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THE *CYANOBACTERIUM* HEMOGLOBIN (CHb) PROMOTES THE GROWTH OF BACTERIA, YEAST AND *BRASSICA NAPUS*, AND ENHANCES SUBMERGENCE RESISTANCE OF *BRASSICA NAPUS*

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Abstract

The coding sequence of *Cyanobacterium* hemoglobin (*CHb*) gene, from Cyanobacterium *Synechocystis* sp. PCC 6803, was inserted into vectors pET-30a, pYES2 and pCAMBIA1300. The expression of *CHb* in *E. coli* (BL21) and yeast (*pep4*) could enhance the growth rate of bacteria and yeast. Constitutive *CHb* over-expression in *Brassica napus* induced earlier germination of transgenic seeds and increased chlorophyll content in transgenic seedlings. Under submergence conditions, the bioassays revealed that *CHb* over-expression protected transgenic plants against prolonged waterlogging. These results suggested that *CHb* gene could promote the growth rate of microbes and *Brassica napus*, and resistance to submergence for *Brassica napus*.

Key words: *Cyanobacterium hemoglobin* (*CHb*), Submergence stress, Promote growth, Germination, Chlorophyll content.

Introduction

Vitreoscilla hemoglobin (VHb) is a soluble protein that functions as the carrying and transporting oxygen, which has similar function as the hemoglobin of animals and human beings (Wakabayashi *et al.*, 1986). In bacteria, oxygen concentration influences the expression of *VHb*, and the expression of *VHb* gene achieves maximum under microaerophilic conditions (Dikshit *et al.*, 1990; Khosla & Bailey 1989). At low extracellular concentrations, the increasing expression level of *VHb* is helpful to trap oxygen and deliver oxygen to terminal respiratory oxidases, which enhance the efficiency of respiratory oxidases and ATP synthesis under hypoxic conditions (Dikshit *et al.*, 1988; Wakabayashi *et al.*, 1986).

It was reported that the expression of heterologous *hemoglobin* could increase the growth rate of tobacco, and its contents of chlorophyll and nicotine (Holmberg *et al.*, 1997). The heterologous expression of *hemoglobin* also gave rise to some meaningful physiological phenomena, such as improving the growth performance, increasing biosynthesis of certain oxygen-required metabolites, enhancing tolerance to submergence and resistance to nitrosation stress (Bülow *et al.*, 1999; Zhang *et al.*, 2007; Wang *et al.*, 2009).

In current study, we cloned Cyanobacterium *Synechocystis* sp. PCC 6803 *hemoglobin* gene (*CHb*), which was 375 bps in length and encodes a 123 amino acids polypeptide chain belonging to the truncated hemoglobin family (Kaneko *et al.*, 1996; Falzone *et al.*, 2002; Tan *et al.*, 2009). Database search showed that a motif 'F-[L]-x(4)-[G]-G-[T]-x(2)-[Y]-x-[G]-[R]-x-[M]-x(3)-H' was identified in hemoglobins and truncated hemoglobins, which might be originated from the same ancestor hemoglobin. Phenotype analysis indicated that the expression of *CHb* was related to earlier maturity of transgenic rapeseed. The common characteristic of hemoglobins is the ability to reversibly bind oxygen, which may play an essential role in growth and development of organisms, especially in bacteria and plants.

In order to investigate the accelerating growth rate function of *CHb* gene, we constructed the expression vectors and then transformed *E. coli*, yeast *pep4* and *Brassica napus*. The results showed that over-expression of *CHb* could enhance growth rate of bacteria and yeast, also had an obvious impact on the seed germination speed, early flowering, and submergence stress.

Materials and Methods

Vector construction and transformation: *CHb* gene was cloned from Cyanobacterium *Synechocystis* sp. PCC 6803, which was 375 bps in length and encoded a 125 amino acids polypeptide chain. The pMD-18T-*CHb* plasmid was digested with *Kpn* I/*Bam*H I to generate *CHb* fragment, which was ligated into the *Kpn* I/*Bam*H I sites of pET-30a and pYES2 to produce the plasmids pET-30a-*CHb* and pYES2-*CHb*.

Plasmid pET-30a-*CHb* and pET-30a empty vector, as control, were transformed into *E. coli* BL21 (DE3) chemically competent cells. The pYES2-*CHb* plasmid was also transformed into yeast *pep4* chemically competent cells. *Pep4* is a yeast strain that can accumulate the inactive vacuolar hydrolases precursors. Therefore, *pep4* contains relatively small amount of the major hydrolases in cytoplasm, such as protease A, protease B, carboxypeptidase Y, large aminopeptidase, repressible alkaline phosphatase, and at least one RNase species (Woolford *et al.*, 1986). Hemoglobin can accumulate relatively high content of the protein. As a control, pYES2 empty vector was also transformed into *pep4* cells. Transformants were selected on DOBA (Dropout base agar-uracil) plates. The positive colonies were selected randomly and grew in Dropout base until middle- to late-log phase. Then yeast colonies were selected and washed with 2 M sorbitol for 2 times. The colonies were transferred to YPD liquid medium containing 2% galactose without dextrose. The cultures grew for another 12 hours to induce the expression.

We constructed binary vector pCAMBIA1300-*CaMV35S-CHb*, and transformed into chemically competent *Agrobacterium tumefaciens* LBA4404 (Tan *et al.*, 2009). The methods of *B. napus* transformation and selection were performed according to the methods of Davis *et al.*, (2009), Harrison *et al.*, (2006), Li *et al.*, (2010) and Zhang *et al.*, (2017).

Western blot analysis: For prokaryotic expression of the recombinant CHb protein, pET-30a-*CHb* plasmid was transformed into *E. coli* BL21 (DE3) chemically competent cells and inoculated in a tube containing 4 mL LB medium with 50 mg/mL kanamycin. The cells were grown at 37°C with continuous shaking (200 rev min^{-1}) until an OD_{600} of 0.6 attainment. The expression of recombinant CHb protein was induced overnight at 28°C by adding 1.0 mM IPTG, 1 mL culture was collected and centrifuged with 5000 rpm for 5 min. The *E. coli* BL21 pellet was resuspended with Laemmli sample buffer and then was boiled for 5 min before analysis with SDS-PAGE. The protein was separated by SDS-PAGE at 12% polyacrylamide gel (Zhang *et al.,* 2008).

The separated proteins in polyacrylamide gel was transferred onto a PVDF membrane, which was blocked with blocked liquid (PBS incubated with 5% skim milk containing 0.05% Tween 20) for 90 minutes to reduce nonspecific binding. The membrane was washed with PBS-Tween buffer three times and incubated with purified primary antibody overnight at 4°C. Next, the membrane was washed five times and incubated with goat anti-mouse IgG/HRP for 90 minutes. After washing three times, the membrane was incubated in 2.0 mM DAB solution prepared in PBS for 5 minutes (Zhang *et al.,* 2008).

The measurement of growth curve: The BL21 (DE3) strains transformed by pET-30a-*CHb* and pET-30a empty vector were grown overnight in LB medium and then subcultured at a 10% inoculum into LB medium. After incubation for 4 h, the cultures were standardized to the same optical density at 600 nm and were used to inoculate fresh LB medium by the addition of IPTG to a final concentration of 1.0 mM (1% inoculum). Then the two strains were inoculated under hypoxia and normal conditions, respectively. The cultures were monitored with a Beijing Rayleigh UV-1601 UV/VIS spectrophotometer and optical density was measured at 600 nm with an interval of 4 h under hypoxia condition or 2 h under enough oxygencondition (Xiong *et al.,* 2000; Kang *et al.,* 2008).

The yeast *pep4* strain (20B12, B/BJ, A4509) transformed by pYES2-*CHb* and pYES2 empty vector were grown overnight in YPD liquid medium (2% bacteriological peptone, 1% yeast extract) containing 2% dextrose and then subcultured at 10% inoculum into YPD liquid medium. After incubation for one day, the cultures were standardized to the same optical density at 600 nm and used to inoculate fresh YPD liquid medium. The cultures were monitored with a Beijing Rayleigh UV-1601 UV/VIS spectrophotometer and the optical density at 600 nm every two hours was measured.

Reverse transcription (RT)-PCR analysis: RNA samples were extracted from induced yeast *pep4* according to TaKaRa RNAiso^{TM} Plus introduction. The first-strand cDNA was synthesized using 5μg of total RNA as template and oligo (dT) as primer following the instruction of the manufacturer (Tiangen Biotech. Co. Ltd. China). PCR reactions were carried out with 300 ng cDNA as a template, and Hemoglobin F: 5'-ggtaccATGTCAACTTTGTATG-3' and Hemoglobin R: 5'-ctgcag TCACTGATTAAGCACG-3' as primers, denatured at 95°C for 4 min and followed by 30 cycles (94°C for 40 s, 57°C for 40 s and 72°C for 40 s). The control yeast *pep4* cDNA was also amplified as a negative control with the same primers. The products of each reaction were electrophoresed by 1% agarose gel and stained with ethidium bromide.

Total plant RNAs were isolated from the 4-week-old leaves of wild type and transgenic *B. napus* (independent lines 18, 21 and 23). The RNA samples were treated with an RNase-free DNase as per manufacturer's instructions (Promega, USA) to remove the DNA in RNA samples. First strand cDNA was synthesized by reverse transcription of purified RNA using M-MuLV reserve transcriptase kit (Fermentas, USA). Amplification was carried out according to the procedure mentioned above. Plant *Actin* was selected as a reference (F: 5'-TGTTGCTATCCAGG CTGTTCTTTC-3'; R: 5'-CTTTAATGTCACGGACGA TTTCC-3'). All amplification products were separated with 1% agarose gel.

DNA extraction and PCR analysis: Using CTAB method, the genomic DNA was isolated from the leaves of three positive transgenic T2 plants (independent lines 18, 21 and 23) and wild type lines. The transgenic plants were detected by PCR using genomic DNA as template and the same primers of *CHb* gene. The amplified samples were separated with 1% agarose gel (Wang *et al.,* 2009).

The growth and germination experiments of transgenic *Brassica napus:* The seeds of transgenic *B. napus* (independent lines 18, 21and 23) and wild type were placed on Whatman filter paper (moistened with sterilized deionized water) in Petri plates. In order to compare the appearance of seed germination, the plates were placed in complete darkness at 28°C (Kelley *et al.,* 2007). Seed germination was recorded for 72 hours (each time/24 hour). The seeds with the emergence of radicle were considered to be germinated. In all experiments, each plate was placed 100 seeds, and each experiment was repeated three times.

Submerged culture and submergence tolerance assay of transgenic *Brassica napus:* In order to verify the submergence resistance of *CHb* overexpressed transgenic plants, the 30-day-old transgenic plants and wild type were put into water containers for 20 days, whose roots were completely submerged in the water during the treatment. The cultivation was carried out according to the description of Wang *et al.,* (2009).

Determination of chlorophyll contents: Measurement of chlorophyll a and b contents was carried out as described by Shen (1988). The *B. napus* leaves of 10-day submerged culture were soaked in the mixed liquid of ether, acetone and water (V:V:V = 4.5:4.5:1) in the darkness for 24 hours and then measured at 645 nm and 663 nm using a spectrophotometer. Finally, the contents of chlorophyll a, b and a+b were calculated (mg/L). Each experiment was repeated three times.

Results

***CHb* gene enhances the growth rate of bacteria:** In order to investigate the effects of *CHb* gene on the growth rate of bacteria, CHb, as a fusion protein, was successfully expressed in *E. coli* (BL21). As shown in Fig. 1, the specific band with about 15 kDa was found, which was larger than the predicted CHb protein (13.9 kDa). It may be due to the CHb fusion protein carrying His-tags at both of the N-terminus and C-terminus. The fusion protein was further testified by Western blot and also detected by anti-6×His monoclonal antibody (Fig. 1).

Compared with non-transformants, under hypoxia oxygen conditions, the transformants increased rapidly from early log phase to stationary phase. In stationary phase, OD_{600} of transformants was 0.35 while the OD_{600} of non-transformants was only 0.2 (Fig. 2A). Moreover, the transformants have similar performance under enough oxygen conditions (Fig. 2B), indicating that *CHb* gene not only improves the growth speed of bacteria, but also increases yield of bacteria under hypoxia and enough oxygen conditions, especially under hypoxia conditions.

***CHb* gene increases the growth speed of Yeast *pep4*:** To confirm successful transformation of *CHb* gene into yeast, we used Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) to examine the expression of *CHb* with *CHb*-specific primers. As shown in Fig. 3, PCR fragments could be detected in transformants, but there were no PCR fragments in non-transformed controls, suggesting that the recombinant plasmid has been transcripted accurately in yeast *pep4*.

To investigate the role of *CHb* gene in regulating the growth of yeast, we compared the optical density between transformants and non-transformants. As shown in Fig. 4, the optical density of transformants increased more rapidly during early log phase than non-transformants. After that, the differences became less significant (Fig. 4). The results indicated that *CHb* gene can enhance the growth rate of yeast *pep4*, but have limited impact on the yield of yeast *pep4*.

CHb overexpression leads to earlier germination of transgenic *B. napus* seeds: To investigate the function of *CHb* gene in plants, *CHb* gene was transformed into *B. napus* according to Li *et al.*, (2010). The expression level of *CHb* was confirmed by RT-PCR. The 375 bp amplification products of *CHb* were detected in all three transgenic lines (independent lines 18, 21 and 23), but there was no fragment in the wild type (Fig. 5A). As shown Fig. 5B, the amplification products of *ACTIN* gene were visualized, which confirmed the expression of *CHb* gene in *B. napus*.

As mentioned above, the *CHb* could enhance the growth rate of *E. coli* and yeast *pep4*. To examine whether *CHb* gene has the same function in *B. napus*, we investigated the seed germination rate between wild type and *CHb* overexpression transgenic lines. As shown in Fig. 6A, the seed germination rate of all 3 *CHb* overexpression transgenic lines was significantly higher than that of wild type, among which the seed germination rate of transgenic line 18 was 20% higher than that of the wild type (Fig. 6A). Furthermore, the roots of all 3 transgenic lines were longer than the wild type at 48 h and 72 h (Fig. 6B and C). These results showed that *CHb* was also advantageous to seed germination and root growth of *B. napus*.

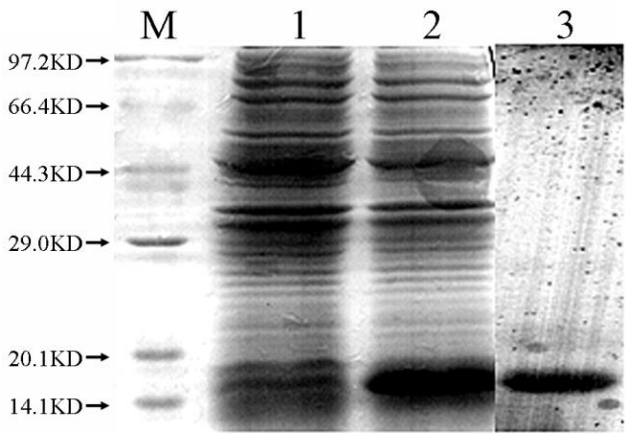

Fig. 1. Expression of pET30a-*CHb* in *E. coli* BL21.
M: Protein marker; Lane 1: SDS-PAGE analysis of total proteins in *E. coli* BL21 transformed by pET-30a and pET30a-*CHb*; Lane 2: after induction overnight. Lane 3: Western blotting analysis of recombinant protein 6xHis-*CHb* in *E. coli* BL21 transformed by pET30a-*CHb*.

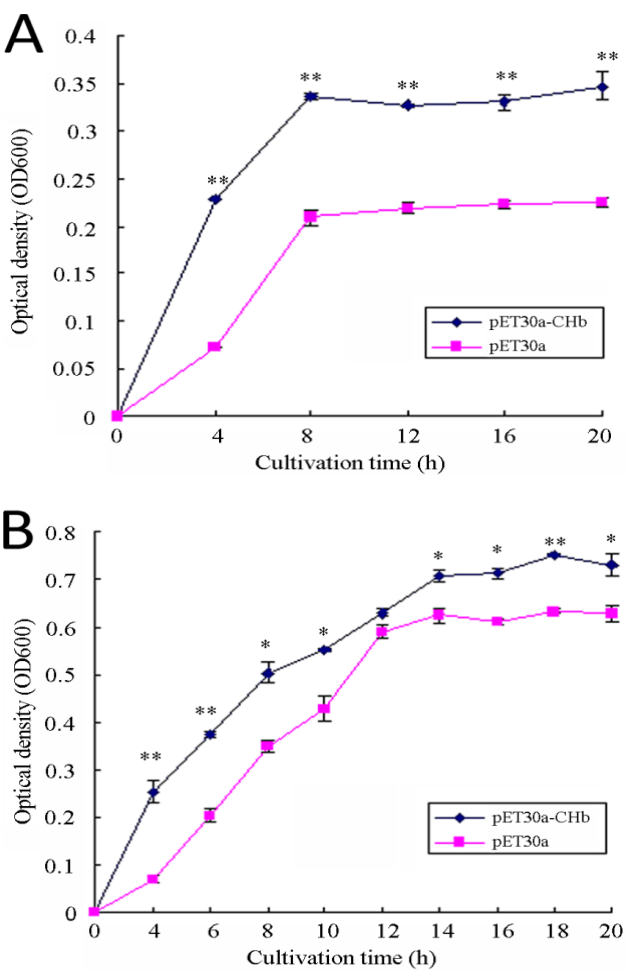

Fig. 2. Comparison of the growth rate and yield of bacteria in *E. coli* BL21.
pET-30a-*CHb* and pET-30a empty vector plasmids in hypoxia (A) and enough oxygen (B). **P*<0.05, ***P*<0.01, Student *t*-test was used to generate *P* value.

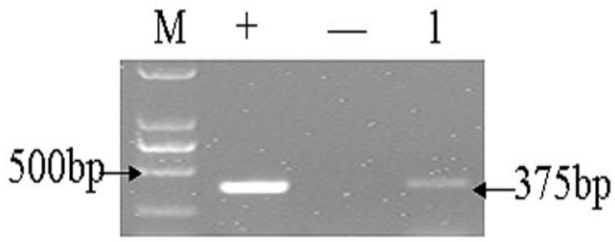

Fig. 3. The expression of *CHb* in yeast *pep4*.
M: DNA marker; Lane +: PCR products of plasmid pCAMBIA1300-*CaMV35S-CHb*; Lane -: PCR products of non-transformed controls; Lane 1: PCR products of transformants.

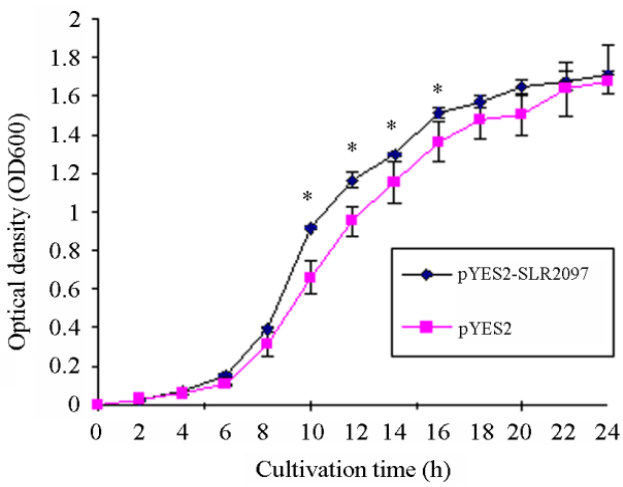

Fig. 4. Comparison of the growth rate and yield of yeast *pep4*.
pYES2-SLR2097 and pYES2 empty vector plasmids in enough oxygen. *p<0.05, Student t-test was used to generate P value.

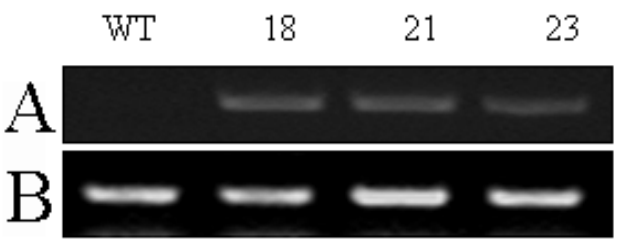

Fig. 5. The expression of *CHb* mRNA in transgenic *B. napus* and wild type.
(A) Analysis of *CHb* expression by RT-PCR, the presence of *CHb* transcript in the transgenic lines (18, 21 and 23) and its absence in the wild type. (B) The presence of ACTIN transcript in both lines is visualized by staining with ethidium bromide.

***CHb* overexpression enhances submergence resistance of transgenic *B. napus*:** Submergence stress often results in the delay of crop growth and dramatically reduces the crop yield. Therefore, submergence tolerance is extremely important for crop improvement. In general, the *CHb* overexpression transgenic *B. napus* were more resistant to submergence stress than the wild type (Fig. 7). Within 10 days of complete root immersion, the oldest leaves of wild type seedling turned yellow, while the leaves of transgenic plant kept growing (Fig. 7A). After 20 days of complete root submergence, many white roots appeared in the hypocotyl of the transgenic plant (Fig. 7B), indicating that a well-developed aerenchyma of the transgenic *B. napus* stem was formed under waterlogged condition. Meanwhile, the *CHb* overexpression transgenic *B. napus* was taller and had more leaves than wild type (Fig. 7B, C).

We measured chlorophyll contents between wild type and transgenic *B. napus* after 10 days of complete root submergence. As shown in Fig. 8, the contents of chlorophyll *a*, *b*, and *a*+*b* of *CHb* overexpression transgenic lines were more than that of wild type. The chlorophyll content of *CHb* overexpression transgenic *B. napus* was about 30% higher than that of the wild type (Fig. 8). These observations suggested that *CHb* gene could be used as an excellent tool for submergence-tolerant cultivars of important agricultural crops.

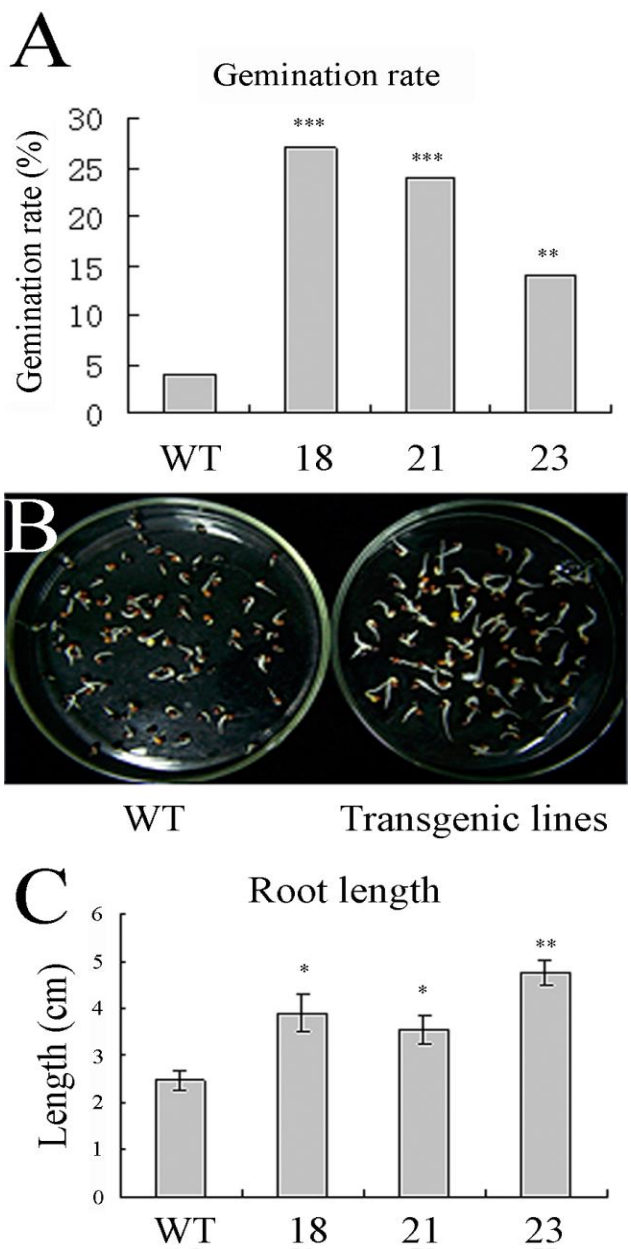

Fig. 6. Germination of wild type (WT) and *CHb* overexpression transgenic *B. napus* seeds.
(A) Seed germination rate at 24h; (B) Seed germination rate at 48h; (C) Root length of wild type and *CHb* overexpression transgenic *B. napus* seedlings at 72h. *p <0.05, **p<0.01, ***p<0.001, Student t-test was used to generate P value.

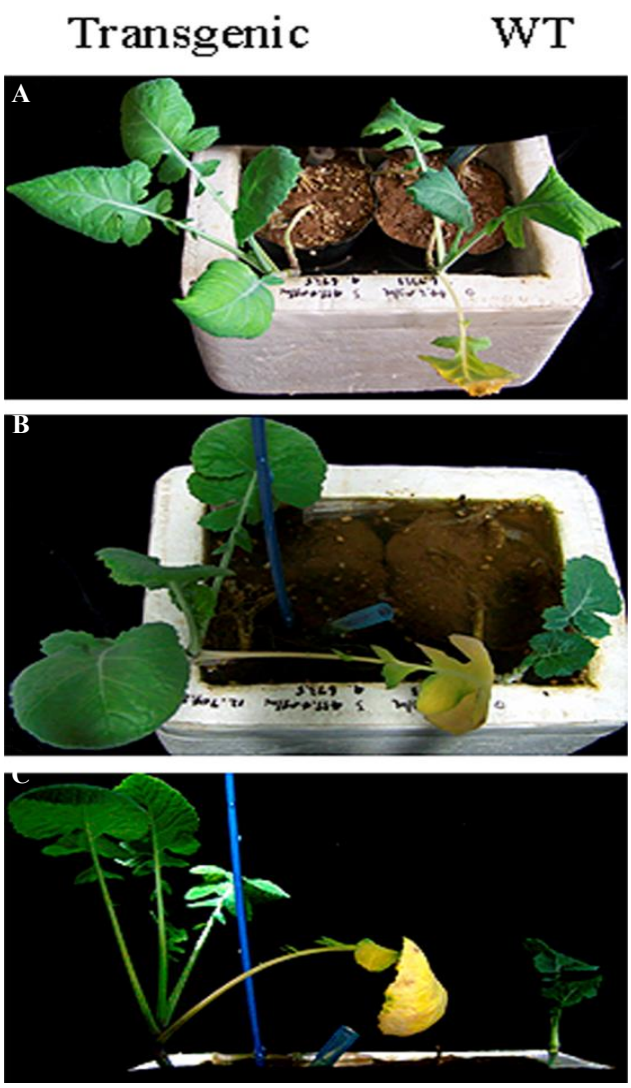

Fig. 7. Morphological differences in response to submergence stress between *CHb* overexpression transgenic *B. napus* and wild type (WT).
(A) The transgenic was more tolerant than WT on the tenth day; (B) In transgenic plant, many white roots had emerged on the tenth day; (C) Transgenic plant was taller and had more leaves than WT on the twentieth day.

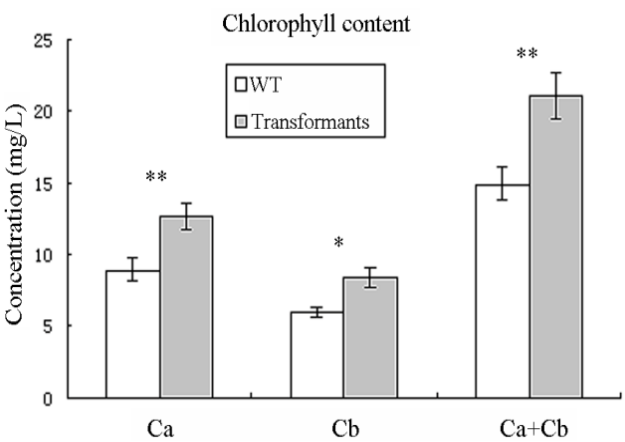

Fig. 8. The chlorophyll contents of transgenic line and wild type (WT) under submergence stress.
Ca: chlorophyll a; Cb: chlorophyll b. *p<0.05, **p<0.01, Student t-test was used to generate P value.

Discussions

It was reported that the heterologous expression of *Vitreoscilla* hemoglobin (*VHb*) in *E. coli* could increase the growth rate of cells and promote the synthesis of host protein under microaerobic conditions (Khosla & Bailey, 1988; Khosla *et al.,* 1990; Zhang *et al.,* 2007). In current study, we found *CHb* gene could promote the rapid growth of bacteria, especially in the late logarithmic phase. We also found that the overexpression of *CHb* gene in *E. coli* and yeast *pep4* (20B12, B/BJ, A4509) could accelerate growth rate. However, the results of Geckil *et al.,* (2001) showed that during the late incubation periods, the cell mass optical density was not completely related to viable cell densities.

In order to verify the role of *CHb* gene, we constructed *CHb* gene overexpression vector and transformed it through *Agrobacterium*-mediated gene transfer. The overexpression of *CHb* gene was confirmed by RT-PCR. In transgenic *B. napus*, the overexpression of *CHb* gene had an obvious effect on the early seed germination, short life cycle (data not shown here). The development of transgenic lines was ahead of wild type, and flowering stage was about one week earlier than the normal time. Previous studies have demonstrated that the increase of nicotine and chlorophyll content in *VHb* overexpression transgenic tobacco was due to the increasing their biosynthesis by more available oxygen (Holmberg *et al.,* 1997; Wang *et al.,* 2009). The *VHb* gene overexpression in tobacco, rice (Cao *et al.,* 2004) and cabbage (Li *et al.,* 2005) can promote the growth of transgenic plants, shorten the germination time, increase productivity of transgenic plants, and shorten the life cycle.

After being submerged by flooding for several days or longer, the crop tends to delay growth and even significantly reduce crop yield (Zhang *et al.,* 2007). It was found that overexpressed *VHb* in *Arabidopsis* and cabbage could increase tolerance to submergence treatment (Li *et al.,* 2005; Wang *et al.,* 2009). Therefore, submergence tolerance of the crop is a very important factor for crop improvement (Nordhaus 2007). In this study, we found that *CHb* overexpression transgenic *B. napus* not only had submergence resistance, but also increased the chlorophyll contents, plant height and leave numbers. Taken together, our work not only illustrates that *CHb* overexpression can promote the growth of microbes and plants, but also demonstrates that submergence resistance of *CHb* overexpression transgenic *B. napus* can be conducive to crop improvement, especially improving the efficiency of oxygen transport or utilization in plants under the hypoxic condition.

Acknowledgement

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