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Research on shanlan rice HKT2 gene cloning and its functions

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ABSTRACT

Soil salinization has been causing crop yields reduction worldwide, which is a great threat to global agriculture. The study of salt tolerance-related genes is the basis of using biological techniques to improve crop plants' salt tolerance. We extract the total RNA from young roots of shanlan rice, and then amplify HKT2 gene segment through PCR, to obtain a sequence of 1593bp. Then by building HKT2 gene overexpression vector, make HKT2 excessively express in Rhine Chlamydomonas CC124. Determine the salt tolerant ability of wild type Rhine Chlamydomonas and transgenic Rhine Chlamydomonas. The experimental results show that shanlan rice HKT2 expression products reduce the salt tolerance of Rhine Chlamydomonas.

KEYWORDS

Shanlan rice; Rhine chlamydomonas; Gene cloning; Salt tolerance.

INTRODUCTION

Soil salinization has been causing crop yields reduction worldwide, which is a great threat to global agriculture. Therefore, improvement of crop salt tolerance is a major research topic in efficient production of crops, while the study of salt tolerance-related genes is the basis of using biological techniques to improve crop plants' salt tolerance.

Salt tolerance of plants may be closely related with HKT proteins, which can mediate transport of Na+ and K+, and regulate plant Na+ homeostasis. As a large class of upland rice, shanlan rice has a very important social, ecological and economic value. In order to study the Na+ and K+ transport mechanism of shanlan rice HKT2 protein, we design a pair of primers according to HKT2 gene conserved sequence. We extract the total RNA from young roots of shanlan rice, and then amplify HKT2 gene segment through PCR. Connect the PCR product into pMD18-T vector, and pick the positive clones to do sequencing. The result shows that it is a sequence of 1593bp, encoding 530 amino acids, which homology with the nucleotide sequence of rice OsHKT2 registered in the GenBank is 94%.

By building HKT2 gene overexpression vector, make HKT2 excessively express in Rhine Chlamydomonas CC124. Determine the salt tolerant ability of wild type Rhine Chlamydomonas and transgenic Rhine Chlamydomonas. The experimental results shows that at 150mmol/L NaCl concentration or 225mmol/L KCl concentration, the growth of wild type Rhine Chlamydomonas will be inhibited, while transgenic Rhine Chlamydomonas is inhibited at 100mmol/L NaCl concentration and 200mmol/L KCl concentration, so it prove that shanlan rice HKT2 expression products reduce the salt tolerance of Rhine Chlamydomonas.

HKT TRANSPORTERS AND CROPS TOLERANT MECHANISM

Effects of soil salinity on plant growth

Salt exists in the lower layers of the soil or groundwater, due to the capillary effect, along with water rise to the surface layer of soil. After water evaporation, the salt will be separated out. This process is called soil salinity. Soil salinization has been causing crop yields reduction worldwide, which is a great threat to global agriculture. Therefore, improvement of crop salt tolerance is a major research topic in efficient production of crops, while the study of salt tolerance-related genes is the basis of using biological techniques to improve crop plants' salt tolerance.

The biggest threat that plants will suffer in salt stress environment is plants may not develop properly or their growth is slow, and growth and differentiation of tissues and organs has been hampered. Studies have shown that, is transfer the plants of vigorous growth in the environment containing high salt concentration, The growth rate of the plant will be reduced over a period time. With the increase of salt concentration, plant leaf area increase speed will decrease. When reaching a certain salt concentration, leaf area growth stopped, meanwhile the weight of the plant roots, leaves and lotus reduced.

Effects of salt ions on plant physiology and biochemistry mainly manifest in two aspects: one is ion stress damage biofilms. When the plants absorb a greater amount of sodium ions, it makes cells thylakoid membrane glycolipid decrease significantly, saturated fatty acid content increase, and unsaturated fatty acid content decrease, and eventually damage the cell membrane of photosynthetic characteristics. Another is that the osmotic pressure of soil reduces the ability of the plant root water absorption. Without water, photosynthesis and metabolism cannot complete, so the plants will be wilting and even dead.

Plant HKT transporters

Salt tolerance of many plants is closely linked with Na+ concentration around the roots, especially for cereal crops such as wheat and rice. The first high affinity Na + transport corridor being report was HKT1 in wheat. This HKT1 in yeast cells and Xenopus oocytes can mediate absorption of high affinity K+ and low affinity Na+. Later, researchers have successively found AtHKT1 in Arabidopsis thaliana and OsHKT1 in rice. Meanwhile, through the study of wheat transgenic lines, found that HKT1 synthesis was inhibited under specific conditions, which proved that HKT1 synthesis is regulated by environment Na + concentration, but Na + concentration has no effect on it. So within the concentration range of low affinity, Na+ as a single ion can be absorbed by the plant.

So far, people have found nine HKT class genes in rice: OsHKT1~OsHKT9. Meanwhile, HKT class genes in other plants were also found, such as HvHKT1 in barley, EcHKT1 in eucalyptus, McHKT1 in Mesembryanthemum crystallinum, etc.

Depending on the genomic, HKT gene family can be divided into two subfamilies: Subfamily I and Subfamily II. Although every HKT gene contains two introns, there is great difference between intron sizes of different subfamily. The introns sizes of the HKT class gene are shown in TABLE 1:

Another important basis to distinguish these two subfamilies is whether the selective sites in the first P-loop region protein of the gene HKT is serine or glycine.

Maser has studied the secondary structure of plant HKT transporter, and speculated that this secondary structure contains four P-loop structures and 8 major transmembrane regions, as shown in Figure 1:

TABLE 1 : Introns sizes of the hkt class gene

Subfamily group	Gene	Intron sizes (bp)	
Subfamily I	AtHKT1;1	1705	1093
	OsHKT1;1	539	300
	OsHKT1;3	799	142
	OsHKT1;4	3603	252
	OsHKT1;5	2217	904
Subfamily II	HvHKT2;1	275	300
	OsHKT2;1	208	405
	OsHKT2;3	107	314
	OsHKT2;4	107	165

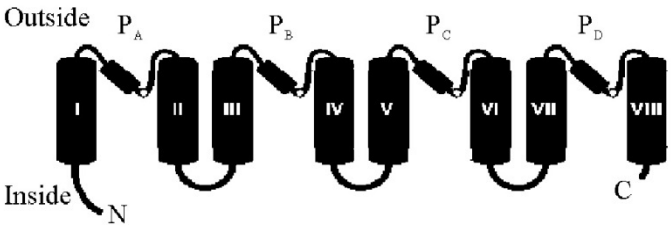

Figure 1 : Structural model of HKT proteins

MATERIALS AND METHODS

Materials

(1) Plant materials

a) Shanlan rice: shanlan rice seeds are collected from Hainan Li regional. Shanlan rice is Li's mountainous upland rice, which is suitable for planting in Arid Zone.

b) Rhine Chlamydomonas: Rhine Chlamydomonas CC124 in this study is from Shanghai Guangyu Biological Technology Co., Ltd. It is a kind of eukaryotic unicellular green algae, with wild-type phenotype. The algal strains can not only grow in liquid culture, but also grow into single colonies in the solid agar medium. Under the optimum liquid culture conditions, cell number doubling time is 5-6h, and by adjusting the light - dark cycle time, can make Chlamydomonas cell division achieve synchronization, so that improve the efficiency of genetic transformation.

(2) Reagents

a) Antibiotic reagent: here we use four kinds of antibiotic reagent, as shown in TABLE 2:

TABLE 2 : Antibiotic reagents

Reagents	Ingredients	Solutions	Volume	Conditions
Ampicillin	0.5g Amp powder	9ml sterile ddH_2O	10ml	-20°C
Kanamycin	0.5g Kan powder	9ml sterile ddH_2O	10ml	-20°C
IPTG	2g IPTG powder	8ml sterile ddH_2O	10ml	-20°C
X-Gal	20mg X-Gal powder	1ml Dimethylformamide	1ml	-20°C

b) Culture medium: components of LB medium and TAP medium are shown in TABLE 3 and TABLE 4:

TABLE 3 : LB solid medium

Component	Amount
NaCl	10g
Tryptone	10g
Yeast extract	5g
Agar	15g
Total volume	1 L

TABLE 4 : TAP solid medium

Solutions	Concentration
K_2HPO_4	119mg/L
KH_2PO_4	61mg/L
NH_4Cl	400mg/L
Tris-Base	2420mg/L
Glacial acetic acid	1 ml/L
$MgSO_4 \cdot 7H_2O$	100 mg/L
$CaCl_2 \cdot 2H_2O$	50 mg/L
Agar	15g/L
Trace	1 mL

Both of the above two formula is for solid medium. If need to compound liquid culture medium, just wipe off the agar. Before the use of the medium, it should do autoclave at 120°C for 20min.

Apparatus

The laboratory apparatus used in this study include: gradient PCR, water bath, pipette, refrigeration centrifuge, gel imager, -80°C ultra-low temperature freezer, DYY-III electrophoresis, vortex, light incubator thermostat, fluorescence microscopy, thermostatic shaker, multi-function microplate glory, etc.

Methods

(1) HKT2 gene cloning

a) Shanlan rice cultivation: perform sterilization for shanlan rice seeds. Soak them in 70% alcohol for 3min, and then washed with distilled water twice. Place in 35-40°C moist environment for germination. After 3 days, place them in moist dish to cultivate for 10 days, in 12h/d light environment.

b) RNA reverse transcription: add the reagents shown in TABLE 5 into centrifuge tube without RNA enzyme:

TABLE 5 : RNA reverse transcription reagents

Reagents	Amount
Template RNA	1μL
Oligo (dT) Primer	1μL
DEPC treated H_2O	Up to 12μL
5× Buffer	4μL
Ribonuclease inhibitor	1μL
10mM dNTP	2μL
Reverse transcriptase	1μL
Total Volume	20μL

Mix the mixture at 42°C for 60min. Terminate the reaction at 70°C heating for 10min, and then put it on ice cooling and reserve at -20°C.

c) PCR amplification: by checking the rice OsHKT2 gene fragment sequence in GeneBank, and design a pair of primer:

Forward primer: 5'-AGCTCCTTTGCTTCAAAATGACGAGCA-3' (HKT2-F)

Reverse primer: 5'-GGCTTCAGCTTCTACCATAGCCTCCA-3' (HKT2-R)

The PCR amplification reaction system is shown in TABLE 6:

TABLE 6 : RNA reverse transcription reagents

Reagents	Amount
cDNA template	2μL
Primer HKT2-F	1μL
Primer HKT2-R	1μL
dNTP mixture (2.5mM)	1μL
10× PCR buffer	2.5μL
Tag DNA polymerase	0.3μL
ddH_2O	25μL

d) Connection: connect the PCR production with pMD18-T vector. The connection system is shown in TABLE 7:

TABLE 7 : Connection system

Reagents	Amount
PCR product	1μL
pMD18-T vector	4μL
Ligation solution I	5μL

Centrifugal and mix the reaction solution, then overnight connection at 16°C.

(2) HKT2 gene overexpression vector construction

a) Primer design: By shanlan rice HKT2 gene fragment sequencing, design a pair of primer:

Forward primer: 5'-CCCATGGCGAGCATTACCAT-3' (HKT2-A)

Reverse primer: 5'-CGGACTAGTCTACCATAGCCTCCAAT-3' (HKT2-B)

According the gene map of target vector pCAMBIA1302, we designed two restriction sites of *NcoI* and *SpeI*. The gene map of vector pCAMBIA1302 is shown in Figure 2:

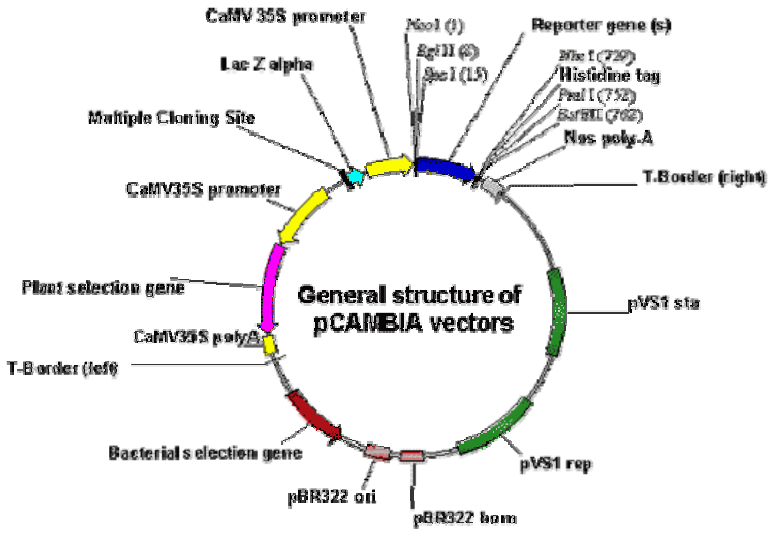

Figure 2 : pCAMBIA1302 vector gene map

Take HKT2 plasmid as template, and do PCR amplification.

b) Overexpression vector pCAMBIA1302-HKT2 construction: do double enzyme digestion of expression vector pCAMBIA1302 and recombinant plasmid pMD18-T-HKT2, at 37°C for 5h. The enzyme digestion system is shown in TABLE 8:

TABLE 8 : Enzyme digestion system

Reagents	Amount
NcoI	1μL
SpeI	1μL
BSA	1μL
10× K buffer	5μL
pCAMBIA1302/pMD18-T-HKT2	20μL
Sterile ddH_2O	18μL
Total Volume	50μL

After enzyme digestion, detect the digestion result by agarose gel electrophoresis.

(3) Rhine Chlamydomonas salt tolerance determination

a) Wild-type CC124 salt tolerance: for Rhine Chlamydomonas in logarithmic growth phase, vaccinate 1% in 50mL TAP liquid medium, at 24°C, 2000~2500lux until logarithmic growth phase. Determine NaCl concentration of 0mmol/L,

50mmol/L, 100mmol/L and 150mmol/L; determine KCl concentration of 0mmol/L, 100mmol/L, 100mmol/L, 200mmol/L and 225mmol/L. Then record the OD_{490} value in different period.

b) Transgenic CC124 salt tolerance: the experimental method and condition is the same as wild-type CC124 salt tolerance. Also record the OD_{490} value of different concentration NaCl and KCl, for further analysis.

RESULTS

HKT2 gene cloning

Take young roots of Hainan shanlan rice breeding in water for ten days as the material. Extract the total RNA by Trizol method, and do agarose gel electrophoresis detection. The result showed that 28s, 18s and 5s of the RNA is completed, so RNA quality is good enough to be used for the subsequent experiments.

Make reverse transcription of the extractive total RNA by reverse transcription kit, and obtain cDNA. Take this cDNA as the template, based on OsHKT2 gene to design the primer and do PCR. Analyze PCR product by agarose gel electrophoresis, the result showed that the segment size is 1593pb, which is consistent with the predicted target fragment. So we inferred it is HKT2 gene fragment, but it still needs further detection. The PCR amplification result of cDNA is shown in Figure 3:

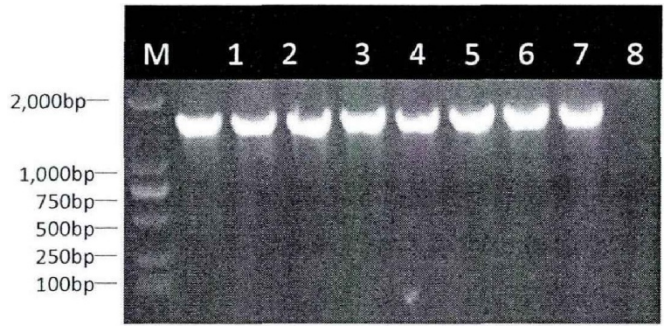

Figure 3 : PCR amplification result of cDNA

The PCR products to be recovered were connected into pMD-18-T vector, and be converted into E. Coli DH5α. Randomly pick the white single colony from LB medium plate, and do colony PCR detection. The amplified fragment is consistent with the cloned fragment in size, indicating that is positive clones, so it can do sequencing.

The sequencing results showed, the 1593bp sequence fragment is HKT2 gene. Its homology with the nucleotide sequence of rice OsHKT2 registered in the GenBank is 94%.

HKT2 gene overexpression vector construction and its transformation

(1) Expression vector construction

For sequenced HKT2 gene and overexpression vector, do enzyme digestion by *NcoI* and *SpeI*, and recover the target fragment, as shown in Figure 4.

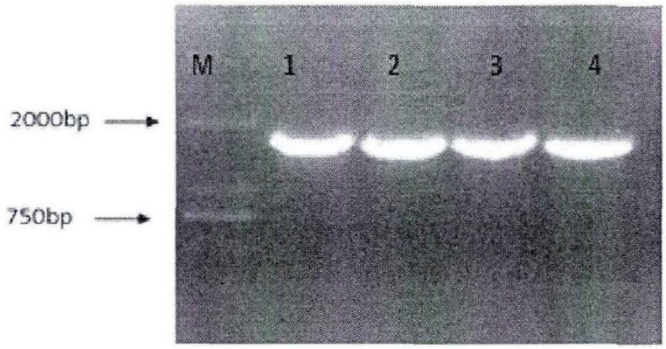

Figure 4 : PCR result of HKT2 gene fragment

Connect by T_4 ligase at 16°C, and transfer into E. coli DH5α after overnight connection. Then cultivate in LB solid culture medium (Kan 50μg/mL). Pick up positive clones and allow amplification culture in LB liquid culture medium. Pick up the plasmid and do *NcoI* and *SpeI1* double enzyme digestion detection, to assure whether vector is constructed successfully or not. Overexpression vector enzyme detection map is shown in Figure 5:

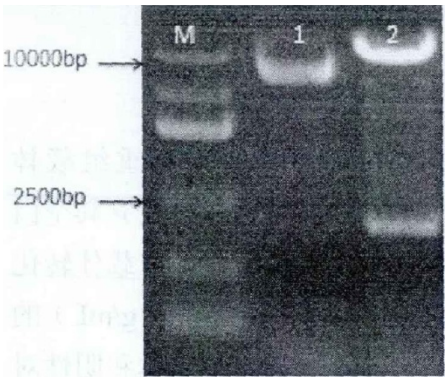

Figure 5 : Restriction analysis of recombinant plasmids

Bacteria liquid PCR amplification examination result is shown in Figure 6:

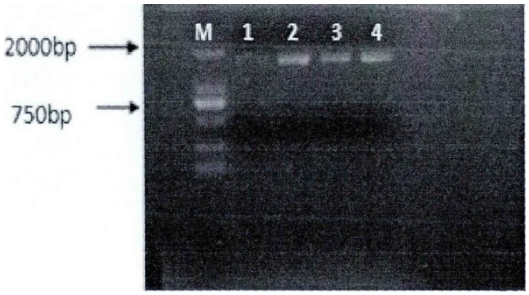

Figure 6 : Bacteria liquid PCR amplification examination result

It can be seen from the above electrophoretogram that, the fragment digested from the recombinant vector is consistent with the original fragment in size, and besides, the amplified fragment from bacteria liquid PCR is also consistent with the original fragment in size. It indicated that the recombinant vector has been successfully connected with target fragment.

(2) Rhine Chlamydomonas CC124 transformation

Transfer the constructed overexpression vector pCAMBIA1302-HKT2 into Rhine Chlamydomonas, and meanwhile set blank control group and negative control group. Evenly smear the algae solution on TAP resistance medium containing 10μg/mL Hygromycin, at 24°C and 200lux continuous illumination. After nine days, rhe recombinant vector and negative control group was still alive due to the Hygromycin resistance, and was able to normally grow single strain of positive transformants. However, the blank control group was dead. The screening process of Rhine Chlamydomonas CC124 transformants is shown in Figure 7:

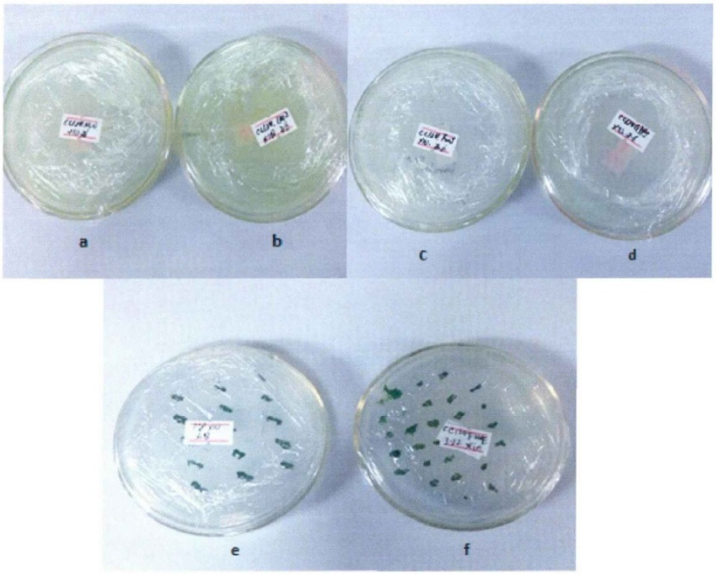

Figure 7 : Screening process of rhine chlamydomonas CC124 transformants

Select four single strains and empty vector, and extract DNA of Rhine Chlamydomonas CC124 transformants. Then take the DNA as template to do PCR detection. Through agarose gel electrophoresis, we found the amplified band is about 1500bp, indicating this four algal strains are successfully transformed. The PCR result of CC124 algal strain is shown in Figure 8:

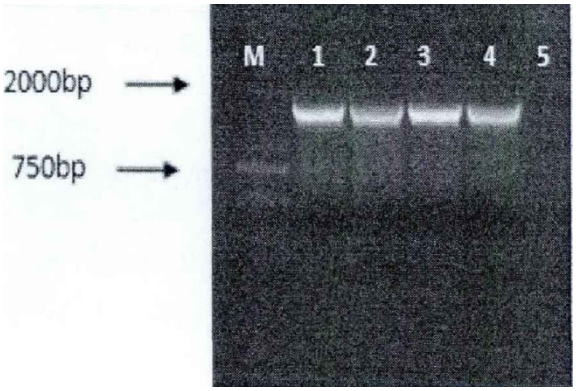

Figure 8 : PCR result of CC124 algal strain

Salt tolerance analysis of rhine chlamydomonas CC124

(1)Wild-type CC124 salt tolerance

In different NaCl concentration environment, the growth curve of wild-type Rhine Chlamydomonas CC124 is shown in Figure 9:

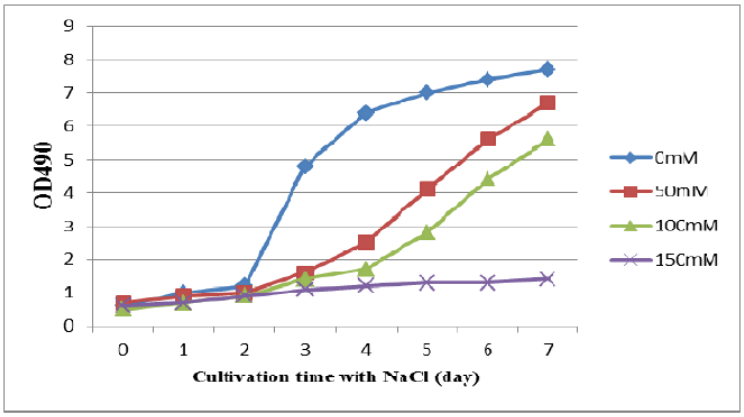

Figure 9 : OD_{490} value in different NaCl concentration

When the concentration reaches 150mmol/L, the increase of OD_{490} is almost stagnant. It indicates that he growth of wild type Rhine Chlamydomonas is inhibited at 150mmol/L NaCl concentration.

In different KCl concentration environment, the growth curve of wild-type Rhine Chlamydomonas CC124 is shown in Figure 10:

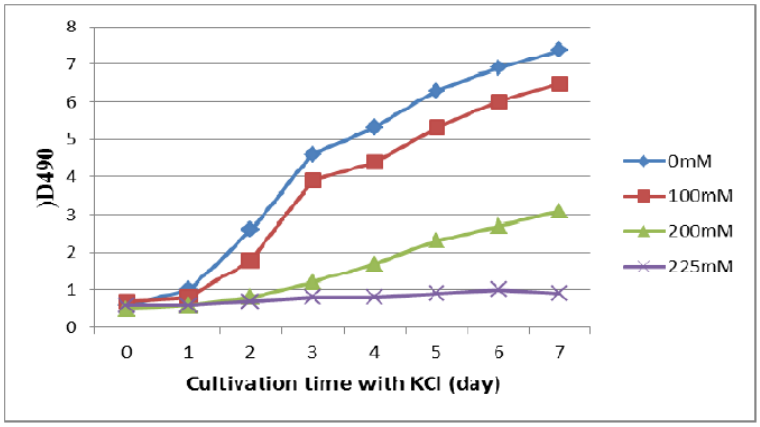

Figure 10 : OD_{490} value in different KCl concentration

When the concentration reaches 225mmol/L, the increase of OD_{490} is almost stagnant. It indicates that he growth of wild type Rhine Chlamydomonas is inhibited at 225mmol/L KCl concentration.

(2) Transgenic CC124 salt tolerance

In different NaCl concentration environment, the growth curve of transgenic Rhine Chlamydomonas CC124 is shown in Figure 11:

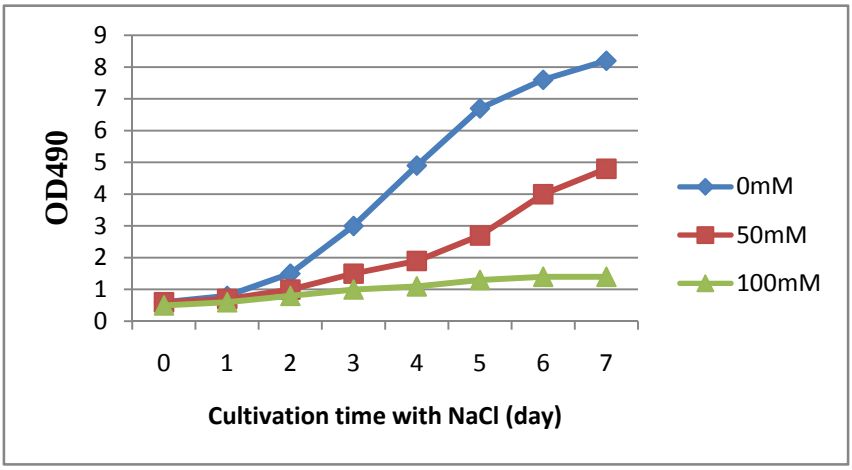

Figure 11 : OD_{490} value in different NaCl concentration

When the concentration reaches 100mmol/L, the increase of OD_{490} is almost stagnant. It indicates that he growth of transgenic Rhine Chlamydomonas is inhibited at 100mmol/L NaCl concentration.

In different KCl concentration environment, the growth curve of transgenic Rhine Chlamydomonas CC124 is shown in Figure 12:

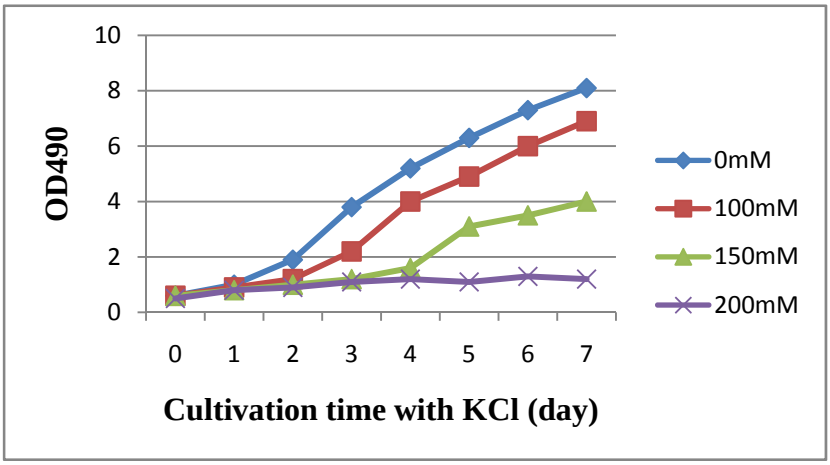

Figure 12

When the concentration reaches 200mmol/L, the increase of OD_{490} is almost stagnant. It indicates that he growth of transgenic Rhine Chlamydomonas is inhibited at 200mmol/L KCl concentration.

CONCLUSION

In this study, through connection between HKT2 and pCAMBIA1302, and transform the recombinant vector into wide-type Rhine Chlamydomonas CC124. Through resistance medium screening, pick out transformants with PCR amplification of target gene, to identify the transformants were positive transformants. It proved that HKT2 gene has successfully expressed in CC124, and provided the basis for salt tolerance analysis of Rhine Chlamydomonas.

Determine the OD_{490} value of wild-type Rhine Chlamydomonas CC124 at different salt concentration, and build its growth curve. The result showed that, at 150mmol/L NaCl concentration or 225mmol/L KCl concentration, the growth of wild type Rhine Chlamydomonas will be inhibited, while transgenic Rhine Chlamydomonas is inhibited at 100mmol/L NaCl

concentration and 200mmol/L KCl concentration, so it prove that shanlan rice HKT2 expression products reduce the salt tolerance of Rhine Chlamydomonas.

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