Enhancing production of ergosterol in *Pichia pastoris* GS115 by over-expression of 3-hydroxy-3-methylglutaryl CoA reductase from *Glycyrrhiza uralensis*

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**KEY WORDS**

*Glycyrrhiza uralensis* Fisch.; 3-Hydroxy-3-methylglutaryl-CoA reductase gene; Over-expression; *Pichia pastoris*; Copy number variation

**Abstract** The rate-limiting enzyme in the mevalonic acid (MVA) pathway which can lead to triterpenoid saponin glycyrrhizic acid (GA) is 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). In order to reveal the effect of copy number variation in the HMGR gene on the MVA pathway, the HMGR gene from *Glycyrrhiza uralensis* Fisch. (GaHMGR) was cloned and over-expressed in *Pichia pastoris* GS115. Six recombinant *P. pastoris* strains containing different copy numbers of the GaHMGR gene were obtained and the content of ergosterol was analyzed by HPLC. The results showed that all the recombinant *P. pastoris* strains contained more ergosterol than the negative control and the strains with 8 and 44 copies contained significantly more ergosterol than the other strains. However, as the copy number increased, the content of ergosterol showed an increasing–decreasing–increasing pattern. This study provides a rationale for increasing the content of GA through over-expressing the GaHMGR gene in cultivars of *G. uralensis*.

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**Abbreviations:** BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; CNV, copy number variation; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; LOD, limit of detection; LLOQ, lower limit of quantitation; MD, minimal dextrose medium; MM, minimal medium; MVA, mevalonic acid; PCR, polymerase chain reaction; RSD, relative standard deviation; YPD, yeast peptone dextrose medium

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1. Introduction

Besides the use as an industrial raw material and tobacco additive, the roots of Glycyrrhiza uralensis Fisch. are widely used in many Chinese herbal remedies for their ability to nourish “Qi”, alleviate pain, tonify the spleen and stomach, eliminate phlegm and relieve cough. The source of this pharmacological activity is a number of active components of which glycyrrhizic acid (GA) is considered the most important. This has led to its adoption as a marker compound of the quality of G. uralensis. Many studies have shown that GA possesses antiinflammatory, antitumor and immune-stimulating activities.

Excessive exploitation of wild G. uralensis plants in the years leading up to 2000 decreased the supply to such an extent that the Chinese government imposed restrictions on their collection. As a result, cultivars have now become the main source of this herb. The Chinese government imposed restrictions on their collection. As a result, cultivars have now become the main source of this herb.

2. Materials and methods

2.1. Construction of the yeast expression vector containing GuHMGR gene

NcoI and SnaBI of pPIC9K (Fig. 1) were selected as the specific enzyme cutting sites to insert the GuHMGR gene. Primer pairs with the specific enzyme sites underlined are as follows:

**HF:** 5′-CGGTACCTGTAATGGGACGTTCC
GCGGAG-3′ (SnaBI)
**HR:** 5′-ATAGCGGCCGCCCTGGAGGCTT
TCGGTATTTGCT-3′ (NcoI)

The cycling parameters of PCR were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, annealing at 64°C for 30 s, extension at 72°C for 2 min; and a final extension at 72°C for 10 min. The amplified fragments were purified and subcloned into pMD19-T (Takara, Japan). The resulting vector (GuHMGR-T) was digested with SnaBI (2 h at 37°C) and NcoI (2 h at 37°C) and then subcloned into pPIC9K (Invitrogen, USA). The resulting recombinant pPIC9K-GuHMGR plasmid was transferred into the disarmed E. coli DH5α and mobilized by electroporation (1500 V, 25 µF, 400 Ω) into the disarmed P. pastoris GS115 (Invitrogen, USA). An aliquot (0.5 mL) of yeast peptone dextrose (YPD) medium was then added and the cells were cultured at 30°C, 200 rpm for 1 h. An aliquot (200 µL) of the suspension was placed on minimal dextrose (MD) solid medium and cultured at 30°C for 2 days. Single colonies were removed and incubated on minimal medium (MM) and MD solid medium simultaneously at 30°C for 2–4 days; the colonies growing on both MM and MD media were selected.

PCR was used to check that the recombinant P. pastoris contained the GuHMGR gene. The single colonies were used as PCR template and primers were as follows: forward primer, 5′-TCTATTGGCCAGCATTTGCTG-3′; reverse primer, 5′-GCAATGAGCATTCTGACATCC-3′. The cycling parameters were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 2 min; and a final extension at 72°C for 10 min.

Selected recombinant P. pastoris was induced to express the GuHMGR gene using BMGY and BMMY liquid media (30°C, 250 rpm). The supernatant from a 96 h culture was examined by 12% SDS-PAGE using Coomassie brilliant blue staining. P. pastoris containing a void vector was used as a negative control.

2.3. Copy number determination

The GAP gene was selected as the internal control gene for real-time PCR. The primer pair of GAP (GenBank accession number: U62648) was as follows: **GF:** 5′-CACAATGCTATCCTGTGCG-3′; **GR:** 5′-GACACACTACAGCCGGCATC-3′. The primer pair of the GuHMGR gene was as previously stated. The cycling parameters were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 2 min; and a final extension at 72°C for 10 min. The amplified fragments were subcloned into pMD19-T and transformed into disarmed E. coli DH5α. Then the standard plasmids pMD19-T-GuHMGR and pMD19-T-GAP were obtained, extracted and diluted to 10^6, 10^7, 10^8, 10^9, 10^10, and 10^11 copy numbers/μL. For real-time PCR analysis, the primer pairs in Table 1 were used with the following cycling parameters: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 60 s, saving at 4°C. Standard curves of Ct (Cycle threshold) on the X-axis and log (concentration of standard plasmid) on the Y-axis were constructed. All recombinant P. pastoris strains were amplified by real-time PCR. The ratio of the Y values of GuHMGR and GAP was taken as the copy number of the GuHMGR gene in each recombinant P. pastoris strain.

![Structure of pPIC9K.](image-url)
2.4. Semi-quantitative RT-PCR analysis

Total RNA was isolated from different recombinant P. pastoris strains using a yeast RNA rapid extraction kit (Beijing BoMaDa Medical Technology Co., Ltd.). To remove plasmid DNA, RNase-free DNase I (Tiangen Biotech Co., Ltd.) was used according to the manufacturer's instructions. Spectrophotometry was used to determine the concentration of RNA. The cycle number was set at 18, 20, 22, 24 and 26, and the optimal cycle number determined by electrophoresis in 1% (w/v) agarose gel. The cycling parameters of RT-PCR were as follows: 50 °C for 30 min; 94 °C for 2 min; optimal cycles of 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 45 s; and a final extension at 72 °C for 10 min; saving at 4 °C. The primer pair, GF (5'-GCTCAATGGCTATCACTGTCG-3') and GR (5'-GATGGTGACAGGGTCTCTCTTGG-3'), was used to amplify the GAP gene as an internal control.

2.5. Assay of ergosterol in recombinant P. pastoris

HPLC analysis of ergosterol in samples was carried out on a Waters 2695 system equipped with a Phenomenex LUNA C18 column (250 mm × 4.6 mm, 5 μm) using a mobile phase of methanol:water 97:3 (v/v) delivered at a flow rate of 1.0 mL/min. The detection wavelength was 283 nm and the injection volume 20 μL.

A stock solution of ergosterol (purity: 97.7%) containing 11.38 mg in 10 mL absolute ethanol was used to prepare a series of standard solutions containing 0.01%, 0.05%, 0.1%, 0.5%, 2%, and 5% of the stock solution in absolute ethanol. Intra-day precision (as relative standard deviation, RSD) was determined by replicate analysis (n=6) of a solution containing 5.559 μg/mL ergosterol. The limit of detection (LOD) and lower limit of quantity (LLOQ) were determined using sequentially more dilute solutions of ergosterol. Recovery was assessed using 9 samples of blank P. pastoris cells accurately weighed (50 mg) and spiked with 2.91 μg, 5.05 μg and 8.41 μg ergosterol.

Recombinant P. pastoris strains containing different copy numbers of the GuHMGR gene were induced to express the gene; P. pastoris GS115 without the GuHMGR gene was used as negative control. All 96 h cultures were collected by centrifugation at 5000 rpm and lyophilized. Samples of the dried powders (50 mg) were extracted into 8 mL ethyl acetate by ultrasonication for 1 h. The ethyl acetate was evaporated to dryness, the residue reconstituted in 2 mL ethanol analyzed for ergosterol.

3. Results

3.1. Construction of the yeast expression vector containing the GuHMGR gene

A 1745 bp fragment was shown by PCR and BLAST analysis to have a 99% identical sequence to that of the GuHMGR gene (GenBank accession number: GQ345405.1). It was successfully inserted at the SmaBI–NotI site of pPIC9K to give the recombinant plasmid pPIC–GuHMGR shown in Fig. 2 where lane 1 is the marker and lanes 2 and 3 are fragments obtained by PCR with the correct length.

3.2. Construction of recombinant P. pastoris containing GuHMGR gene

The linearized pPIC–GuHMGR was transformed to P. pastoris GS115. Most single colonies of recombinant P. pastoris simultaneously growing on MM and MD media were shown by PCR to have the correct fragment length. After inducing for 96 h, the negative control was dark yellow while the recombinant P. pastoris was yellowish-white. SDS-PAGE (Fig. 3) showed a band between 86 and 47 kDa in samples from recombinant P. pastoris which was not present in the negative control. These results demonstrate that the construction and inducible expression of recombinant P. pastoris strains containing the GuHMGR gene were successful.

3.3. Copy number determination of the GuHMGR gene in transgenic P. pastoris

PCR and sequencing showed that the standard plasmids were correct. The melting curves of the GAP and GuHMGR genes were both unimodal suggesting that the primers used in real time PCR were specific. Using real time PCR, two fragments with 220 and 237 bp were obtained which sequencing and BLAST analysis showed were the GuHMGR gene and the GAP gene of P. pastoris, respectively. Their standard curves were described by the equations \( Y = -2.609 X + 32.21 \) (\( R^2 = 0.995 \)) and \( Y = -2.994X + 35.27 \) (\( R^2 = 0.997 \)), correspondingly. The copy numbers of the GuHMGR gene in different transgenic P. pastoris strains were found to be 1, 2, 4, 8, 13 and 44 (Table 2).

3.4. Semi-quantitative RT-PCR analysis of transgenic P. pastoris

Semi-quantitative RT-PCR was employed to detect the relative abundance of the GuHMGR gene in the transgenic P. pastoris strains. The cycle number was fixed at 20 based on a
preliminary experiment. RT-PCR analysis revealed the GuHMGR gene was expressed in all transgenic P. pastoris strains at different levels (Fig. 4b) whereas expression of the GAP gene was roughly similar (Fig. 4a). As shown in Fig. 4c, the relative expression of the GuHMGR gene in the recombinant P. pastoris strain containing 4 copies was higher than in the other strains consistent with the results listed in Table 3 ($P<0.05$ for the strain containing 4 copies versus all other strains).

3.5. Assay of ergosterol in recombinant P. pastoris

The retention time of ergosterol in HPLC was 8.16 min. The standard curve was linear over the concentration range 0.1–50.0 μg/mL and described by the equation $Y=3.8184 \times 10^{-5} X−7.9302 \times 10^{-3}$ ($R^2=0.9999$). The LLOQ ($S/N$ of 10) was 2.22 ng and the LOD ($S/N$ of 3) 0.89 ng. The assay was precise (RSD 0.059%, $n=6$) with recovery in the range 97.3%–101.0% (RSD 0.55%–0.82%). The content of ergosterol in all samples is shown in Fig. 5. The results of independent $t$-tests of the content of ergosterol in different recombinant P. pastoris strains ($n=3$) are listed in Table 4. The content of ergosterol was 1.07–2.51 times higher in the negative control but with increase in the copy number of GuHMGR gene; the content of ergosterol showed an increasing–decreasing–increasing pattern. For strains with copy number <4, the content of ergosterol was similar; at copy number 8, the content of ergosterol was highest (2.5 times the negative control); for copy number 13, the content of ergosterol was only 1.04 times negative control; and for copy number 44, the content of ergosterol was 1.8 times negative control. Clearly, the copy number of the GuHMGR gene influences the level of ergosterol in transgenic P. pastoris.

4. Discussion

In our previous studies, we found functional genes in G. uralensis such as those of HMGR and SQS were subject to CNV23–25. We were therefore interested to analyze the relationship between the

![Figure 3](image-url)  
Figure 3 SDS-PAGE analysis of the expression of the GuHMGR gene. Lane 1: marker; lanes 2–4: recombinant P. pastoris containing the GuHMGR gene; lane 5: negative control.

![Figure 4](image-url)  
Figure 4 Semi-quantitative RT-PCR analysis of the expression of the GuHMGR gene in different recombinant P. pastoris strains. (a) RT-PCR results of the GAP gene; (b) RT-PCR results of the GuHMGR gene (the numbers are the copy numbers of the GuHMGR gene in the recombinant P. pastoris strains); (c) relative expression of the GuHMGR gene in recombinant P. pastoris strains with different copy numbers.

### Table 2  
Copy numbers of the GuHMGR gene in different recombinant P. pastoris.

| No. of strain | Average $C_t$ value | $Y$ value in standard curve | Copy number of GuHMGR (GuHMGR/GAP) |
|--------------|---------------------|-----------------------------|-----------------------------------|
|              | GuHMGR | GAP  | GuHMGR | GAP  | GuHMGR |
| 1            | 23.10  | 21.18 | $1.2 \times 10^4$ | $1.7 \times 10^4$ | 1 |
| 2            | 17.29  | 15.51 | $1.0 \times 10^6$ | $2.5 \times 10^6$ | 2 |
| 3            | 24.26  | 20.83 | $4.7 \times 10^3$ | $2.3 \times 10^4$ | 4 |
| 4            | 20.38  | 16.81 | $9.4 \times 10^4$ | $8.0 \times 10^5$ | 8 |
| 5            | 22.36  | 18.06 | $2.0 \times 10^4$ | $2.7 \times 10^5$ | 13 |
| 6            | 25.12  | 19.07 | $2.5 \times 10^3$ | $1.1 \times 10^5$ | 44 |

### Table 3  
Independent $t$-test results of expression of the GuHMGR gene in different recombinant P. pastoris strains by RT-PCR.

| Copy number | $P$ value |
|-------------|------------|
| 1           | 0.425 0.000 0.736 0.994 0.019 |
| 2           | 0.425 0.000 0.264 0.421 0.082 |
| 4           | 0.000 0.000 0.000 0.000 0.000 |
| 8           | 0.736 0.264 0.000 – 0.741 0.010 |
| 13          | 0.994 0.421 0.000 0.741 – 0.018 |
| 44          | 0.019 0.082 0.000 0.010 0.018 – |

In our previous studies, we found functional genes in G. uralensis such as those of HMGR and SQS were subject to CNV23–25. We were therefore interested to analyze the relationship between the...
copy number of functional genes and the content of GA acid in *G. uralensis*.

CNV arises from deletions, insertions, duplications, and more complex variations ranging from 1 kb to submicroscopic sizes26. Genes with CNV have wide distribution, hereditability, relative stability and high heterogeneity. CNV can lead to changes in gene dosage and phenotypic character and, to date, many reports have documented a close relationship between CNV and human disease27.

In this study, recombinant *P. pastoris* strains containing 1, 2, 4, 8, 13 and 44 copies of the *GuHMGR* gene were constructed. RT-PCR analysis revealed that the *GuHMGR* gene was expressed in all transgenic *P. pastoris* strains at different levels with the strain containing 8 copies, showing highest expression as indicated by the content of ergosterol. However, with increasing copy number, the content of ergosterol did not increase in a linear fashion but showed an increasing–decreasing–increasing pattern with the strains containing 8 and 44 copies containing higher levels of ergosterol than those containing 1, 2, 4 and 13 copies.

The reason for the non-linear dependence of expression on copy number is unclear but one possibility is that it involves gene silencing caused by integration sites of exogenous genes. Recently, several studies have demonstrated feedback inhibition in over-expressing exogenous genes in plants1,32 and it may be that an increase in the *GuHMGR* gene results in feedback inhibition of upstream steps of the MVA pathway to reduce the accumulation of ergosterol. Then when an enzyme level decreases, feedback inhibition may be interrupted leading to reopening of the metabolic pathway which could explain why the level of ergosterol is increased in the *P. pastoris* strain containing 44 copies of the *GuHMGR* gene.

In this study, the dependence of the content of ergosterol on the copy number of the *GuHMGR* gene suggests that an increase in the latter could lead to an increase in the production of GA in *G. uralensis*. However, it must be recognized that the production of GA involves a very complex metabolic network which is regulated and controlled by many key enzymes, of which HMGR is but one. Nevertheless we maintain that the current results provide an important basis for further studies aimed at increasing the GA content of *G. uralensis* and exploring its biosynthesis in vitro. In addition, other herbs used in Chinese medicine such as *Glycyrrhiza glabra* and *Glycyrrhiza inflata* also produce GA and this work is relevant to further studies of its biosynthesis in these medicinal plants.

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References

1. Zeng L, Li SH, Lou ZC. Morphological and histological studies of Chinese licorice. *Acta Pharm Sin* 1988;23:200–8.
2. The State Pharmacopoeia Committee of China. *The pharmacopoeia of the People’s Republic of China. Part1*. Beijing: China Chemical and Technology Press; 2010, p. 80–1.
3. Cheng JM, Lin HJ, Hsu YH, Hung MS, Lin JC. A quantitative bioassay for HIV-1 gene expression based on UV activation effect of glycyrrhetic acid. *Antiviral Res* 2004;62:27–36.
4. Hower G, Bzltina L, Michaelis M, Kondratenko R, Balina L, Tolstikov GA, et al. Antiviral activity of glycyrrhetic acid derivatives against SARS-coronavirus. *J Med Chem* 2005;48:1256–9.
5. Kim HK, Park Y, Kim HN, Choi BH, Jeong HG, Lee DG, et al. Antimicrobial mechanism of b-glycyrrhetinic acid isolated from licorice, *Glycyrrhiza glabra*. *Biotechnol Lett* 2002;24:899–902.
6. Shibata S. A drug over the millennia: pharmacognosy, chemistry, and pharmacology of licorice. *Yakugaku Zasshi* 2000;120:849–62.
7. van Rossum TG, Vulto AG, Hop WC, Brouwer JT, Niesters HG, Schalm SW. Intravenous glycyrrhizin for the treatment of chronic hepatitis C: a double blind, randomized, placebo-controlled phase IIb trial, *J Gastroenterol Hepatol* 1999;14:1093–9.
8. Hayashi H, Fukui H, Tabata M. Examination of triterpenoids produced by callus and cell suspension cultures of *Glycyrrhiza glabra*. *Plant Cell Rep* 1988;7:508–11.
9. Hayashi H, Huang P, Inoue K. Up-regulation of soyasaponin biosynthesis by methyl Jasmonate in cultured cells of *Glycyrrhiza glabra*. *Plant Cell Physiol* 2003;44:404–11.
10. Hayashi H, Sakai T, Fukui H, Tabata M. Formation of soyasaponins in licorice cell suspension cultures. *Phytochemistry* 1990;29:3127–9.
11. Ayabe S, Takano H, Fujita T, Furuya T, Hirota H, Takahashi T. Triterpenoid biosynthesis in tissue cultures of *Glycyrrhiza glabra var. glanduliera*. *Plant Cell Rep* 1990;9:181–4.
12. Harker M, Holmberg N, Clayton JC, Gibbard CL, Wallace AD, Rawlins S, et al. Enhancement of seed phytoester levels by expression of an N-terminal truncated *Hevea brasiliensis* (rubber tree) 3-hydroxy-3-methylglutaryl-CoA reductase. *Plant Biotechnol J* 2003;1:113–21.
13. Aquil S, Husaini AM, Abdin MZ, Rather GM. Overexpression of the HMGR-CoA reductase gene leads to enhanced artemisinin biosynthesis in transgenic *Artemisia annua* plants. *Planta Med* 2009;75:1453–8.
14. Bach TJ. Synthesis and metabolism of mevanoic acid in plants. *Plant Physiol Biochem* 1987;25:163–7.
15. Friesen JA, Rodwell VW. The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductases. Genome Biol 2004;5:248–54.
16. Yang ZB, Park H, Lacy GH, Cramer CL. Differential activation of potato 3-hydroxy-3-methylglutaryl coenzyme a reductase genes by wounding and pathogen challenge. Plant Cell 1991;3:397–405.
17. Chappell J, Nable R. Induction of sesquiterpenoid biosynthesis in tobacco cell suspension cultures by fungal elicitors. Plant Physiol 1987;85:469–73.
18. Schaller H, Grausem B, Benveniste P, Chye ML, Tan CT, Song YH, et al. Expression of the Hevea brasiliensis (H.B.K) Mull. Arg 3-hydroxyl-3-methylglutary coenzyme A reductase 1 in tobacco results in sterolover production. Plant Physiol 1995;109:761–70.
19. Dai ZB, Cui GH, Zhou SF, Zhang XN, Huang LQ. Cloning and characterization of a novel 3-hydroxy-3-methylglutaryl-coenzyme a reductase gene from Salvia miltiorrhiza involved in diterpenoid tanshinone accumulation. J Plant Physiol 2011;168:148–57.
20. Ausubel FM, Kinston RE, Seidman JG, Strahl K, Brent R, Seidman JG, et al. Short protocols in molecular biology. 4th ed. Beijing: Science Press; 2005, p. 25.
21. Zhu XQ. Researches on the influences of CNVs of functional genes HMGR, SQS and β-AS on their expression in Glycyrrhiza uralensis [Dissertation]. Beijing: University of Chinese Medicine; 2012, p. 40–1.
22. Waterham HR, Digan ME, Koutz PJ, Lair SV, Cregg JM. Isolation of the Pichia Pastoris glyceradehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene 1997;186:37–44.
23. Liu Y, Liu DJ, Liu CS, Liao CL, Cheng XL. Mechanism of genuineness of liquorice Glycyrrhiza uralensis based on CNVs of HMGR, SQS1 and β-AS gene. Acta Pharmaceut Sin 2012;47:250–255.
24. Liu Y, Xu QX, Wang XY, Liu CS, Chen HH. Researches on the influence of 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene polymorphism on catalytic efficiency of its encode enzyme in Glycyrrhiza uralensis. China J Chin Mater Med 2012;37:3784–8.
25. Liu Y, Xu QX, Wang XY, Liu CS, Chen HH. Analysis on correlation between 3-hydroxy-3-methylglutary-coenzyme A reductase gene polymorphism of Glycyrrhiza uralensis and content of glycyrrhizic acid. China J Chin Mater Med 2012;37:3789–92.
26. Kang TW, Jeon YJ, Jang E, Kim JJ, Kim JH, Park JL, et al. Copy number variations (CNVs) identified in Korean individuals. BMC Genomics 2008;9:492–9.
27. Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, et al. Global variation in copy number in the human genome. Nature 2006;444:444–54.
28. Beckmann JS, Estivill X, Antonarakis SE. Copy number variants and genetic traits: closer to the resolution of phenotypic to genotypic variability. Nat Rev Genet 2007;8:639–46.
29. Stranger BE, Forrest MS, Dunning M, Ingle CE, Beazley C, Thorne N, et al. Relative impact of nucleotide and copy number variation on gene expression phenotypes. Science 2007;315:848–53.
30. Hastings PJ, Lupski JR, Rosenberg SM, Ira G. Mechanisms of change in gene copy number. Nat Rev Genet 2009;10:551–64.
31. Larkin PJ, Miller JAC, Allen RS, Chitty JA, Gerlach WL, Frick S, et al. Increasing morphinan alkaloid production by over-expressing codeinone reductase in transgenic Papaver somniferum. Plant Biotechnol J 2007;5:26–37.
32. White PJ. The regulation of K^+ influx into roots of rye (secale cereale L.) seedlings by negative feedback via the K^+ flux from shoot to root in the phloem. J Exp Bot 1997;48:2063–73.