生物工程学报
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Chin J Biotech 2008, September 25; 24(9): 1526-1530
Chinese Journal of Biotechnology ISSN 1000-3061

研究报告

转 *trxS* 基因大麦发芽种子水解酶活性的变化

卫丽[1], 孔维威[2], 尹钧[1]

1 河南农业大学, 郑州 450002
2 南京农业大学生命科学院, 南京 210095

摘　要: 利用转基因技术是改良大麦品种品质的有效途径。研究了转 *trxS* 基因对大麦种子发芽过程中水解酶活性的影响, 结果表明转基因种子中α-淀粉酶、自由态β-淀粉酶和极限糊精酶的活性比未转基因种子高; 转基因种子醇溶蛋白和谷蛋白中巯基的含量提高, 说明该基因能够表达, 为大麦育种和品质改良提供新的途径。

关键词: ***trxS*** 基因, 发芽, α-淀粉酶, β-淀粉酶, 极限糊精酶, 巯基

Change of Hydrolase Activity in Germinating Seeds of *trxS* Transgenic Barley

Li Wei[1], Weiwei Kong[2], and Jun Yin[1]

1 *Henan Agricultural University*, *Zhengzhou* 450002, *China*
2 *College of Life Sciences*, *Nanjing Agricultural University*, *Nanjing* 210095, *China*

Abstract: Genetic modification of barley variety can be an efficient way to improve beer quality. The objective of this study was to understand the effect of *trxS* gene on hydrolases activities in transgenic and non-transgenic barley seeds. The results showed that α-amylase, free β-amylase and limit dextrinase activity were increased in transgenic seeds in comparison with non-transgenic seeds. Sulfhydryl content of protein in transgenic seeds was also higher than that in non-transgenic seeds, suggesting that *trxS* gene could express in barley seeds, which opens a new way for breeding new barley varieties to improve beer quality.

Keywords: *trxS* gene, germination, α-amylase, β-amylase, limit dextrinase, sulfhydryl

Thioredoxins that discovered in *E. coli* are small ubiquitous disulfide reductases (14 kD average) possessing a characteristic active site WCGPC and were identified as the hydrogen donor for ribonucleotide reductase [1]. Thioredoxins have been found with many functions. Compared with other living organisms, plants are unique in possessing several types of trxs that are located in different cellular compartments. Among which, thioredoxinh has been implicated in a wide range of biological functions. It has been shown that thioredoxinh is able to reduce low-molecular–mass cystine-rich proteins, including thionins, protease inhibitors and chloroform/methanol-soluble proteins that are inhibitors of exogeneous a-amylases[2]. Gliadins and glutenins, the major storage proteins of wheat endosperm, were reduced by the NADP/thioredoxin system (NTS). During seed germination thioredoxinh functions as a signal to enhance metabolic process for germination and seedling development[3,4]. Recent research with transgenic barley indicated that thioredoxinh of the starchy endosperm is a member of network to communicate with the embryo and the aleurone to accelerate germination and release key hydrolytic

Received: January 18, 2008; **Accepted:** April 16, 2008
Corresponding author: Jun Yin. Tel: +86-371-63558203; E-mail: xmzxyj@126.com

enzymes[5]. The reduction of thioredoxin increased the solubility of proteins of wheat starchy endosperm such as α-amylase inhibitor, as a result of conversion from disulfide(S-S) to sulfhydryl(-SH) form, lost biochemical inhibiting activity and was easier to be degraded by proteolysis[6].

A putative *S* gene has been isolated from the pollen of the grass *Phalaris coerulescens* [7], a database search showed that *S* gene has high homology with thioredoxinh proteins at its conserved C terminus. This was functionally confirmed by demonstration of thioredoxin-like activity in the *E. coli* expressed C terminal protein[8]. The sequences of their cDNA are highly homologous, and their expressed products have similar functions[9]. In 2000, we transformed *trxS* gene into barley by particle bombardment and got homologous lines[10,11]. It was reported that the trxh activity in transgenic barley seeds was higher than that in non-transgenic seeds[12]. The objective of this study was to understand the impact of *trxS* gene on hydrolases activities in transgenic barley seeds in order to find a new way to develop varieties for beer malting industry.

1 Materials and methods

1.1 Plant materials

TrxS gene was transferred into Jinyin No.6 (*Hordeum vulgare cv.* Jinyin 6) by particle bombardment and homologous line JY_6-6-19-4 was obtained by successive selection. Dry mature seeds from JY_6-6-19-4 and corresponding non-transgenic (as CK) lines were surface-sterilized with 70% aqueous ethanol for 5 min and then washed 3 times with sanitized water. The grains were incubated at 25°C for 6 days for germination, the germinated seeds were freezen in liquid nitrogen and then stored at −80°C.

1.2 Hydrolases activities

The activities of α-amylase, free β-amylase and bound β-amylase were measured at 540 nm as described according to Li[13]. The activity of limit dextrinase was measured following Liang *et al*.[14]with some modification: 0.2 gram of de-embryo grain was ground in a mortar and pestled, 5 mL 0.2 mol/L NaAc (pH5.5) was added and extracted for 16 hours, then centrifuged for 10 min at 13 000 r/min. The supernatant was used for analyzing the activity of limit dextrinase. In 0.1 mL enzyme extraction, 0.1 mL 0.4% amylopectin was added and incubated at 40°C for 20 min, then 2 mL DNS (3,5-Dinitrosalicylic acid) was added to end the reaction, followed by immersing in boiling water for 5 min, and cooled immediately in ice water, the final volume was 20 mL by adding sterilized water. Absorbance at 540 nm was recorded as the activity of limit dextrinase. The result was identified by three-time repetition (the following experiments were the same).

1.3 Determination of sulfhydryl content

Sulfhydryl content in hordein and glutelin was measured according to Huang *et al*[15]. The ratio OD_{412}/OD_{595} was used to express the sulfhydryl content per unit protein.

2 Results

2.1 Changes of α-amylase activity

As an initial enzyme for catalyzing starch degradation, α-amylase is very limited in mature seeds. During seed germination the changes of α-amylase activity in transgenic and non-transgenic barley seeds were different (Table 1). On 1d after seed germination, α-amylase activity was very low, but on 2~3d it increased and reached the highest on 4d and then reduced gradually, the difference of α-amylase activity between transgenic and non-transgenic barley seeds was significant (P=0.0189, t-paired detection, the following was the same).

Table 1 Changes of α-amylase activity in transgenic (T) and non-transgenic (CK) germinated barley seeds

Day after germination(d)	CK(*OD*)	T(*OD*)
1	0.000	0.002
2	0.006	0.037
3	0.042	0.135
4	0.222	0.292
5	0.153	0.196
6	0.066	0.072

2.2 Changes of free β-amylase and bound β-amylase activities

β-amylase has two forms, free β-amylase and bound β-amylase. It is composed and accumulated during seed development and won't further accumulate in seed germination process[16]. Table 2 showed that free β-amylase activity in transgenic barley seeds was lower than that in non-transgenic seeds on 1d after seed germination, while it was higher than that in non-transgenic seeds on 2~5 d after germination, respectively; the difference of free β-amylase activity between transgenic and non-transgenic seeds was significant(P=0.0289).

Bound β-amylase is abundant and accounts for 75% of total β-amylase in barley seed. It can be converted

into free β-amylase. The result showed that the activity of bound β-amylase was very high on 1 d after seed germination, but reduced dramatically from 2 d after seed germination (Table 2). The bound β-amylase activity in transgenic barley seeds was lower than that in non-transgenic seeds on 2 d after germination, indicated that the whole release of bound β-amylase in transgenic barley grains was 1 d earlier than that in non-transgenic barley grains. The difference of bound β-amylase activity between transgenic and non-transgenic barley seeds was not significant (P=0.3324).

Table 2 Changes of free β-amylase and bound β-amylase activities in transgenic (T) and non-transgenic (CK) germinated barley seeds

Day after germination (d)	Free β-amylase activity (*OD*)		Bound β-amylase activity (*OD*)	
	CK	T	CK	T
1	0.780	0.773	0.422	0.418
2	0.823	0.933	0.089	0.024
3	1.341	1.434	0.020	0.020
4	1.109	1.181	0.021	0.021
5	0.955	1.008	0.020	0.020
6	0.697	0.684	0.020	0.019

2.3 Changes of limit dextrinase activity

Limit dextrinase is a key enzyme to decompose starch. Very small amount of limit dextrinase was reported to present in barley at the time of grain ripening[17]. The changing trend of limit dextrinase activity was similar between transgenic and non-transgenic barley seeds during seed germination (Table 3). The limit dextrinase activity in transgenic seeds was higher than that in non-transgenic seeds and reached significant level (P=0.008).

Table 3 Changes of limit dextrinase activity in transgenic (T) and non-transgenic (CK) germinated barley seeds

Day after germination(d)	CK(*OD*)	T(*OD*)
1	0.174	0.182
2	0.183	0.192
3	0.181	0.208
4	0.193	0.24
5	0.206	0.221
6	0.205	0.227

2.4 Changes of sulfhydryl content in protein

Thiol-disulfide is an important factor to control physiological activity and three-dimensional structure of protein. During seed germination, the thiol-disulfide in protein is broken and becomes sulfhydryl, therefore, the increase of sulfhydryl makes protein more soluble. Trxh could convert thiol-disulfide into sulfhydryl. The change of sulfhydryl content indicates if the foreign gene is expressed in transgenic barley seed. Hordein and glutelin are main storage proteins in barley seed. Table 4 showed that the sulfhydryl content in hordein increased during seed germination, while the sulfhydryl content of transgenic barley seeds increased more rapidly than that in non-transgenic seeds and reached significant level (P=0.045). Sulfhydryl content in glutelin was very low on 1~2 d after seed germination, and increased from 3~6 d after seed germination, while the difference was not significant (P=0.134) between transgenic and non-transgenic seeds. The results indicated that the introduction of *trxS* gene could improve the converting of thiol-disulfide into sulfhydry in hordein and glutelin.

Table 4 Changes of the sulfhydryl content in hordein and glutelin in transgenic and non-transgenic germinated barley seeds

Day after germination(d)	Sulfhydryl content in hordein (OD_{412}/ OD_{595})		Sulfhydryl content in glutelin (OD_{412}/ OD_{595})	
	CK	T	CK	T
1	0.955	0.911	0.782	0.778
2	1.737	1.87	0.917	0.918
3	2.444	2.929	1.898	2.168
4	9.500	16.074	4.500	5.433
5	9.583	24.944	10.014	12.680
6	10.156	24.889	8.892	26.667

3 Discussion

The activity of hydrolase plays an important role in the content of fermentable sugar generated from starch hydrolyzation in beer brewing. Improving the degradation of starch would improve beer yield. The main hydrolases are α-amylase, β-amylase and limit dextrinase. α-amylase limited in mature barley seed is synthesized during seed germination, it can hydrolyze α-1, 4-glycosidic bond into small molecular maltose. These results in this study showed that the activity of α-amylase was improved in transgenic barley seed, which is similar with Kobrehel's finding [3]. α-amylase inhibitors exist in barley seed and can combine with α-amylase at the beginning of seed germination to form composites and reduce α-amylase activity. Trxh could redox thiol-disulfide of α-amylase inhibators and change its structure and made it lose biochemical inhibiting activity, so α-amylase was released and its activity would be increased.

β-amylase is a kind of abundant enzyme in barley seed catalyzing starch into maltose and limit dextrine.

It has two forms in dry barley grains: free of active form and bound of less-active form, the latter accounts for about 75% of total β-amylase. Both forms may be aggregated or disulfide bonded via a Cys residue near the C terminus to other grain proteins[18]. Bound β-amylase could combine with other kinds of proteins by thiol-disulfide (S-S), and then loses activity. It is also released during germination by a disulfide reductase or by proteolysis of cysteine proteases. The release of bound β-amylase into free β-amylase could improve the whole activity of β-amylase. It was reported that during seed germination the activity of free β-amylase increased and the activity of bound β-amylase reduced and disappeared gradually. On the other hand, β-amylase contains sulfhydryl (S-H) and the reduction of sulfhydryl is essential to keep the activity of β-amylase. Trxh could deoxidize thiol-disulfide into sulfhydryl. From above research we knew that the release of bound β-amylase was important to increase free β-amylase activity during seed germination. The whole release of bound β-amylase in transgenic barley grains was earlier than that in non-transgenic barley grains. The results indicated the expression of *trxS* gene and accelerated the release of bound β-amylase in germinating barley seeds, which might due to the increase of β-amylase activity during grain germination. A similar result has been reported by Sopanen *et al*[19].

The co-operation of α-amylase and β-amylase can't hydrolyze starch completely into fermentable sugar, which just improve hydrolyzing speed but not change the composing of reaction products. It needs the join of limit dextrinase to hydrolyze starch completely. Limit dextrinase is a type of hydrolase produced during seed germination, hydrolyzing α-1, 6-glycosidic bond exclusively[20]. The activity of limit dextrinase is very low in mature barley grain, and it is increased during seed germination [21,22]. Limit dextrinase has three forms in germinating barley seeds. The transfer of bound form to active form was controlled by thiol-protein hydrolyse. The reduction of limit dextrinase was related to thioredoxin. The increase of limit dextrinase in malted barley will increase the hydrolysis of wort dextrins to fermentable sugars and end up with an increase in the yield of beer. The result in the present study showed that the activity of limit dextrinase in transgenic seeds was also higher than that in non-transgenic seed.

Protein is also a main storage in seed, accounting for 8%~15% of seed dry weight. According to Osborne's method, it can be divided into albumin, globulin, hordein and glutelin. The hydrolysis of protein plays a key role in beer quality, such as the speed of wort filtration, diastaic power and sol-nitrogen. During germinating of barley seed the degradation of protein is related to the activity of protease. The redox state of protease is benefit with its function. To further identify the expression of *trxS* gene, the sulfhydryl content in hordein and glutelin were measured. The result showed that the sulfhydryl content in transgenic seeds was higher than that in non-transgenic seed, indicating that the thiol-disulfide (S-S) in these proteins was transferred to sulfhydryl (S-H) under the redox of trxh. The transformation of *trxS* gene could enhance the hydrolases activities of transgenic barley seeds. The increase of the degradation of starch and protein in transgenic barley seeds provides a new way for barley high quality breeding, which will benefit beer malting industry.

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