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Characterization and Expression Profiles of Galaetinol Synthase and Raffinose Synthase in Rubber Tree

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Abstract Galactinol synthase (GolS) and Raffinose synthase (RS) are key enzymes for the synthesis of Raffinose family oligosaccharides (RFOs) which are widely involved in the growth development and resistances to stresses. To explore the function of *HbGolSs* and *HbRSs* during leaf development and tapping period in Hevea brasiliensis, in this study, based on the established library data platform of rubber tree genome, we isolated and identified the family members of *HbGolSs* and *HbRSs*, then we analyzed their expression profiles with emphasis through on-line molecular biological tools and PRISM software as well. The results demonstrated that *HbGolS1* and *HbRS1* were prime member in their separate family, *HbGOlS1* was highly expressed in latex and leaf, and with leaf development, its level gradually increased till the ultimate in mature leave, especially, it was dramatically up-regulated by tapping but markedly inhibited by Ethrel treatment; Different from it, *HbRS1* was little influenced by tapping or Ethrel treatment although it was specially expressed in latex. Together, this study demonstrated the expression characteristics of *HbGolS1* and *HbRS1* with leaf development and tapping, which has laid a foundation for further exploring the metabolism of RFOs and their roles in rubber trees.
Keywords Adversity stress; Galaetinol synthase; Raffinose synthase

When plants are under environmental stress, they can rapidly synthesize and accumulate different kinds of osmotic regulatory substances to maintain normal physiological functions in plant cells (Sun et al., 2013). Raffinose family oligosaccharides (RFOs), for example, is widely involved in plant abiotic stress resistance, especially osmotic stress resistance. The key enzymes catalyzing the synthesis of RFOs are GolS (Galactinol synthase, EC 2.4.1.123) and RS (Raffinose synthetase, EC 2.4.1.82). This is because the Galactinol (Gol) catalyzed by GolS is not only the only known source of galactosyl donors in plants, but also the raffinose product catalyzed by GolS and RS is the smallest trisaccharide and the initial substrate for the synthesis of RFOs. Studies have shown that the *GolS* gene in *Boea hygrometrica* ((Bunge) R. Br.) is closely associated with drought tolerance and recovery (Li, 2017; Gu, 2018; Qin et al., 2019); *At5g40390* (RS5) is the only functional gene of raffinose accumulation induced by abiotic stress in Arabidopsis leaves (Taji et al., 2002). Other studies have shown that RFOs, a class of small molecular osmotic regulatory substances, accumulates less in the body under normal growth conditions, but its content will increase rapidly under stress (Shimosaka and Ozawa, 2015). For example, RFOs in cucumber (*Cucumis sativus* L.) is the main form of phloem assimilate transport (Nishizawa et al., 2008). Therefore, Gol and Raf are not only osmotic protective substances to maintain the integrity and function of cell membranes, but also effective scavengers of reactive oxygen species, participating in the regulation of various abiotic stress resistance during cell metabolism, including chloroplast photosynthesis, salt resistance, low temperature and drought resistance (Wu et al., 2009).

Hevea brasiliensis ((Willd. ex A. Juss.) Muell. Arg) is a tall tropical tree, adapted to high temperature, high humidity, low wind speed and other natural environments. However, China's rubber planting areas are often threatened by cold waves, droughts, typhoons and other natural disasters. In addition, due to its special method of

rubber extraction, the laticifer cells in the whole surface of rubber extraction are subjected to huge osmotic pressure stress and ROS stress in the process of tapping and degreasing (D'Auzac and Jacob, 1989). The study of other plants showed that the RS and GolS in adversity stress plays an important role, but how rubber laticifer cells to shoulder such large changes in osmotic pressure is an important scientific problem such as drop changes of about 10 atmospheric pressures inside and outside the laticifer cells, the response of abiotic stress adaptation mechanism is closely related with its high yielding.

This study focused on analyzing the expression characteristics of *HbGolS1* and *HbRS1* genes encoding GolS and RS in rubber latex with tapping and Ethrel treatment and with leaf development, and analyzed the functions of GolS and RS in rubber latex and leaves of rubber trees (Gao et al., 2018). The results will be helpful to understand the role of GolS and RS in rubber tree adaptation to mechanical injury of tapping and osmotic differential stress of degreasing, which is of great significance to reveal the resistance mechanism of rubber tree, and provide target genes for molecular breeding to improve the stress resistance of rubber tree by regulating GolS and RS.

1 Results and Analysis

1.1 GolS and RS gene sequences of Hevea brasiliensis

2 GolS genes (*HbGolS1*, *HbGolS2*) and 2 RS genes (*HbRS1*, *HbRS2*) were retrieved from HeveaDB by using GolS and RS gene family sequences in *Arabidopsis thaliana*, and their DNA sequences and CDS sequences were analyzed by online software GSDS2.0. The CDS sequences of the 2 GolS genes in rubber tree were slightly different, but the CDS sequences of the 2 RS genes were significantly different. Ten amino acid sequences of *Arabidopsis GolS* gene family were obtained by GeneBank data download, and phylogenetic tree was constructed with the amino acid sequences of GolS gene family of rubber tree. The results showed that the 12 amino acid sequences of the two species could be divided into two subgroups, among which *HbGolS1* and *ATGOLS1-7* and *AtGolS10* were clustered into group I, while *AtGolS8* and *AtGolS9* were clustered into group II (Figure 1A). 4 Arabidopsis RS amino acid sequences were obtained by GeneBank data download. Phylogenetic tree results showed that the six amino acid sequences of the two species were divided into two subgroups (Figure 1), in which *HBRS1-2* and *ATRS4-5* were clustered into group I, while *AtRS1* and *AtRS2* were clustered into group II (Figure 1B). According to the gene structure of GolS and RS in *HbGolS1* and *HbGolS2*, the exons of *HbGolS1* and *HbGolS2* are four, and the gene structure is similar. However, *HbRS1* and *HbRS2* have significant differences in gene structure. *HbRS1* has 4 exons and the length difference is small, while *HbRS2* has 5 exons and the length difference is significant (Figure 1C).

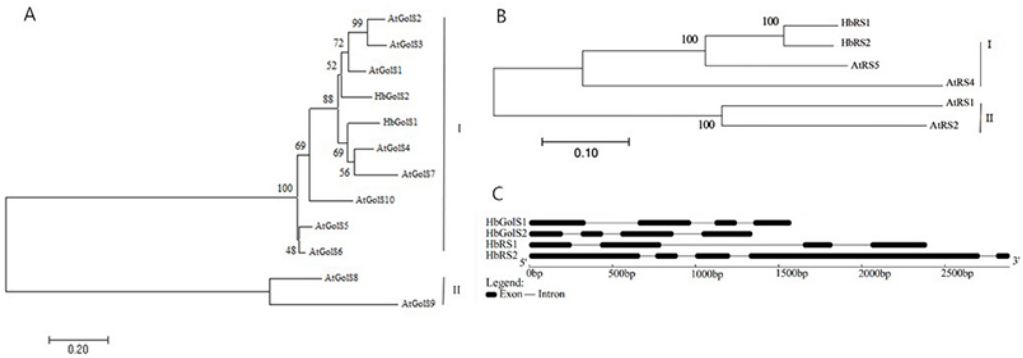

Figure 1 Gene structure and phylogenetic analysis of HbGolS1-2 and HbRS1-2

1.2 Physicochemical properties of GolS and RS in Hevea brasiliensis

The physicochemical characteristics of GolS and RS in rubber trees were analyzed by online software. The results showed that the total length of amino acid sequence of GolS gene family in rubber trees was 324~340 aa, the total proportion of negatively charged amino acids was 12.4%~14.5%, and the total proportion of positively charged amino acids was 9.7%~10.5%. The lipid solubility index is 73.40~77.18, the relative molecular weight is 37.62~38.86 ku, the average hydrophilic coefficient is -0.440~0.235, the theoretical isoelectric point is 5.09~5.28,

the instability index is 41.65~44.97. The total length of amino acid sequence of RS family is 779~786 aa, the total proportion of negatively charged amino acids is 11.4%-11.6%, the total proportion of positively charged amino acids is 9.1%~9.3%, the lipid solubility index is 82.04~84.17, and the relative molecular weight is 86.14~86.96 ku. The average hydrophilic coefficient is -0.160~0.130, the theoretical isoelectric point is 5.09~5.28, and the instability index is 30.38~31.67. There is no significant difference between *HbRS1* and *HbRS2* and *HbGolS1* and *HbGolS2* in physicochemical properties.

1.3 Protein structure of GolS and RS in Hevea brasiliensis

GolS and RS were analyzed by ProtComp 9.0 software, and the results showed that all members of GolS and RS were located in cytoplasm (Table 1). SOPMA software was used to further analyze the secondary structure of GolS and RS, and the analysis results showed that alpha helix, extended strand existed, beta turn, and random coil in the secondary structure of all HbGolS and *HbRS* proteins. And alpha helix and random coil structures account for more than 84% in GolS and 71% in RS (Table 1). CDD analysis showed that all *HbGolS* and *HbRS* proteins had conserved domains, which were GolS (PLN00176) and RS (PLN02711). Amino acid homology of the 2 members of GolS family was up to 75.0%. The amino acid homology of RS family members reached 93.3%.

Table1 Location and Structure characteristics of GolS and RS members in rubber tree

Protein name	Subcellular localization			The secondary structure composition			
	Cytoplasmic	Chloroplast	Vacuolar	Alpha helix (%)	Extended strand (%)	Beta turn (%)	Random coil (%)
HbGolS1	7.98	1.12	0.51	38.53	11.76	3.53	46.18
HbGolS2	7.98	1.12	0.51	41.67	10.49	2.78	45.06
HbRS1	7.10	1.38	0.77	27.47	21.82	6.68	44.03
HbRS2	7.03	1.41	0.80	27.86	21.25	7.25	43.64

1.4 Tissue specific expression of GolS and RS in rubber tree

The results of tissue expression analysis showed that *HBGOLS1-2* and *HBRS1-2* showed different expression trends in different tissues. *HbRS1* was only expressed in high abundance in latex and a little in leaf tissue. *HbGolS1* was mainly expressed in leaves and latex with specific high abundance, especially in leaves, which was much higher than other tissues, and its expression level in leaves was more than twice that in latex (Figure 2A). In order to analyze the characteristics of high abundance expression of *HbGolS* and *HbRS* genes in leaves, the changes in leaf development stages (Bronze, color-change, pale-green and mature stage) were observed. The results showed that the expression level of *HbGolS1* increased rapidly with the development of leaves in the four development stages. The expression level of *HbGolS1* in the pale-green stage was 48 times higher than that in the bronze stage, and mature stage was 642 times higher than that in the bronze stage. The expression of *HbGolS2* increased gradually with the development of leaves, and the mature stage was 9 times higher than the bronze stage. In contrast, the expression levels of RS *HbRS1* and *HbRS2* were not high, increasing slowly before the pale-green stage, but decreasing rapidly in the mature stage. It can be seen from the results that RS and GolS work together in latex, while in leaf development, GolS is mainly more active, and it must play an important defense function with leaf development (Figure 2B).

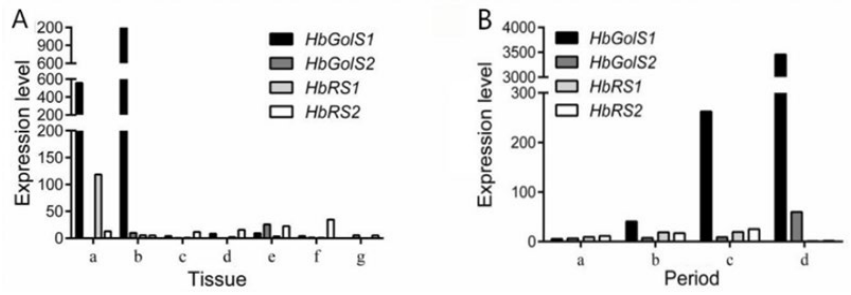

Figure 2 Expression profiles of HbGolS1-2 and HbRS1-2 in different tissues and different development stages of leaves

Note: A: a: Latex; b: Leaf; c: Root; d: Bark; e: Female flower; f: Male flower; g: Seed; B: a: Bronze; b: Color-change; c: Pale-green; d: Mature

1.5 GolS and RS were induced by tapping and Ethrel treatment

Tapping and Ethrel treatment are both severe stresses on laticifer cells of rubber trees, such as coping with changes in osmotic pressure, the surge of reactive oxygen species and the metabolic demands of material and energy. In order to maintain normal functions, drastic metabolic changes occur in laticifer cells. Gene expression abundance is bound to be related to its function. Since we mainly focus on the situation in the laticifer, and only *HbGolS1* and *HbRS1* in *HbGolS* and *HbRS* family are highly expressed in latex. Therefore, only *HbGolS1* and *HbRS1* were analyzed in the tapping experiment and Ethrel treatment experiment. The results showed that *HbGolS1* and *HbRS1* were much more active in *HbGolS1* than *HbRS1* under the two treatments in Strain 73397 (Figure 3): *HbGolS1* gene with high expression in latex was significantly upregulated with tapping, and the expression level was 3 times higher at the third cut, and the expression level remained more than 2 times in the following 27 days (9 tapping*3 days =27 days) (Figure 3A). After Ethrel treatment (within 24 h), *HbGolS1* gene expression decreased rapidly by 4.5 times (Figure 3B). In contrast, *HbRS1* gene expression levels did not change significantly under both treatments (Figure 3). Therefore, *HbGolS1* gene must play a more important role in the 2 treatments, and the mechanism of action is significantly different.

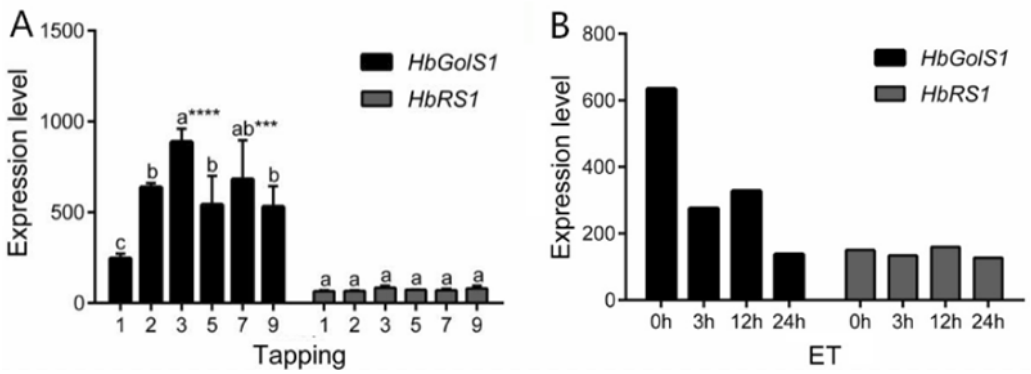

Figure 3 Expression profiles of HbGolS1 and HbRS1 in response to tapping and Ethrel treatment

2 Discussion

Galaetinol synthase (GolS) and raffinose synthase (RS) are the first and second rate-limiting enzymes for the synthesis of raffinose family oligosaccharides (RFOs) during the resistance to various abiotic stresses. It is involved in plant defense and response to abiotic stress such as low temperature, drought, non-selective contact herbicide (methylviologen, MV), mechanical injury and reactive oxygen species (Maruyama et al., 2009; Zuther et al., 2004). Existing studies have mostly reported the relationship between RFOs accumulation and resistance in seeds or leaves, for example, *At5g40390* is the only functional gene of raffinose accumulation induced by abiotic stress in Arabidopsis leaves (Kim et al., 2011; Egert et al., 2013). By analyzing the structure and physicochemical properties of GolS and RS family members in laticifer of rubber tree, we found that they have typical characteristics of conserved plant GolS and RS and are soluble proteins expressed in cytoplasm. The main results of expression analysis showed that :(1) *HbGolS1* was a specific and abundant GolS in laticifer cells of rubber tree. It was significantly up-regulated by tapping, but inhibited by Ethrel treatment, suggesting that GolS might be involved in the defense against stress in different ways. (2) *HbRS1* is the only raffinose synthase gene in laticifer cells, but its expression level changed little with the treatment of tapping and Ethel, indicating that raffinose may not be the oligosaccharide to resist stress in latex, which is consistent with the low level of raffinose in latex (D'Auzac and Jacob, 1989). Therefore, *HbGolS1* was preliminarily inferred to be the rate-limiting enzyme in the biosynthesis pathway of RFOs in rubber tree, but raffinose was probably not the ultimate anti-stress substance, and GolS was likely to be used for the synthesis of other regulatory substances. How laticifer cells from rubber trees participate in abiotic stress defense by regulating RFOs metabolism needs further research (Farcuh et al., 2017; Zuo, 2017).

As a tropical cash crop, rubber trees often suffer from low temperature or drought stress, especially after tapping, with the discharge of latex, the inner membrane system of laticifer cells is seriously damaged. Therefore, whether

the reactive oxygen species (ROS) can be quickly cleared and balanced is an important regulatory point related to whether it will die and whether it can fully play its potential for rubber production (Nishizawa et al., 2008; Huang and Qin, 2012). For example, production is regulated by the transcription factor DREB1A (Maruyama et al., 2009; Han et al., 2020). Raffinose family oligosaccharides (RFOs), as an important carbohydrate in plants, can be used as osmotic regulatory substances to maintain the osmotic pressure of tissues and enhance the ability of plants to resist stress. Therefore, it is speculated that their function in laticifer cells is to enhance the resistance to adversity, which is consistent with the expression characteristics of *HbGolS1*. However, since the transcription level of *HbRS* did not change much with tapping and Ethrel treatment, it is likely that RS is not regulated at the transcription level, and may be regulated by enzyme activity or other means. Therefore, the utilization of GolS and RS in rubber trees needs further research. Considering the high expression of *HbGolS1* in mature leaves, their role in leaf stress regulation should not be ignored. In addition, we have expressed *HbRS1* and *HbGolS1* proteins *in vitro*, hoping to analyze the specificity of their functions in the laticifer and leaves of rubber tree and reveal the significance and mechanism of RFOs biosynthesis.

3 Materials and Methods

3.1 Experimental materials

Hevea brasiliensis 'Reyan7-33-97' was used in this study, which was located in Team 2 (19°33′34″N, 109°30′12″E) of the Experimental Site of Chinese Academy of Tropical Agricultural Sciences, Danzhou City, Hainan Province. The expression databases of different tissues (latex, leaf, root, bark, female flower, male flower and seed), different stages of leaf development (Bronze, color-change, pale-green, mature stage), ethylene stimulation (1.5% Ethrel, 0, 3 h, 12 h and 24 h), and tapping treatment (1, 2, 3, 5, 7 and 9 for continuous tapping of uncut trees) were constructed, and stored in our laboratory.

3.2 HbGolSs and HbRSs gene sequences

Based on the nucleotide/amino acid sequences of Arabidopsis GolS and RS family members, the rubber tree GolS and RS family members were retrieved from the HeveaDB (http://hevea.catas.cn/home/index). Mega7.0 neighbor-joining method was used to construct phylogenetic trees of *Arabidopsis thaliana*, rubber tree GolS and RS. Using GSDS2.0 software (http://gsds.cbi.pku.edu.cn/) to analyze the introns and exons of rubber GolS and RS family genes (Long et al., 2014; Zhou et al., 2014).

3.3 Physicochemical properties of HbGolSs and HbRSs proteins

Based on the amino acid sequences of GolS and RS gene families in rubber trees, the physicochemical properties, protein length, theoretical isoelectric point, relative molecular weight, instability index, lipid solubility index, average hydrophilic coefficient and subcellular localization of GolS and RS in rubber tree were analyzed by online software ProtParam tool (https://web.expasy.org/protparam/) (Long et al., 2014; Zhou et al., 2014).

3.4 Structural characteristics of HbGolSs and HbRSs proteins

According to the amino acid sequence of GolS and RS gene families, the subcellular localization of GolS and RS proteins was analyzed by ProtComp 9.0 software. SOPMA software was used to further analyze the secondary structure of GolS and RS in rubber tree—α helix, extension band, β-turn and random coil. Conservative domains of rubber tree GolS and RS family members were analyzed by CDD software (Long et al., 2014; Zhou et al., 2014).

3.5 Expression analysis of *HbGolSs* and *HbRSs* genes

Through PRISM data analysis software and HeveaDB, the expression characteristics of GolS and RS genes in rubber latex under different tissues, different stages of leaf development, ethylene stimulation and tapping treatment were analyzed.

Authors' Contributions

LJL is the experimental designer and executor of this study. LJL and WZY completed data analysis and wrote the first draft of the paper. LXY, FYJ, ZMX and YJH participated in experimental design and analysis of experimental results. QYX is the designer and

principal of the project, directing experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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