber elongation factor (REF) [9,11], small rubber particle protein (SRPP) [12], and hydroxymethylglutaryl coenzyme A reductase (HMGR) [13]. However, the expression of the genes involved in other metabolic pathways within the latex cells can be essential in latex regeneration, especially in the case of ethylene-stimulated latex production [3]. For example, the expression of a sucrose transporter, *HbSUT3 (=HbSUT1B)* [14,15] and two aquaporins [16,17] revealed to be significantly stimulated in the latex by ethylene treatment, and correlated with the effect of yield stimulation. In contrast, the expression of REF is little affected by ethylene treatment, suggesting REF is not a limiting factor in the case of ethylene-stimulated latex regeneration [3]. On the whole, the knowledge we have about the molecular regulation of rubber productivity is still scarce.

In a rubber plantation of Mengla County in Yunnan Province of China, a rubber tree of clone PR107 planted in 1963 produced 126 kg of dry rubber in 2002, over 20-fold higher than the average rubber trees [18]. We honored this tree as a super-high-yielding (SHY) PR107 tree, and designated it as SY107. To our knowledge, SY107 is the most productive rubber tree ever reported in the literature, and is a precious material for investigating the molecular regulation of rubber yield. The rubber trees presently exploited for rubber production are propagated by grafting auxiliary buds (scions) of elite clones onto unselected seedlings (rootstocks), and are simply named after the elite clones that provide the scions. Since rubber tree is a cross-pollinated crop, rootstock plants obtained by germinating open pollinated seeds harvested from the fields are highly heterozygous. This can lead to stock-scion interactions. Large intrACLonal variations observed in growth and yield of bud-grafted clones of Hevea are attributed to the genetic heterogeneity of the rootstocks [19]. Therefore, the occasional optimum combination of scion and rootstock may account for the SHY performance of SY107. Other hypotheses include special micro-environments, or beneficial genetic mutations in the scion. Whatever the real reason is, realization of the ultimate SHY phenotype will depend largely on the regulation of gene expression in the laticifers. The effects of gene expression on biological responses have been reported both in plants [20] and in animals [21]. Therefore, to investigate the high-yielding molecular mechanisms of SY107, it is necessary to compare the latex transcriptome of SY107 with that of its average counterparts, and identify the differentially expressed (DE) genes. cDNA-amplified fragment length polymorphism (cDNA-AFLP) is a sensitive, reproducible, and high throughput technique for isolating DE-genes and is also a powerful tool in understanding the molecular mechanisms of a complex phenotype in non-model organisms [22-28]. A radioactive-labeling cDNA-AFLP analysis has been successfully applied to identify the genes that are differentially expressed in latex compared to leaf tissues [29]. Here, a silver-staining cDNA-AFLP procedure established for latex transcript profiling [30] was exploited to extensively compare the latex transcriptome of SY107 with that of the average PR107 trees. All the 384 selective primer pairs for two restriction enzyme pairs (*Apol/Msel* and *TaqI/Msel*) were employed, and such analysis can theoretically cover more than 90% of the latex entire transcriptome [30].

### Results

#### Latex yields and physiological parameters

Measured for three rounds of Etherl (an ethylene generator) stimulation (9 tappings in total), the mean latex yield of the SY107 tree was 4.12 kg per tapping, whereas it was only 0.32 kg and 0.83 kg for the two controls 107A and 107B, respectively. To have a preliminary knowledge about the physiological difference in the latex between SY107 and its average controls, four latex physiological parameters were determined. Table 1 shows the values of total solid content (TSC) and inorganic phosphorus (Pi) content were not significantly different between SY107 and the controls. TSC has dual effects on rubber productivity: limiting latex flow at a certain higher threshold but revealing an insufficient rubber synthesis activity at a certain lower threshold [31]. Pi participates in the regulation of glycolysis and in the formation of ATP, which is correlated with rubber production [32]. Sucrose content in SY107 was in a value of abundance range [33,34], but significantly lower than that in the control trees (Table 1), suggesting a more active sucrose catabolism in the laticifers of SY107. The thiol content of SY107 was significantly lower than that of the controls, but still maintained a value within the normal range [33,34]. The latex thiols play roles in maintaining the normal functions of the laticifers [35].

| Physiological parameters | PR107 rubber trees | Control trees |
|--------------------------|--------------------|--------------|
| Total solid content (%)  | SY107: 39.5 ± 0.8 a | Control: 37.9 ± 1.3 a |
| Sucrose (mM)             | SY107: 20.95 ± 0.16 a | Control: 34.20 ± 0.12 b |
| Inorganic phosphorus (mM)| SY107: 11.72 ± 0.32 a | Control: 12.14 ± 0.18 a |
| Thiols (mM)              | SY107: 0.596 ± 0.062 a | Control: 0.744 ± 0.021 b |

* Different letters in the same row indicate statistical significance at *P*<0.05 while the same letter indicates no statistical significance.

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Differential expression of TDFs

Most discernible bands appeared in the range of 150-700 bp, and beyond this range fewer bands were observed (Figure 1). For each primer combination, usually 80-90 transcript-derived fragments (TDFs) were visualized and a total of more than 35,000 TDFs were counted for all primer combinations. A total of 1038 differentially expressed (DE) TDFs were recovered and sequenced successfully, corresponding to about 3% of all visualized transcripts. After online BLAST analysis, 158 putative contaminated TDFs were discarded, which showed significant homology to the sequences of animals, fungi and bacteria. This left 880 TDFs for subsequent analysis.

All the TDFs in the format of FASTA were clustered using CAP3, and yielded 746 unique TDFs with average length of 306 bp, which included 98 contigs and 648 singletons. These DE-TDFs were BLASTN searched against the 48768 Unigenes assembled recently from the rubber leaf and latex transcriptomes [7]. According to the criterion of sharing ≥98% nucleotide identity as the same gene, out of the 746 DE-TDFs we isolated, 477 ones corresponded to 594 assembled Unigenes. The expression patterns for 60.7% (453) of the TDFs were further verified by semi-quantitative RT-PCR (sqPCR). The sqPCR-verified TDFs were annotated by similarly searching using the BLASTX program against the GenBank non-redundant public sequence database. To increase the rate of TDF annotation and reduce the redundancy of the acquired DE-TDFs, all sqPCR-verified TDFs were further clustered with the 20126 high-quality Sanger ESTs of SY107 latex [36], and further annotated according to the assembled contigs. The TDFs that showed above 98% sequence identity among their aligned portions (≥ 40 bp) with the same contig, and displayed similar expression patterns, were due to derive from the same mRNA transcript. In these cases, only the longest ones were characterized further. Finally, only 352 TDFs were subjected to further analysis, which may represent most of the non-redundant genes differentially expressed between the SY107 and its average controls (Tables 2 and 3).

qPCR validation of differential expression

To further validate the cDNA-AFLP expression patterns, 18 TDFs were selected for qPCR analysis. As shown in Figure 2, 13 of the 18 TDFs examined revealed the same expression profiles as in cDNA-AFLP analysis. However, the expression of the genes encoding an USP-like protein (T12M1-1), a dehydrin (T8M3-3) and a COX5C family protein (T9M8-1) showed different patterns. They were down-regulated in qPCR analysis, but up-regulated in cDNA-AFLP analysis (Figure 2). In addition, in cDNA-AFLP analysis an alcohol dehydrogenase gene (T15M1-1) was strikingly up-regulated especially with comparison to 107A, whereas in qPCR analysis, this gene displayed a similar expression between SY107 and 107A (Figure 2). The discrepancies between the results of qPCR and cDNA-AFLP analysis are probably due to gene family complexity or the ways to normalize the template amount. In cDNA-AFLP analysis, equal aliquots of total RNA were used for different samples at the beginning, or equal double-stranded cDNAs used at a later stage [28,30,37]. In qPCR analysis, an internal control gene, UBC2 in this study, was exploited to normalize the differences of the cDNA templates used. Nevertheless, the general approach used in this study proved reliable in identifying the genes differentially expressed between the SY107 and the average trees: 72.2% of the cDNA-AFLP patterns were confirmed by qPCR analysis (Figure 2).

Functional classification of DE-genes

Of the 352 non-redundant DE-genes, 215 (61.1%) showed significant homology to genes with known or partially known functions. Fifty-one (14.5%) were homologous to predicted proteins or proteins with unknown functions, and were categorized as 'unclassified' (Tables 2 and 3). The final 86 (24.4%) didn’t show significant matches to available public sequences.

The 215 DE-genes with known or partially known functions were grouped into ten functional categories (Figure 3 ; GenBank_Accn: JZ479023-JZ479237). Transcription and protein synthesis, the largest category, represented 24.7% of the annotated sequences. The genes involved in defense and stress were the second largest category (15.3%), and followed by the category of primary and secondary metabolism (14.0%). These three categories add up to 54.0% of the total sequences, revealing the major biological differences that occur in the laticifers between SY107 and the average trees. Protein destination and storage comprises 10.2% of the total sequences, taking up the fourth place. Other categories include signal transduction (8.8%), cell structure, growth and division (7.9%), unclear classification (7.4%), transporters and intracellular traffic (7.4%), energy (3.7%), and RB (0.5%). The category of unclear classification includes the genes with only partially known functions, and unable to be assigned into any definite functional category. Most of the DE-genes were up-regulated (73.5%) in SY107 compared with the average trees. There are two exception categories, viz., defense and stress, and rubber biosynthesis. For the category of defense and stress, 63.6% of the DE-genes were down-regulated. In the category of rubber biosynthesis, only one DE-gene was identified and revealed to be down-regulated.

Predicted function of DE-genes

In total, 30 DE-genes were in the category of primary and secondary metabolism (Tables 2 and 3), of which 24 were up-regulated. Some of the up-regulated genes were involved in basic metabolic functions, such as carbohydrate metabolism, amino acid metabolism, fatty acid synthesis, lipid degradation, vitamin metabolism, and alcohol fermentation (Table 2). The other up-regulated genes played roles in secondary metabolism, such as the metabolism of amines and steroids, chlorocatechol degradation, and anthocyanin synthesis. The down-regulated DE-genes were involved in lipid metabolism, phenylpropanoid synthesis, nucleotide hydrolysis, and polysaccharide breakdown.

Of the eight DE-genes in the energy category, six were up-regulated and involved in glycolysis, electron transport chain and chlorophyll b synthesis (Table 2). The two down-regulated genes were in the photorespiratory C2 cycle and electron...
Figure 1. Example of a cDNA-AFLP fingerprint after silver staining. Seven selective primer combinations (A6M1-A6M7) are shown as an example. Lanes labeled SY, A and B were derived from latex RNA samples of SY107 and the two controls, 107A and 107B, respectively. Lanes labeled M indicates the 100-bp DNA ladder.

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Table 2. Annotation and functional classification of the DE-TDFs up-regulated in the latex of SY107 trees compared to its average counterparts.

| TDF      | Length(bp) | Genbank accession | Annotation (species)                                      | E-value |
|----------|------------|-------------------|----------------------------------------------------------|---------|
| 01 Primary and secondary metabolism |            |                   |                                                          |         |
| T15M1-1  | 517        | XP_002513255      | alcohol dehydrogenase, putative [R. communis]            | 2E-94   |
| T2M2-2   | 253        | XP_002515027      | steroid dehydrogenase, putative [R. communis]           | 1E-18   |
| T4M11-3  | 180        | XP_002511334      | copper amine oxidase, putative [R. communis]            | 6E-29   |
| T7M5-2   | 283        | AAN63056          | dihydroflavonol reductase [P. tremuloides]              | 4E-32   |
| T16M8-1  | 483        | XP_002514996      | ketoacyl-ACP Reductase (KAR) [R. communis]               | 1E-59   |
| T13M4-3  | 80         | XP_002525172      | dolichylphosphatase, putative [R. communis]             | 2E-08   |
| T14M16-1 | 532        | XP_002511378      | anthranilate phosphoribosyltransferase, putative [R. communis] | 2E-87   |
| T10M2-5  | 116        | ABB89014          | CXE carboxylesterase [A. arguta]                       | 3E-09   |
| T9M11-1  | 360        | XP_002520220      | UDP-glucosyltransferase, putative [R. communis]         | 1E-50   |
| A5M8-2   | 337        | XP_002524657      | stachyose synthase precursor, putative [R. communis]    | 1E-54   |
| A7M6-2   | 258        | NP_180811         | dienelactone hydrolase family protein [A. thaliana]     | 9E-32   |
| T8M10-3  | 99         | ACN66755          | thiazole biosynthetic enzyme [C. papaya]                | 7E-12   |
| A4M2-1   | 292        | XP_002531475      | mannose-6-phosphate isomerase, putative [R. communis]  | 2E-28   |
| T13M2-6  | 186        | BAH03299          | GDP-D-mannose-3',5'-epimerase [P. persica]              | 1E-20   |
| A1M5-3   | 209        | XP_002528703      | O-glycosyl hydrolase, putative [R. communis]            | 3E-32   |
| T6M6-2   | 345        | XP_002520975      | tryptophan synthase alpha subunit, putative [R. communis] | 4E-49   |
| A8M8-1   | 236        | AAQ22256          | gamma-aminobutyrate transaminase subunit precursor isozyme 2 [S. lycopersicum] | 8E-27   |
| T15M3-4  | 141        | XP_002518302      | inorganic pyrophosphatase, putative [R. communis]       | 9E-09   |
| T3M4-3   | 302        | XP_002520803      | biotin carboxyl carrier protein of acetyl-CoA carboxylase [R. communis] | 2E-06   |
| T2M8-1   | 492        | ACN6881           | VTC2-like protein [Malus x domestica]                   | 8E-74   |
| A6M1-2   | 167        | XP_002513571      | spermidine synthase 1, putative [R. communis]           | 3E-14   |
| T6M1-1   | 248        | XP_002526709      | asparagine synthetase, putative [R. communis]           | 5E-31   |
| T4M7-1   | 298        | AAY85187          | pyridoxal kinase [G. max]                               | 6E-33   |
| A5M10-2  | 390        | XP_002518755      | methylcrotonoyl-CoA carboxylase beta chain [R. communis] | 1E-52   |
| 02 Energy |            |                   |                                                          |         |
| T9M8-1   | 327        | XP_002524666      | Cytochrome c oxidase polypeptide Vo-2 [R. communis]     | 8E-19   |
| T1M4-1   | 356        | XP_002526025      | phosphofructokinase, putative [R. communis]             | 7E-52   |
| A1M9-2   | 119        | XP_002516495      | phosphofructokinase, putative [R. communis]             | 2E-13   |
| T9M16-1  | 255        | XP_002515391      | chlorophyll a oxygenase, putative [R. communis]         | 4E-41   |
| T9M11-2  | 181        | XP_002529227      | cytochrome P450, putative [R. communis]                 | 4E-22   |
| T4M12-3  | 186        | XP_002521002      | cytochrome P450, putative [R. communis]                 | 1E-23   |
| 03 Cell, structure, growth and division |            |                   |                                                          |         |
| T13M2-1  | 508        | XP_002520329      | cyclin A, putative [R. communis]                        | 2E-32   |
| T2M8-2   | 471        | ABA96359          | Nse1 non-SMC component of SMC5-6 complex family protein [G. sativa (japonica cultivar)] | 3E-45   |
| T8M10-1  | 333        | AAK84479          | putative auxin growth promotor protein [S. lycopersicum] | 3E-49   |
| A2M1-2   | 298        | XP_00252631       | villin 1-4, putative [R. communis]                      | 3E-42   |
| T7M8-3   | 186        | XP_002512241      | DNA-damage-repair/toleration protein DRT102 [R. communis] | 7E-20   |
| T16M5-3  | 173        | XP_002520219      | gibberelin-regulated protein 3 precursor [R. communis] | 2E-23   |
| T12M5-3  | 258        | AAR07598          | fiber protein Fb19 [G. barbadense]                     | 1E-29   |
| T12M12-2 | 364        | AAC33277          | cotton fiber expressed protein 2 [G. hirsutum]          | 7E-17   |
| T9M11-3  | 129        | XP_002530862      | glucan endo-1,3-beta-glucosidase precursor [R. communis] | 2E-15   |
| T16M10-2 | 233        | C05812            | profilin [H. brasiliensis]                              | 1E-37   |
| A4M6-1   | 385        | BAC78627          | caffeic acid O-methyltransferase [R. chinensis var. spontanea] | 4E-50   |
| T15M10-2 | 176        | XP_002525818      | o-methyltransferase, putative [R. communis]             | 1E-12   |
| T2M15-1  | 510        | XP_002511717      | cinnamoyl-CoA reductase, putative [R. communis]         | 1E-59   |
| T5M4-3   | 308        | XP_002297654      | 10-formyltetrahydrofolate synthetase [P. trichocarpa]   | 9E-39   |
| T13M10-3 | 185        | XP_002232819      | histone h2a, putative [R. communis]                     | 2E-26   |
| 04 Transcription and protein synthesis |            |                   |                                                          |         |
| T4M10-3  | 186        | NP_566412         | KH domain-containing protein / zinc finger (CCCH type) family protein [A. thaliana] | 3E-17   |
| T8M5-1   | 337        | ACT78956          | zinc finger protein [G. arboreum]                       | 4E-16   |
| A2M10-1  | 257        | XP_002532252      | zinc finger protein, putative [R. communis]             | 2E-20   |
| TDF  | Length(bp) | Blast searching Genbank accession | Annotation (species) | E-value |
|------|------------|----------------------------------|----------------------|---------|
| T14M5-1 | 288 | NP_568590 | zinc finger (C3HC4-type RING finger) family protein [A. thaliana] | 2E-14 |
| A8M2-2  | 189 | ABH02645 | MYB transcription factor MYB95 [G. max] | 1E-18 |
| T7M16-4  | 125 | BAD28879 | myb family transcription factor-like [O. sativa Japonica Group] | 2E-13 |
| T15M3-2  | 358 | XP_002308691 | AP2/ERF domain-containing transcription factor [P. trichocarpa] | 2E-31 |
| T6M2-1  | 358 | XP_002515379 | transcription factor, putative [R. communis] | 1E-26 |
| T14M16-4  | 176 | XP_002531463 | homeobox protein, putative [R. communis] | 1E-13 |
| T16M11-1 | 303 | ACLS1015 | squamosa promoter-binding protein [C. trifoliata] | 8E-24 |
| T7M10-3  | 166 | XP_002527359 | transcription initiation factor Iia (tflia), gamma chain [R. communis] | 1E-20 |
| T4M12-2  | 232 | ABD32320 | DNA-directed RNA polymerase, subunit C11/M9 [M. truncatula] | 3E-34 |
| T6M6-1  | 416 | XP_002518700 | DNA-directed RNA polymerase II subunit, putative [R. communis] | 7E-67 |
| T4M2-1  | 314 | XP_002529501 | Heterogeneous nuclear ribonucleoprotein A1, putative [Ricinus communis] | 7E-23 |
| T9M10-1  | 328 | NP_174334 | CPSF30, RNA binding/calmodulin binding/endonuclease[Arabidopsis thaliana] | 1E-45 |
| T3M2-1  | 394 | XP_002530100 | U4/U6 small nuclear ribonucleoprotein Pp4, putative [R. communis] | 4E-64 |
| T11M10-1  | 342 | XP_002528794 | nonsense-mediated mRNA decay protein, putative [R. communis] | 2E-26 |
| T4M6-1  | 212 | BAF01501 | Sm-like protein [A. thaliana] | 4E-12 |
| A2M2-2  | 161 | XP_002513402 | yth domain-containing protein, putative [R. communis] | 3E-10 |
| A3M2-1  | 115 | XP_002513402 | yth domain-containing protein, putative [R. communis] | 2E-07 |
| T11M10-2  | 335 | NP_567592 | GCN5-related N-acetyltransferase (GNAT) family protein [A. thaliana] | 1E-10 |
| A1M7-1  | 340 | XP_002512829 | DNA binding protein, putative [R. communis] | 6E-14 |
| T8M9-1  | 288 | XP_002525167 | DNA-binding protein S1FA, putative [R. communis] | 5E-07 |
| T3M16-2  | 191 | XP_002511085 | DNA binding protein, putative [R. communis] | 2E-15 |
| T5M2-2  | 432 | XP_002515833 | nucleic acid binding protein, putative [R. communis] | 7E-70 |
| T6M12-1  | 361 | XP_002532850 | nucleic acid binding protein, putative [R. communis] | 6E-60 |
| T5M7-2  | 217 | XP_002512575 | nucleic acid binding protein, putative [R. communis] | 8E-27 |
| T14M10-1  | 230 | XP_002512132 | eukaryotic translation initiation factor 5a, putative [R. communis] | 1E-37 |
| T15M1-3  | 297 | ACG38773 | eukaryotic translation initiation factor 2 gamma subunit [Z. mays] | 2E-18 |
| T9M4-2  | 309 | XP_002511200 | elongation factor 1-beta, putative [R. communis] | 1E-24 |
| T5M4-1  | 307 | XP_002509506 | ribosomal protein L27, putative [R. communis] | 1E-49 |
| T15M6-2  | 291 | AAR3886 | 40S ribosomal protein S17 [C. annuum] | 4E-42 |
| T10M9-1  | 297 | XP_002521598 | 40S ribosomal protein S12, putative [R. communis] | 1E-26 |
| T11M13-1  | 514 | XP_002526494 | 40S ribosomal protein S11, putative [R. communis] | 1E-74 |
| T15M1-4  | 185 | ACM90156 | 40S ribosomal protein S15-like protein [J. curcas] | 9E-23 |
| T3M4-2  | 204 | XP_002519689 | 40S ribosomal protein S14, putative [R. communis] | 3E-22 |
| T10M1-1  | 352 | XP_002511695 | 40S ribosomal protein S13, putative [R. communis] | 3E-19 |
| T15M3-1  | 629 | XP_002530136 | 60S ribosomal protein L18a, plant, putative [R. communis] | 2E-24 |
| T11M6-3  | 230 | XP_002530279 | 60S ribosomal protein L12, putative [R. communis] | 3E-34 |
| A6M2-1  | 329 | ACJ02347 | 60S ribosomal protein L17 [V. fordii] | 2E-36 |
| T12M13-1  | 258 | NP_00151284 | 60S ribosomal protein L5-1 [Z. mays] | 2E-38 |
| T15M7-1  | 158 | XP_002526873 | 60S acidic ribosomal protein P0, putative [R. communis] | 2E-21 |
| T16M3-1  | 355 | XP_002527874 | 60S ribosomal protein L35a, putative [R. communis] | 9E-36 |
| A2M4-1  | 332 | XP_002515180 | prolyl-RNA synthetase, putative [R. communis] | 4E-68 |

**5 Protein destination and storage**

| T2M7-1  | 326 | XP_002533817 | ubiquitin-conjugating enzyme E2 [J. putative [R. communis] | 9E-36 |
| T3M5-2  | 140 | XP_002265672 | ubiquitin-protein ligase [V. vinifera] | 4E-16 |
| T11M2-1  | 470 | NP_565522 | ubiquitin-protein ligase [A. thaliana] | 8E-43 |
| T16M2-1  | 169 | XP_002517911 | ubiquitin conjugating enzyme, putative [R. communis] | 3E-15 |
| A8M12-1  | 402 | ACV49920 | ubiquitin-conjugating enzyme variant [C. sinensis] | 2E-26 |
| A4M12-3  | 328 | XP_002513950 | ubiquitin-protein ligase, putative [R. communis] | 3E-28 |
| A5M7-1  | 493 | XP_002521028 | ring finger protein, putative [R. communis] | 3E-38 |
| T13M2-3  | 411 | XP_002280271 | similar to ubiquitin fusion protein [V. vinifera] | 2E-54 |
| T12M7-1  | 224 | NP_565253 | RHA2B (RING-H2 FINGER PROTEIN 2B) [A. thaliana] | 2E-21 |
| T12M2-2  | 134 | XP_002308717 | f-box family protein [P. trichocarpa] | 2E-13 |
| A5M11-2  | 160 | ACB67912 | F-box-containing protein 2 [Malus x domestica] | 1E-10 |
| A7M12-1  | 245 | NP_198752 | EDL2 (EID1-like F-box protein 2) [A. thaliana] | 1E-36 |
Table 2 (continued).

| TDF   | Length(bp) | Genbank accession | Annotation (species)                              | E-value |
|-------|------------|-------------------|---------------------------------------------------|---------|
| A4M10-1 | 479        | XP_002530834      | 26S proteasome non-ATPase regulatory subunit [R. communis] | 2E-49   |
| T1M12-1 | 440        | XP_002526992      | BTB and MATH domain-containing protein [R. communis] | 9E-63   |
| T9M9-1  | 234        | XP_00251285       | protein-I-isoaspartate O-methyltransferase, putative [R. communis] | 2E-34   |
| T7M14-2 | 219        | NP_171698         | peptidyl-prolyl cis-trans isomerase cyclophilin-type family protein [A. thaliana] | 9E-28   |
| T14M7-2 | 566        | XP_002285871      | peptidase isomerase 1 [V. vinifera]                 | 1E-89   |
| T4M10-1 | 435        | XP_002532339      | protein translocase, putative [R. communis]         | 2E-41   |

06 Transporters and intracellular traffic

| A1M5-1 | 700 | ACV66986          | plasma membrane aquaporin 2 [H. brasiliensis]         | 1E-54   |
| A7M5-1 | 220 | XP_002516442      | zinc/iron transporter, putative [R. communis]         | 3E-21   |
| T4M6-2  | 205 | XP_002522484      | vesicle transport V-snare protein vti1a, putative [R. communis] | 1E-20   |
| T3M10-2 | 232 | EF067334          | sucrose transporter 3 [Hevea brasiliensis]            | 1E-70   |
| A5M11-1 | 272 | XP_002336439      | magnesium transporter [P. trichocarpa]                 | 2E-35   |
| T11M8-1 | 312 | ACX37450          | plasma membrane intrinsic protein (PiP2;1) [H. brasiliensis] | 7E-66   |
| T12M2-1 | 265 | BAB80396          | ADP-ribosylation factor [O. sativa Japonica Group]    | 1E-11   |
| T13M10-1 | 326 | XP_002516060      | mitochondrial carnitine/acylcarnitine carrier protein[R. communis] | 6E-08   |
| A3M15-1 | 102 | XP_002523748      | vacuolar protein sorting vps41, putative [R. communis] | 6E-11   |
| A4M11-1 | 298 | BAFO1013          | putative Rab5-interacting protein - like [A. thaliana] | 8E-32   |

07 Signal transduction

| A5M6-2 | 372 | XP_002526147      | brassinosteroid LRR receptor kinase precursor [R. communis] | 3E-33   |
| T7M1-2  | 298 | XP_002529785      | dimethylamine monooxygenase, putative [R. communis]       | 2E-28   |
| T6M11-1 | 264 | XP_002510007      | receptor protein kinase CLAVATA1 precursor [R. communis]  | 3E-37   |
| T11M1-2 | 320 | XP_002510650      | receptor protein kinase, putative [R. communis]           | 2E-44   |
| T15M13-1 | 81  | AAP88291          | protein kinase [C. sativus]                              | 2E-06   |
| A6M5-1 | 633 | XP_002527541      | histidine kinase 1, 2, 3 plant, putative [R. communis]    | 2E-70   |
| A9M9-3  | 94  | AAA41123          | putative casein kinase II, alpha chain 2 CK II [A. thaliana] | 2E-09   |
| T7M5-1  | 451 | XP_002510808      | steroid binding protein, putative [R. communis]          | 2E-28   |
| T3M9-1  | 361 | AAC2626          | rac GTPase activating protein 3 [L. japonicus]            | 3E-10   |
| T16M7-4 | 185 | XP_002529557      | receptor for activated protein kinase C [R. communis]     | 4E-26   |
| T3M7-1  | 346 | XP_002513509      | nucleolar GTP-binding protein, putative [R. communis]     | 4E-40   |
| T7M7-2  | 211 | XP_002513080      | annexin, putative [R. communis]                          | 1E-26   |
| T15M6-1 | 383 | BAD8034           | adenylyl cyclase-like [O. sativa Japonica Group]          | 3E-10   |
| T8M10-4 | 99  | XP_002521342      | developmentally regulated GTP-binding protein [R. communis] | 4E-11   |
| A3M1-2  | 89  | XP_002524007      | phospholipase C 3 precursor, putative [R. communis]       | 6E-06   |

08 Defense and stress

| T4M11-1 | 194 | XP_002530396      | heat-shock protein, putative [R. communis]              | 1E-19   |
| T5M6-1  | 507 | XP_002531089      | chaperone protein DNAJ, putative [R. communis]          | 9E-58   |
| T2M8-3  | 256 | XP_002531285      | wound-induced protein WIN1 precursor [R. communis]       | 3E-11   |
| T10M15-3 | 236 | CAA11041          | latex allergen [H. brasiliensis]                         | 1E-27   |
| T7M9-1  | 223 | ACI04518          | translationally controlled tumor protein [H. brasiliensis] | 6E-22   |
| T12M1-1 | 443 | ABB13620          | USP-like protein [A. sinicus]                           | 1E-36   |
| T8M3-3  | 286 | ABS12334          | dehydrin [P. tremula var. davidiana]                     | 5E-19   |
| T14M4-1 | 334 | XP_002318626      | disease resistance protein [P. trichocarpa]              | 4E-10   |
| T10M9-2 | 221 | XP_002515414      | glutathione peroxidase, putative [R. communis]           | 7E-36   |
| T7M9-2  | 152 | XP_002303583      | glutathione peroxidase [P. trichocarpa]                  | 2E-06   |
| T16M4-4 | 132 | XP_002527520      | trehalose-6-phosphate synthase, putative [R. communis]   | 1E-08   |
| T11M3-2 | 192 | XP_002527520      | trehalose-6-phosphate synthase, putative [R. communis]   | 3E-27   |

09 Rubber biosynthesis

10 Unclear classification

| A3M12-2 | 513 | NP_192096         | toB protein-related [A. thaliana]                       | 4E-58   |
| T1M12-3 | 104 | XP_002522792      | neigor of COX4, putative [R. communis]                  | 5E-05   |
| T4M8-1  | 503 | XP_002524557      | XPA-binding protein, putative [R. communis]             | 2E-60   |
| T5M2-3  | 328 | XP_002528475      | leucoanthocyanidin dioxygenase, putative [R. communis]  | 4E-09   |
| T5M8-3  | 215 | XP_002515000      | ankyrin repeat-containing protein, putative [R. communis] | 4E-26   |
| T13M7-2 | 349 | XP_002517559      | hydrolase, putative [R. communis]                       | 1E-52   |
transfer (Table 3). Fifteen up-regulated and two down-regulated DE-genes were grouped in cell structure, growth and division (Tables 2 and 3). Of the up-regulated genes, three were involved in DNA synthesis and repair, and cell cycle; the others were implicated in cytokkeleton, cell wall structure modification, cell growth and division. The two down-regulated genes were predicted in DNA replication and cell growth.

The largest functional category, i.e. transcription and protein synthesis included 53 DE-genes, the majority (44) of which was up-regulated (Tables 2 and 3). Fifteen genes encoded various types of transcription factors (TFs), including six zinc-finger containing TFs, two MYB or MYB-related family TFs, one HB family TF, one SBP family TF, one AP2/ERF family TF, two general TFs, and two unknown type of TFs. Through regulating the expression of a variety of target genes, these types of TFs are implicated in almost all known biological processes in higher plants, such as cell proliferation and differentiation, growth and development, and various responses to environmental stimuli [38-41]. Nineteen genes were directly involved in protein synthesis, including fourteen ribosomal proteins, four translation factors, and one aminoacyl tRNA synthase. Twelve genes were involved in mRNA synthesis, processing and stability control. The remaining seven genes encoded nucleic acid (DNA, RNA or both)-binding proteins, being putative regulators of transcription or translation.

The category of protein destination and storage included 22 DE-genes, only four of which were down-regulated (Tables 2 and 3). Most genes in this category encoded proteins involved in ubiquitin-mediated protein degradation [42], such as ubiquitin-conjugating enzymes, ubiquitin-protein ligases, F-box proteins, ring-finger proteins, and 26S proteasome and peptidase. The other genes were involved in protein folding, protein damage repair, and protein targeting. In the category of transporters and intracellular traffic, 16 DE-genes were included, of which six were down-regulated (Tables 2 and 3). Of the up-regulated genes, two aquaporins, one sucrose transporter, four vesicle trafficking proteins, two ions transporters, and one carnitine carrier protein were identified. The down-regulated genes included two sucrose transporters, one putative sugar transporter, one Rab protein, one cation transport protein, and one grave disease carrier protein.

Nineteen genes were assigned in the category of signal transduction, most (15) of which were up-regulated (Tables 2 and 3). Some important signaling components were included, such as protein kinases, phosphatases, receptors, and G proteins, which play roles in a number of well-known signaling pathways [43-47]. Thirty-three DE-genes were grouped in the category of defense and stress, consisting of the second largest category. It is the only category that has a larger quantity of down-regulated genes (21) than up-regulated (12) (Tables 2 and 3). The 12 up-regulated genes consisted of a variety of stress and defense-related genes (Table 2), such as molecular chaperons, detoxification enzymes, osmoprotectant-biosynthesis related proteins, disease resistance proteins and so on. Interestingly, in this category, nearly all down-regulated genes belonged to the family of heat shock proteins (Table 3). The exception one was a phosphosulfolactate synthase-related protein (A4M5-1), which also reveals to be a novel heat shock protein [48]. Only one DE-gene was identified in the category of rubber biosynthesis, and it was a down-regulated acetyl-CoA C-acetyltransferase (Tables 2 and 3).

Discussion

Rubber plantations in China are located at 18-24 °N, entirely beyond the traditional rubber-growing tract in the world, and are often subjected to serious damages caused by typhoons and cold waves [49]. Correspondingly, the productivity of rubber tree in China is somewhat lower and usually 3-4 kg of dry rubber tree-1 yr-1. In such a growing condition it is spectacular to have this super-high-yielding PR107 tree (SY107) [18]. As calculated from the latex yield per tapping (Table 1), the SY107 produced 74.2 kg of dry rubber in 2006, the year when we conducted this study. This yield decreased markedly as compared to that (126 kg) in 2002 [18], but was still much higher than that of other rubber trees by average (5-6 kg of dry rubber tree-1 yr-1) growing in the same region and that.
Table 3. Annotation and functional classification of the DE-TDFs down-regulated in the latex of SY107 trees compared to its average counterparts.

| TDF     | Length (bp) | Blast searching | Genbank accession | Annotation (species) | E-value |
|---------|-------------|-----------------|-------------------|----------------------|---------|
| **01 Primary and secondary metabolism** |             |                 |                   |                      |         |
| T1M1-3  | 191         | XP_002514215    | ceramide glucosyltransferase, putative [R. communis] | 8E-16                |         |
| T3M10-3 | 74          | XP_002519980    | ethanolamine-phosphate cytidylyltransferase, putative [R. communis] | 6E-05                |         |
| T14M13-3| 167         | XP_002527433    | phosphatidylcholine diacylglycerol acyltransferase [R. communis] | 3E-19                |         |
| T9M16-2 | 206         | XP_002313788    | phenylcoumaran benzyl ether reductase 3 [P. trichocarpa] | 6E-26                |         |
| A6M6-1  | 641         | XP_002518196    | beta-amylase, putative [R. communis] | 1E-107               |         |
| T14M4-2 | 256         | XP_002513415    | proteasome-activating nucleotidase, putative [R. communis] | 6E-24                |         |
| **02 Energy** |       |                 |                   |                      |         |
| T13M13-1| 452         | XP_002516221    | adrenodoxin, putative [R. communis] | 4E-36                |         |
| T18M4-2 | 301         | XP_002517532    | D-glycerate 3-kinase, putative [R. communis] | 3E-39                |         |
| **03 Cell structure, growth and division** |       |                 |                   |                      |         |
| T3M1-1  | 313         | XP_002517787    | Angio-associated migratory cell protein, putative [R. communis] | 3E-13                |         |
| A7M4-1  | 206         | XP_002526324    | DNA gyrase subunit A [R. communis] | 2E-16                |         |
| **04 Transcription and protein synthesis** |       |                 |                   |                      |         |
| A7M9-2  | 183         | XP_002514762    | transcription factor, putative [R. communis] | 2E-17                |         |
| T16M13-1| 577         | AAD26942        | zinc-finger protein 1 [D. glomerata] | 2E-26                |         |
| A1M9-3  | 98          | ABF95225        | zinc-finger family protein [O. sativa (japonica cultivar-group)] | 2E-11                |         |
| T9M13-2 | 245         | NP_00150575    | transcription elongation factor SPT4 [Z. mays] | 2E-29                |         |
| T6M11-3 | 160         | XP_002530669    | RNA splicing protein mrs2, putative [R. communis] | 9E-12                |         |
| T9M13-3 | 152         | XP_00251442    | ccr4-associated factor, putative [R. communis] | 2E-17                |         |
| A1M13-1 | 466         | XP_002520438    | RNA binding motif protein, putative [R. communis] | 4E-29                |         |
| T5M16-3 | 79          | XP_002523184    | GTP-dependent nucleic acid-binding protein engD [R. communis] | 3E-06                |         |
| A1M15-1 | 415         | ACS96446        | 60S ribosomal protein L18a [J. curcas] | 5E-73                |         |
| **05 Protein destination and storage** |       |                 |                   |                      |         |
| T7M4-4  | 196         | XP_002512947    | ubiquitin-protein ligase, putative [R. communis] | 6E-21                |         |
| A2M9-1  | 210         | Q43207          | peptidylprolyl isomerase [T. aestivum] | 2E-28                |         |
| A4M15-1 | 275         | XP_002533551    | ubiquitin ligase protein cop1, putative [R. communis] | 3E-31                |         |
| A8M12-2 | 238         | XP_002526029    | groes chaperonin, putative [R. communis] | 1E-16                |         |
| **06 Transporters and intracellular traffic** |       |                 |                   |                      |         |
| T6 M15-2| 324         | XP_002529745    | sugar transporter, putative [R. communis] | 9E-25                |         |
| A8M2-1  | 193         | ABJ51934        | sucrose transporter 2A [H. brasiiliensis] | 5E-05                |         |
| T13 M1-2| 390         | ABK60189        | sucrose transporter 5 [H. brasiiliensis] | 1E-02                |         |
| T3M5-1  | 307         | NP_001150271    | ras-related protein Rab-6A [Z. mays] | 7E-47                |         |
| A3M14-1 | 238         | XP_002527996    | cation transport protein chaC [R. communis] | 2E-32                |         |
| T7M3-3  | 349         | XP_002532663    | grave disease carrier protein, putative [R. communis] | 4E-34                |         |
| **07 Signal transduction** |       |                 |                   |                      |         |
| A3M10-1 | 772         | XP_002527010    | protein phosphatase, putative [R. communis] | 1E-126               |         |
| T10M4-3 | 152         | BAA94510        | protein kinase 2 [P. nigra] | 6E-19                |         |
| A5M2-1  | 411         | NP_173259       | calcium-binding protein, putative [A. thaliana] | 2E-43                |         |
| T6M16-1 | 303         | NP_567979       | cyclase family protein [A. thaliana] | 7E-28                |         |
| **08 Defense and stress** |       |                 |                   |                      |         |
| A6M7-1  | 246         | XP_002520482    | heat-shock protein, putative [R. communis] | 5E-37                |         |
| A4M1-1  | 312         | XP_002529170    | heat-shock protein, putative [R. communis] | 7E-12                |         |
| A4M1-2  | 120         | XP_002520482    | heat-shock protein, putative [R. communis] | 2E-14                |         |
| T2M11-3 | 216         | XP_002520484    | heat-shock protein, putative [R. communis] | 2E-29                |         |
| T3M10-1 | 218         | XP_002516106    | heat-shock protein, putative [R. communis] | 9E-26                |         |
| T3M15-2 | 360         | XP_002532054    | heat shock protein, putative [R. communis] | 1E-37                |         |
| T2M3-2  | 123         | XP_002532054    | heat shock protein, putative [R. communis] | 4E-05                |         |
| T6M1-2  | 179         | XP_002520481    | heat shock protein, putative [R. communis] | 1E-23                |         |
| A5M9-2  | 327         | XP_002526950    | heat-shock protein, putative [R. communis] | 3E-29                |         |
| T7M11-1 | 317         | XP_002521081    | heat-shock protein, putative [R. communis] | 6E-46                |         |
| T8M1-1  | 387         | XP_002530396    | heat-shock protein, putative [R. communis] | 3E-28                |         |
of the two control trees (5.76 and 14.94 kg of dry rubber tree⁻¹ yr⁻¹, respectively) selected in this study at its proximity.

As the first step to investigate the mechanisms of high productivity in SY107, we compared four physiological parameters of the latex (the cytoplasm of rubber-producing laticifers) between SY107 and its controls. The four latex parameters, i.e. total solid content (TSC), sucrose, thiols and inorganic phosphorus (Pi) contents, revealed direct correlations with the production of certain rubber clones under certain conditions [33,50,51]. SY107 showed similar TSC and Pi contents to the controls, whereas its sucrose and thiols contents were significantly lower than the controls (Table 1).

Sucrose is the raw material for laticiferous metabolism, particularly for rubber synthesis [52]. The sucrose content in the latex reflects the balance of sucrose loading and utilization in the laticifers. Sucrose utilization is always guided by the activity of a neutral/alkaline type of invertase, and thus constitutes one of the limiting factors for rubber production [52,53]. We hypothesize that the lower sucrose content in SY107 is due to more active sucrose utilization. This hypothesis was supported by a higher latex invertase activity (8.53 units/ml c-serum) in SY107 over the control trees (6.36 units/ml c-serum). In the latex, thiols are both protectors of the membrane systems and the activators of some key enzymes [33]. The lower latex thiols in SY107 may reflect higher consumption of thiols in SY107 to counteract the enhanced production of reactive oxygen species (ROS) under the conditions of a more active laticiferous metabolism [3]. Although the analysis of latex physiological parameters gave a hint to the mechanisms of high productivity in SY107, differences are not known in the molecular events that occur in the laticifers between SY107 and its controls.

The cDNA-AFLP technique allowed transcription changes to be surveyed with no prior assumption about which genes might be differentially expressed in the latex between SY107 and its controls. With two enzyme combinations selected, about 35000 cDNA fragments were visualized on the gels, and to our knowledge, this is one of the most extensive cDNA-AFLP analyses ever done in biological samples. A total of 352 genes were found to be differentially expressed, of which 215 showed significant homology to genes with known or partially known functional categories, which indicates that SY107 and its controls differ in different physiological and biochemical pathways.

The up-regulation of a sucrose transporter HbSUT3 (T3M10-2) suggests an enhanced sucrose loading to the laticifers of SY107. HbSUT3 has been demonstrated to be actively involved in sucrose loading to laticifers and rubber productivity [14,15]. Moreover, two other sucrose transporters (HbSUT5, T13M1-2; HbSUT2A, A8M2-1) were also differentially expressed between SY107 and the control trees, further implicating the importance of the regulation of sucrose loading. In latex, both HbSUT5 and HbSUT2A are less expressed than HbSUT3 [14], but their roles in regulating sucrose loading deserve further studies. Sucrose catabolism in the latex is also an important factor affecting rubber productivity [52], and a neutral/alkaline invertase HbNIN2 was identified as the key gene responsible for sucrose catabolism in the latex, and its expression was much higher in SY107 than the control trees (Tang et al., unpublished data). In this study, the cDNA-AFLP analysis failed to detect TDFs for HbNIN2. This may simply be due to our insufficient isolation of DE-TDFs since in silico restriction mapping indicates the suitability of HbNIN2.

Table 3 (continued).

| TDF   | Length (bp) | Blast searching | Annotation (species) | E-value |
|-------|-------------|-----------------|----------------------|---------|
| A5M15-1 | 538          | XP_002526012    | heat shock protein binding protein, putative [R. communis] | 6E-27   |
| A7M3-1  | 227          | XP_002516783    | heat shock protein 70Kd, putative [R. communis] | 5E-32   |
| A8M6-2  | 281          | CAA72128        | heat shock protein 70 [C. sativus] | 1E-36   |
| T3M6-3  | 175          | CAI4864.2       | heat shock protein 101 [T. turgidum subs. durum] | 7E-25   |
| T2M3-1  | 353          | XP_002517628    | small heat-shock protein, putative [R. communis] | 2E-22   |
| A4M11-3 | 262          | CAI12387        | Hsp20.1 protein [S. peruvianum] | 2E-18   |
| T2M11-1 | 431          | AAD41409        | cytosolic class II low molecular weight heat shock protein [P. dulcis] | 4E-49   |
| T9M5-2  | 274          | ABW69469        | low molecular weight heat shock protein [G. hirsutum] | 1E-25   |
| T6M16-2 | 232          | XP_002530362    | Hsp90 co-chaperone AHA1, putative [R. communis] | 9E-23   |
| A4M5-1  | 489          | AAV35619        | phosphosulfocodetate synthase-related protein [S. lycopersicum] | 2E-43   |

09 Rubber biosynthesis
A2M8-1  217  BAF98277  acetyl-CoA C-acetyltransferase [H. brasiliensis]  4E-08
10 Unclear classification
A7M8-2  231  CAP16621  polyprotein [M. acuminata subs. malaccensis]  2E-33
T2M5-1  351  XP_002511089  nucleotide binding protein, putative [R. communis]  1E-55
11 Unclassified
10 DE-TDFs in total
12 No hit
38 DE-TDFs in total

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mRNA for the cDNA-AFLP analysis. An INDETERMINATE DOMAIN transcription factor (TF) AtIDD8 was reported in Arabidopsis to regulate sugar transport and metabolism by binding directly the promoters of a sucrose synthase gene (SUS4) [54]. It still needs to investigate whether in the laticifers some of the DE-TFs identified in this study (Tables 2 and 3) play roles in regulating the expression of HbSUT3 and HbNIN2 in similar ways to AtIDD8.

In the latex, rubber synthesis is thought to make better use of the energy and reducing power generated by carbohydrate fermentation rather than the Krebs cycle and oxidative phosphorylation [3], stressing the significance of glycolysis in rubber production. Phosphofructokinase (PFK) is one of the rate-limiting enzymes of glycolysis. In this study two PFK TDFs (T11M4-1; A1M9-2) were up-regulated, indicating an increased glycolysis in the laticifers of SY107. Also, the genes encoding a mannose-6-phosphate isomerase (A4M2-1) and an O-glycosyl compound hydrolase (A1M5-3) were identified, the former of which transforms mannose 6-phosphate into fructose 6-phosphate and then into glycolysis [55], and the latter hydrolyzes glycosidic linkages, such as those in cellulose and hemicellulose to release smaller sugars [56]. The up-regulation of these two genes suggests that SY107 has a higher capacity to mobilize other types of sugars as well as sucrose for subsequent glucidic metabolism. An alcohol dehydrogenase (T15M1-1) was observed up-regulated, which under the conditions of hypoxia facilitates the maintenance of glycolysis and the alleviation of cytosolic acidification [57], and favors latex regeneration [53]. In addition, a vacuolar-type inorganic pyrophosphatase (T15M3-4) was up-regulated, which functions in efficient turnover of pyrophosphate (PPi) produced during isoprene anabolism [3,58], and thus activates isoprene synthesis and glycolysis.

The expressional regulation of the genes that are directly involved in the pathways running from acetyl-CoA to the cis-polyisoprene (rubber), and hence called rubber-biosynthesis...
pathway genes (RB-genes) is thought to be critical in affecting rubber productivity [6, 59]. Several RB-genes, such as cis-prenyl transferase (CPT), small rubber particle protein (SRPP), rubber elongation factor (REF) and HMG-CoA reductase (HMGR), have been extensively studied, and the expression of REF in the latex was even found to correlate positively with the productivity of rubber clones (varieties) [11]. Although in silico restriction mapping confirmed the suitability of all known RB-genes for the cDNA-AFLP analysis, only one RB-gene (acetyl-CoA C-acetyltransferase, A2M8-1) was isolated in this study, and found down-regulated. Therefore, we set out to determine whether the failure to identify more RB-genes is due to insufficient DE-TDF isolation or just reflects their actual expression patterns between SY107 and other trees. As determined by qPCR, none of the eight RB-genes examined (HMG-CoA synthase, HMGR, phosphomevalonate kinase, IDP isomerase, FPP synthase, REF, SRPP and CPT) was significantly higher or lower expressed in SY107 than in the two controls (Figure 4). In another independent study, we compared the transcriptomes of three tissues (latex, bark and leaf) between two rubber clones (Reyan8-79 and Reyan7-33-97) using 454 GSFLx technology (Li et al. unpublished). Again, the differential expression of RB-genes in the latex of the two clones accounted little for the higher productivity of Reyan8-79 than Reyan7-33-97, further strengthening the idea that the SHY characteristics of SY107 has little correlation with the expression of RB-genes, but with the differential expression of the other genes involved in sucrose availability and catabolism, and other kinds of metabolisms within the latex cells.

Latex productivity of a rubber tree depends mainly on two factors: the duration of latex flow after tapping and the capability of latex regeneration between two consecutive tappings [3]. Latex regeneration is subjected to multiple biological processes, such as the regulation of transcription & translation, intracellular trafficking and signaling pathways, and water movement across the laticifers that is critical in controlling latex flow. In this study, the DE-genes involved in transcription and protein synthesis were most abundantly represented, the majority of which were up-regulated in SY107 (Tables 2 and 3), suggesting that during latex regeneration a much more enhanced gene expression and protein synthesis occurs in the latex of SY107. Also, latex regeneration requires the replenishment of large amounts of various organelles,
especially the laticifer-specific ones (rubber particles, lutoids and Frey-Wyssling complexes), which adds up to 50-70% of the fresh latex volume [3]. The up-regulation of three important proteins, i.e. vacuolar protein sorting vps41 (A3M15-1), vesicle transport V-snare protein vti1a (T4M6-2) and ADP-ribosylation factor (T12M2-1), involved in intracellular trafficking, biogenesis and development of organelis [60-62], agrees with the finding that SY107 has a much stronger capability of latex regeneration. In addition, signaling pathways, especially those of ethylene, jasmonate and wounding, are actively implicated in the regulation of latex regeneration [63]. In this study, nineteen genes encoding signaling components, including different types of kinases, receptors, G proteins and annex, were identified, most of which were up-regulated (Tables 2 and 3). The function of annex in signal transduction and amplification has been well described in plants [64], and here the up-regulation of an annex protein (T7M7-2), together with the up-regulation of many other signaling components, corroborates the idea that the stimulation of multiple signaling pathways contributes to the enhanced productivity in SY107. Moreover, the up-regulation of two aquaporins (A1M5-1; T11M8-2) was noticed in this study. One aquaporin (HbPIP2;1, T11M8-2) has been confirmed to have a role in regulating the water conduction between the laticifers and the inner liber tissues, and its expression is correlated positively with the ethylene stimulation of latex yield [16], suggesting that up-regulation of aquaporins contributes to an improved latex flow and thus the higher productivity in SY107.

Rubber harvesting, including the act of tapping and ethylene stimulation, produces osmotic and oxidative stresses [65]. The capability of a rubber tree, especially the rubber-producing cells - the laticifers - to tolerate, adapt and alleviate such stresses is essential for its sustainable rubber productivity. Among the 34 DE-genes in the category of defense and stress, 23 encode different families of heat-shock proteins (Hsps). The roles of Hsps in protein protection and cellular homeostasis have been made clear under cellular stress [66]. Remarkably, 21 of the 23 Hsps are down-regulated, whereas the other two Hsps, along with 11 other stress-related genes, are up-regulated (Tables 2 and 3). In plants, the over-expression of Hsp genes and other stress-related genes under stress conditions is usually realized at the cost of depressing other multiple metabolic genes, and thus affects plant normal growth and development [67,68]. The down-regulation of most DE-genes in the category of defense and stress and up-regulation of the vast majority of DE-genes on the whole suggest that the laticifers of SY107 are subjected to less stresses than other trees. This idea is further strengthened by the finding of the down-regulation of two Hsp70 proteins (A7M3-1 and A8M6-2), which are reported to be significantly over-expressed under stress conditions and can act as sensitive biomarkers of cell stress in humans [69], animals (snail species of the genus Tegula) [70] and plants.

Figure 4. Expressional comparison of 8 rubber-biosynthesis-pathway genes between SY107 and its control trees by qRT-PCR. Abbreviations: HMGS, 3-hydroxy-3-methylglutaryl-coenzyme synthase; HMGR, 3-hydroxy-3-methylglutaryl -coenzyme reductase; PMK, phosphomevalonate kinase; IPPI, isopentenyl diphosphate isomerase; FDS, isopentenyl diphosphate synthase; REF, rubber elongation factor; SRPP, small rubber particle protein; CPT, cis-prenyl transferase. The values of relative expression are presented as the mean ± SE of three biological replicates. No genes showed significantly differential expression between SY107 and the two controls, as determined by t test.

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(Fucus serratus and Lemna minor) [71]. In Arabidopsis, most of the ROS metabolism-related genes are significantly regulated in stress conditions [72]. So, the result that no DE-TDFs for such kind of genes were isolated in this study further reveals a lower stress level in the latex of SY107. Together, these results suggest that SY107 has a better capability to lower stress levels in time and then enables to invest more effort into the metabolic pathways related to latex regeneration, and thus display a phenotype of higher productivity. Moreover, the up-regulation of multiple components of ubiquitin-mediated protein degradation and protein degradation-related signaling pathways was noticed (Table 2). A higher rate of protein turnover is essential for higher metabolism of plants [73,74], and it corroborates the extraordinarily active metabolism that occurred in the laticifers of SY107.

This is the first report of revealing markedly different yield levels of rubber trees through a global transcription comparison and analysis. The profiles of the large number of DE-genes identified in this work suggest that the SY107 tree has sufficient molecular basis for its super-high yielding, especially in the aspects of improved sucrose loading capability, RB-preferred sugar utilization, enhanced general metabolisms, and timely stress alleviation. However, it is noteworthy that RB-pathway genes seem to have little correlation with the high yielding phenotype. Next generation sequencing technologies, e.g. Solexa, SoLiD and 454, provide new tools to further exploring the mechanisms of super rubber productivity, since the powerfulness of these techniques in transcriptome profiling has been well documented [75]. Anyway, the results presented here provide a valuable guide for molecular rubber breeding aimed to enhance rubber production.

Materials and Methods

Ethics Statement

Our field studies and sampling have been approved and assisted by the owner of the rubber plantation, Yunan State Farms Group Co., LTD.

Plant materials and growth conditions

Clone PR107 rubber trees used for cDNA-AFLP analysis have been cultivated in a plantation in Mengla County, Xishuangbanna Dai Autonomous Prefecture, Yunnan Province, China. These trees, including the super-high-yielding tree (SY107) and four average PR107 trees in its proximity, were planted in 1963 and opened in 1970. Since 2000 these trees have been tapped in a system of two half spiral cuts, every 4 days and with 4% Ethrel (an ethylene generator) stimulation (2×S/2DU. d4. ET4%). Pa. 20/y (15d). The four average PR107 trees were divided into two groups (107A and 107B) of two trees according to latex yields.

Determination of latex yield, physiological parameters and invertase activity

The latex yields were determined from May to July 2006. In each month, the latex yields for three consecutive tapings after one round of Ethrel stimulation were measured. To assay the physiological parameters, an aliquot of 20 ml of latex per tree was collected 5 min after tapping in a 50-ml centrifuge tube placed in ice, and transported to the laboratory for immediate analysis. The parameters of total solid content (TSC), pH, thiols, inorganic phosphates, and sucrose contents were determined according to Eschbach et al. (1984) [33]. A fraction of fresh latex was centrifuged for 30 min at 4 °C and 45,000×g, and the clear middle phase (cytoplamic serum, c-serum) was collected for invertase activity analysis. The invertase activities were determined according to the procedure of Tupy (1969) [76] with modifications. Briefly, a 200-μl reaction mixture contained 50 μl c-serum, 0.1 mol/l of NaF and 0.06 mol/l of sucrose. The mixture was incubated at 30 °C for 1 h, and stopped by adding 2 volumes of alcohol and boiling for 5 min. Then, distilled water was added to adjust the mixture to a final volume of 1.5 ml, and centrifuged to collect the clear solution for determining the reducing sugars by the method of Miller (1959) [77]. One unit of invertase activity is defined as the amount of enzyme that which produces 1.0 μmoles of reducing sugars per minute at 30 °C. The number of units given in the results is expressed per ml of c-serum.

RNA extraction, cDNA synthesis and cDNA-AFLP analysis

During the collection of latex for determining physiological parameters, an aliquot of 5 ml of latex per tree was sampled concomitantly for RNA extraction, cDNA synthesis and cDNA-AFLP analysis. Experimental manipulations were conducted according to the methods previously described [30,78]. Total RNA concentration was determined spectrophotometrically and adjusted to a final concentration of 1 μg μl⁻¹. Poly(A⁺) RNA was enriched from a 50-μg aliquot of total RNA for each sample using polyTract® mRNA isolation systems III (Promega, Madison, WI). The enriched poly (A)⁺ RNA was reverse-transcribed with oligo(dT) 25V (V=A, C or G) primer into first strand cDNA by using the RevertAidTM First Strand cDNA synthesis kit (Fermentas, Lithuania). The first strand cDNA was then transformed into double-stranded (ds) cDNA using DNA polymerase I (Takara Bio, Dalian) and RNase H (Fermentas, Lithuania) according to the manufacturers’ manuals. The resulting ds-cDNA was extracted with phenol:chloroform: isoamyl alcohol (25:24:1), and then ethanol precipitated with linear polyacrylamide as the carrier [79]. The ds-cDNA precipitate was dissolved in double distilled water (ddH₂O) for template preparation.

The ds-cDNA was digested by one of the two restriction enzyme pairs selected, Apol/Msel and TaqI/Msel, and used for the preparation of pre-amplification templates. All 384 possible selective primer combinations for the two restriction enzyme systems (Table S1) were employed to perform transcriptional profiling. The details of cDNA-AFLP analysis were previously described [30].

Isolation and sequencing of transcript-derived fragments (TDFs)

All bands longer than 70 bp were compared in the three samples (SY107, 107A and 107B), and those differentially expressed (DE) TDFs, varying in length from 70-800 bp, were
exercised from the gels, re-amplified, and sequenced. The DE-
TDFs were classified into two categories with the expression
level in SY107 compared to that in the two controls (107A and
107B): up-regulated and down-regulated, with a criterion of ≥2-
fold difference in band intensities. The bands corresponding to
differentially expressed TDFs between SY107 and the controls
(107A and 107B) were excised from the PAGE gel. The gel
bands were soaked in 20 µl of sterile 10 mM Tris-HCl buffer
containing 1 mM EDTA, pH 8.0, initially at 95 °C for 30 min and
then hydrated overnight at 4 °C, and followed by centrifugation
at 10,000 g for 2 min to collect the supernatant. An aliquot of 2
µl supernatant was included in a 40 µl mixture for re-
amplification, using the corresponding selective primers (Table
S1) and the same conditions that were in pre-amplification. The
amplified fragments were fractionated on a 1.5% agarose gel.
The band with the target size was isolated and then sequenced
using ABI PRISM™ 3730 DNA Sequencer in Shanghai
GeneCore BioTechnologies Co., Ltd (Shanghai, China).

Sequence analysis
To analyze a large set of TDF data, we built a local stand-
dalone EST analysis platform. First, PHRAP package [http://
www.phrap.org/phredphrapconsed.html] was used to pre-
process the TDF data. ABI files derived from the ABI
PRISMTM 3730 DNA Sequencer were converted into text files
using PHRED with default parameters [80,81]. Vector
sequences were then eliminated by CROSS_MATCH with the
parameters of -minmatch 10 -miniscore 20. All TDFs, in the
format of FASTA, were clustered using CAP3 with the
parameters of -o 40 –p 90 [http://genome.cs.mtu.edu/cap/
cap3.html], with 21 as the overlap parameter [82]. BLAST
analysis was conducted to the filtered unique TDFs with stand-
dalone BLAST [ftp://ftp.ncbi.nlm.nih.gov/blast/executables/
release/] package using BLASTX as the engine, NR [ftp://
ftp.ncbi.nlm.nih.gov/blast/db/] as the database, and E-value of
1e-5 as the cutoff point for acceptance of similar functions.
Sequences were manually assigned to functional categories as
described by Bevan et al. [83], and assisted by the relevant
scientific literature and the information reported for each
sequence by the Gene Ontology consortium, when available,
or reported by the Swiss-Prot [http://expasy.org/sprot/], KEGG
[http://www.genome.jp/kegg/] [84] and TAIR databases [http://
www.arabidopsis.org/].

QPCR Analysis
For qPCR, first-strand cDNA was synthesized using 2.5 µg of
DNase I-treated total latex RNA as described in Li et al. [85].
The PCR reaction was performed in a LightCycler 2.0 system
(Roche Diagnostics, Germany). Oligonucleotide primer pairs
(forward and reverse) for 18 randomly selected target genes
(Table S2) and eight rubber biosynthesis pathway genes
(Table S3) were designed according to the known DNA
sequences from TDFs. The UBC2b gene, determined to be the
most suitable internal control for individual rubber trees [85],
was used as the reference gene. The relative abundance of
transcripts was calculated according to the instructions of the
LightCycler Relative Quantification Software 4.05: Expression
=\( e^{\Delta C_{t}(UBC2b)} \times C_{t}(target
gene) \). All amplified fragments were cloned, and
sequenced for target confirmation. For each target gene, three
independent qPCR analyses were performed. The experimental
details were described previously [85].

Statistical Analysis
The Student’s t test was used to compare the expression of
the selected genes or TDFs in latex between SY107 and its
controls using the algorithm embedded into Microsoft Excel
2007. The term “significant” is used in the text only when the
change in question has been confirmed to be significant (P <
0.05).

Supporting Information
Table S1. Adaptors and primers used for cDNA-AFLP
analysis.
(DOC)
Table S2. Primers used in quantitative real-time PCR (qRT-
PCR) for validation of cDNA-AFLP results.
(DOC)
Table S3. Primers used in qRT-PCR for expressional
comparison of 8 rubber-biosynthesis-pathway genes
between SY107 and its control trees.
(DOC)

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Author Contributions
Conceived and designed the experiments: CRT. Performed the
experiments: XHX HPL YJF JHY JYQ. Analyzed the data: XHX
CRT. Contributed reagents/materials/analysis tools: HBL.
Wrote the manuscript: XHX CRT.
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