ABSTRACT: 25

DEVELOPMENT OF TRANSGENIC SOYBEAN PLANTS WITH SORGHUM C4-SPECIFIC PEPC GENE

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To establish a high frequency regeneration system for gene transformation of soybean [*Glycine max* (L.) Merr.], cotyledonary nodes and embryonic tips from three soybean cultivars 'Heinong 35', 'Heinong 41' and 'Heinong 58' were used as explants. 'Heinong 35' was considered one of the best cultivars for *in vitro* culture and high-frequency regeneration. Regeneration systems of cotyledon nodes and embryonic tips from three varieties were established in this research. The effect of 6-benzylaminopurine (6-BA) on soybean regeneration was studied. The results indicated that the optimal bud induction medium of cotyledonary nodes for 'Heinong 41' was MSB5 plus 1.0 mg·L-1 6-BA and 0.2 mg·L-1 indolebutyric acid (IBA) and the optimal shooting induction medium of the embryonic tip was MSB5 plus 0.2 mg·L-1 6-BA and 0.2 mg·L-1 IBA. In comparison with 'Heinong 35' and 'Heinong 58' regeneration systems, Heinong 41 was superior in shoot regeneration frequency, shoot regeneration number and shoot elongation number indicating that 'Heinong 41' is an excellent receptor for soybean genetic transformation. Sorghum C4-specific full-length pepc gene was transformed into soybean cultivar 'Heinong 41' by Agrobacterium-mediated gene transfer. Transgenic soybean lines were obtained. PCR-Southern and southern blot analysis indicated that the pepc gene has been integrated into the soybean genome. Tests showed that net photosynthetic rates, stomatal conductivity and transpiration rate of T0 and T1 transgenic soybean lines were higher than the non-transformed donor, but the intercellular CO_2 concentration was lower. The net photosynthetic rates of T0 and T1 transgenic soybean lines increased by 37.4% and 20.7%, respectively compared to the donor variety. The transgenic soybean line T1-3-3 had the highest net photosynthetic rate and yield per plant. It indicated that the photosynthetic characteristics of the transgenic soybean lines with the pepc gene have been improved compared to their controls.

Establishment of Regeneration System of Soybean and Development of Transgenic Soybean Plants with Sorghum C4-Specific *PEPC* Gene

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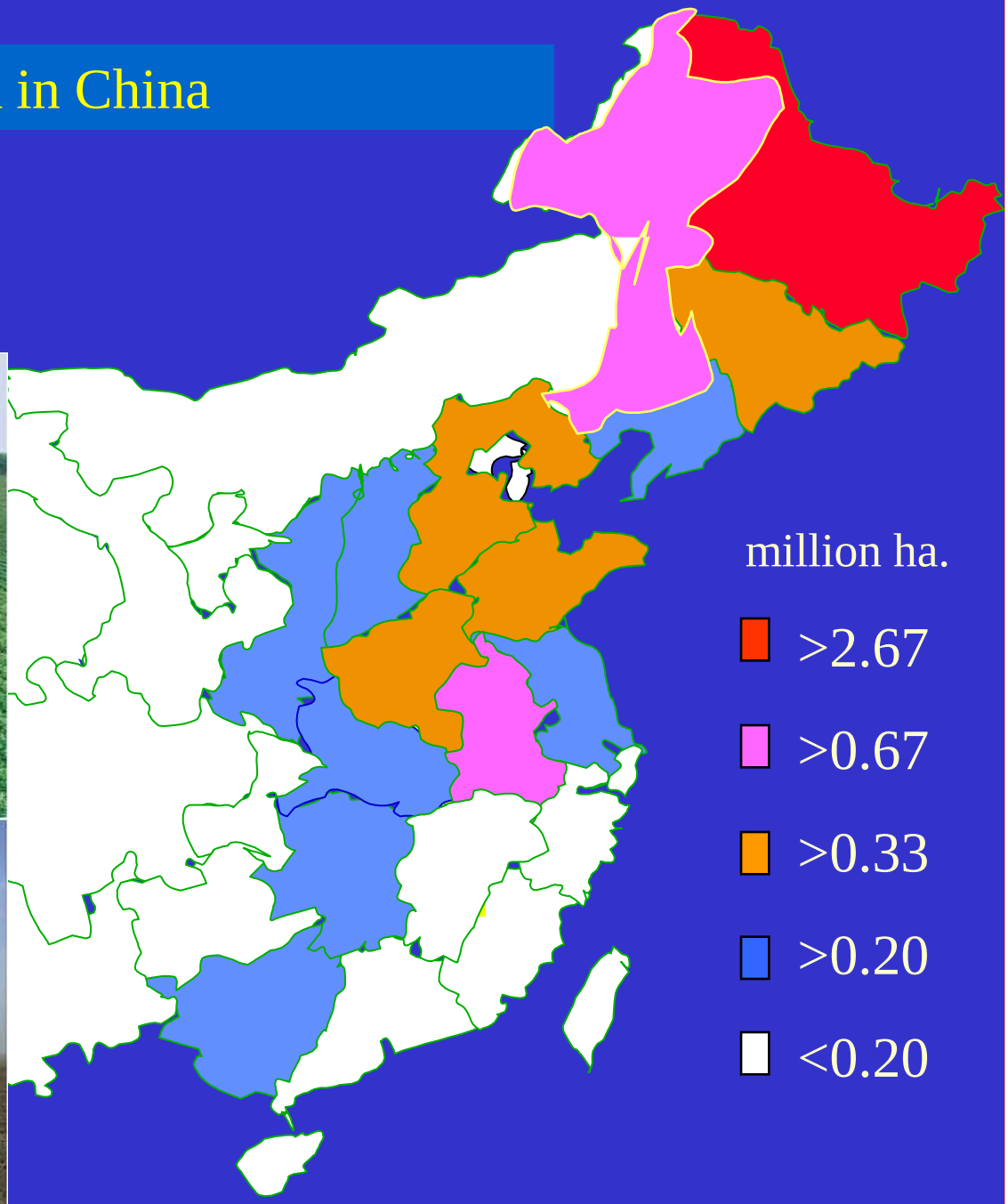
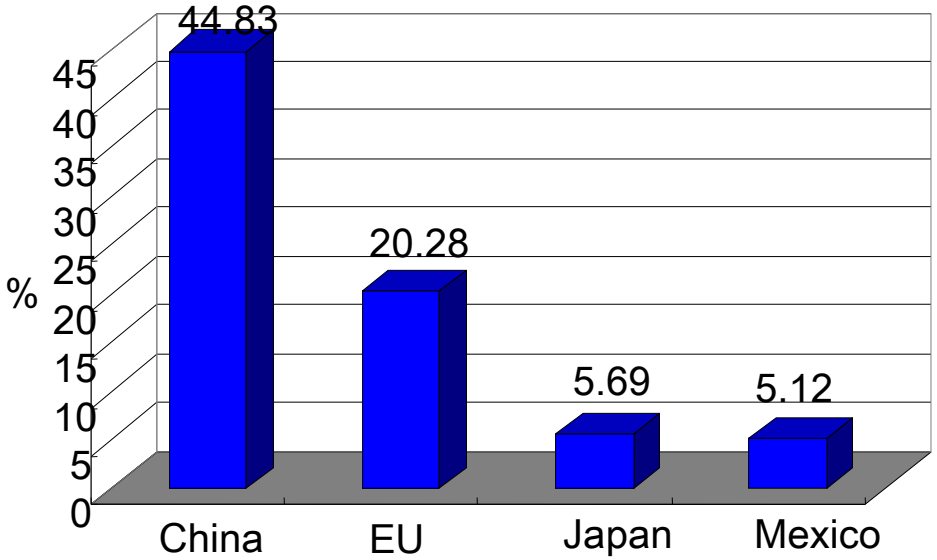
- Soybean production in China and other regions of the world
- Review of *PEPC* gene transformation
- Establishment of regeneration system of soybean
- Development of transgenic soybean plants with sorghum C4-specific *PEPC* gene

Soybean Production in Heilongjiang, China

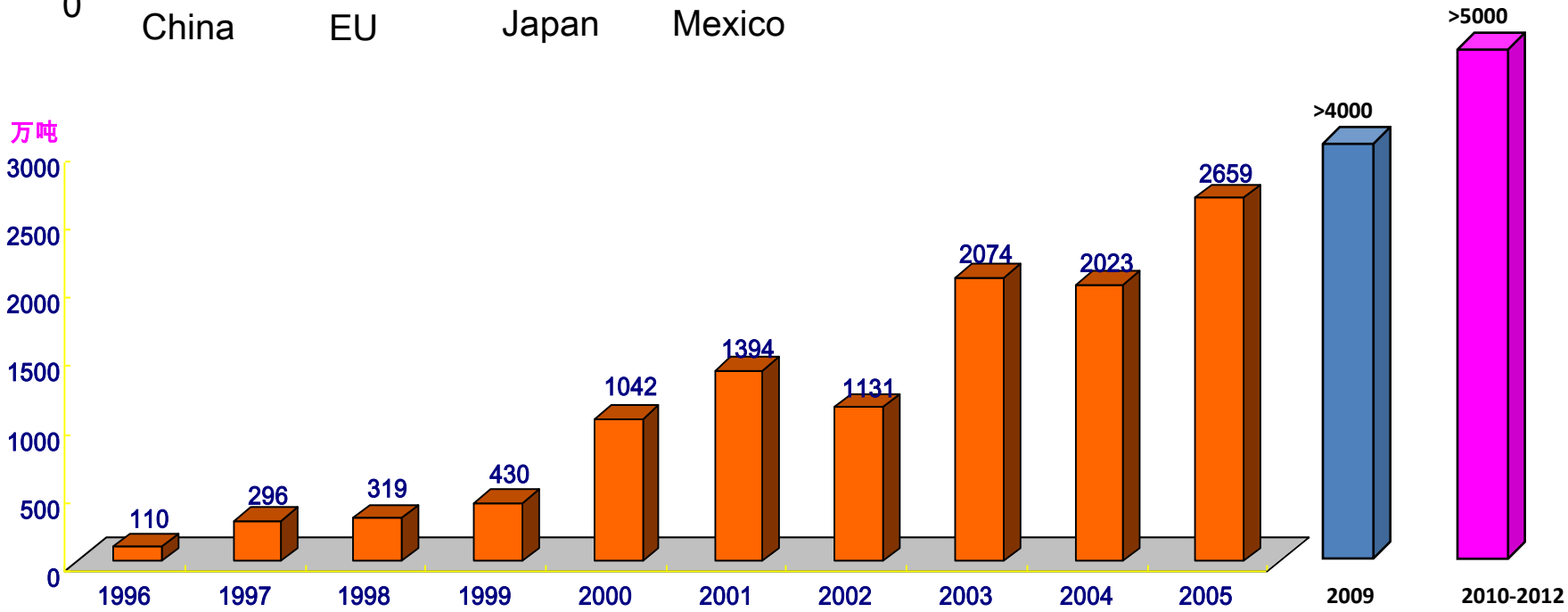
Soybean Production in Heilongjiang, China

Soybean Distribution in China





The importing rate of major consuming countries
(2000/2001 - 2011/2012)

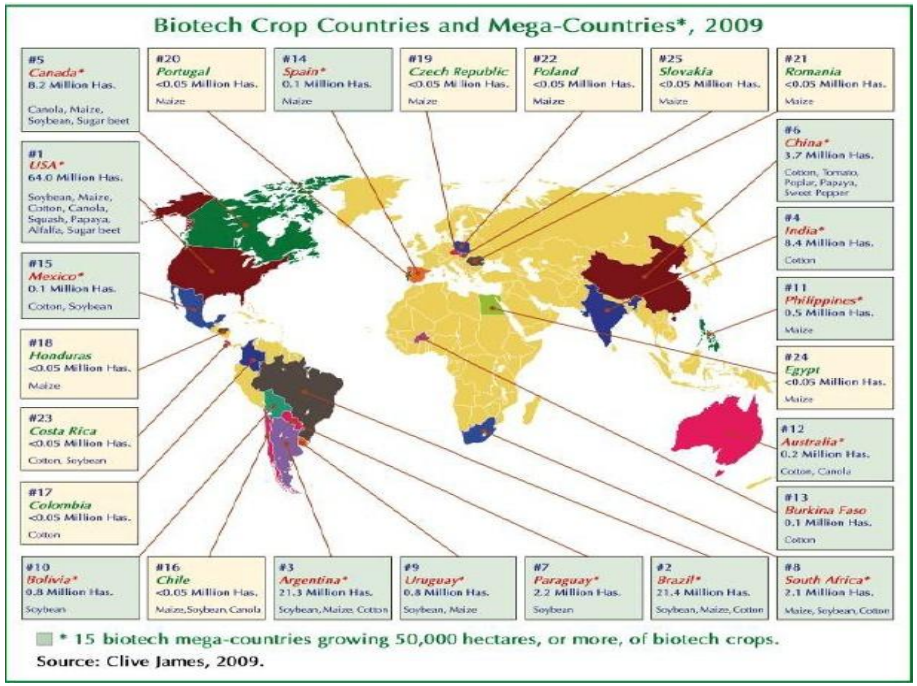


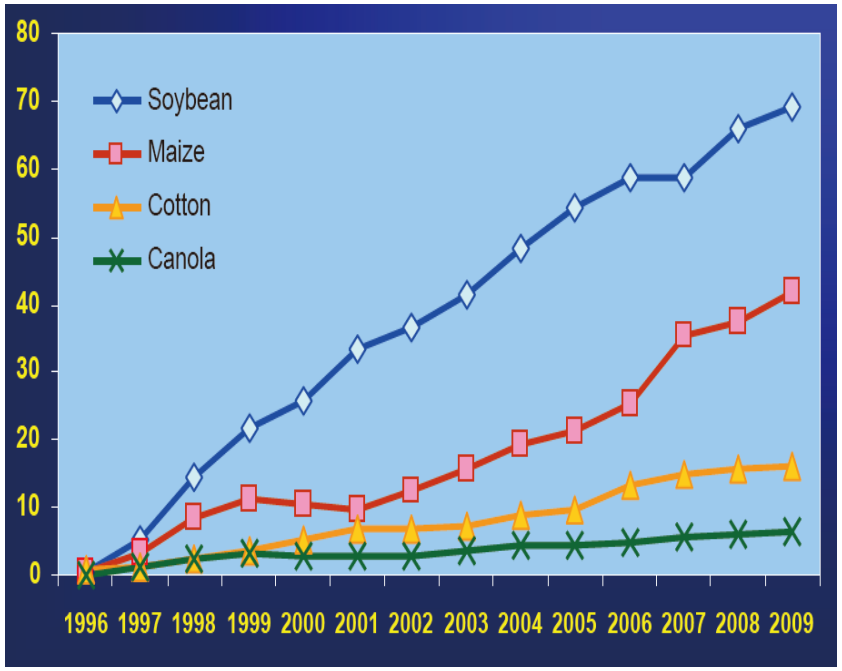
Annual imports of soybean in China

Transgenic technology is considered as one of technologies with the fastest application speed in human history.

Transgenic technique can realize direct transfer of gene between different species, and it plays irreplaceable role in crop variety improvement for high yield, high seed quality, herbicide resistance, pest resistance, stress tolerance and so on, which is hard to be dealed with conventional breeding approaches.

The Current Situation of Transgenic Crops





The world planting area of transgenic crops increase 95 times from1996 to 2011, and transgenic crops are planted in 29 countries so far. The planting area is about 134 million ha.

soybean (50%) >corn (31%) >cotton (12%) >canola (5%) in 2010

Review on *PEPC* Gene Transformation

Crop: C3、C4、CAM

Hatch-Slack (1966) discovered C4 photosynthetic pathway
Aoyaki and Bassham(1986) , Hatch(1987) intended to improve C3 crops and to increase photosynthetic capacity of major crops (eg. rice, wheat, soybean).

After the “green revolution” and the usage on heterosis, improving photosynthetic characteristics become a major pathway to breaking through yield potential barrier.
Soybean is C3 plant, has a low photosynthetic efficiency and low photorespiration. If we transfer the key enzyme of C4 pathway into C3 plant, it will be possible to improve photosynthetic capacity and to increase photosynthetic efficiency and crop yield of soybean .

PEPC is an important ubiquitous cytosol enzyme. It participates in CO2 assimilation and fixes $HCO3^-$ together with phosphoenolpyruvate (PEP) .

Rikisi (1988)made the three-way-cross combination:
At riplex rosea (C4) / At riplexputula (C3) // At riplex rosea(C4)
some C4-like lines were achieved

In recent years, with the development on biological technique, C4 genes from C4 plant, like corn and sorghum were cloned and transferred into dicots plants, such as petunia (Tagu et al.,1988), tobacco (Tagu et al.,1991), potato (Gehlen et al.,1996); also transferred into monocots, such as rice (Ku et al.,1999) and Cruciferae-Moricandia arvensis (Tanabe et al,.2000).

Cui Jilin (2000) intended to screen rice germplasm with high photosynthetic efficiency by using the same chamber effect of rice and corn, He developed wide compatibility seeds with fitting broad spectrum light intensity although failure to achieve the goal.

In recent years, scientists in China began to study the breeding and physiology about transferring C4 nitrogen fixation genes, and reached significant achievements. International Rice Institute invited many scientists worldwide and organized a seminar on the topic of building C4-rice, targeting at realizing a new “green revolution” (Dennis, 2006).

Ku (1996) firstly transferred corn C4-specific *PEPC* gene into rice, obtained the transgenic plants with a significant high level of *PEPC* enzyme activity, maximally increasing 110 fold, the content of *PEPC* enzyme was account for 12% of the soluble proteins.

Zhang Fang (2003) transferred the *PEPC* gene of sorghum into rice, resulting in the increase of photosynthetic efficiency.

Chen Xuqing (2004) cloned *PEPC* gene from corn, and transferred it into wheat. The *PEPC* enzyme activity increased 3-5 times in some of leaves of transgenic plant.

Soybean Transformation

Sorghum C4 *PEPC*

Lepiniec,et al.(1993) made a system analysis of the structure, function and molecular evolution of the Sorghum C4 *PEPC* genes family.

One of the three genes is C4 type, which is inducted by light and expressed in mesophyll cells of green leaf.

There are 10 exon and 9 intron in the gene.

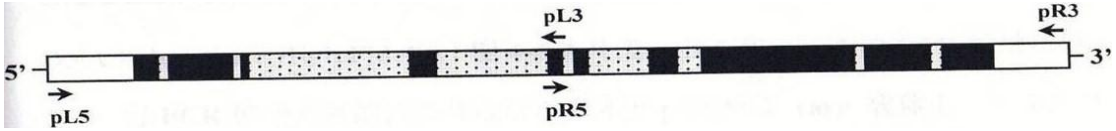


Fig. C4 *PEPC* gene structure of sorghum

□ flanking sequence ■ exon ⬚ intron

pL5&pL3 , primers of amplifying gene 5'-left,
pR5&pR3, primers of amplifying gene 3'-right.

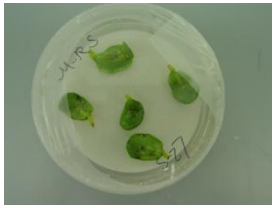
Procedure Flow Chart

Seed germination

B_5/B_5
pH5.8
Genotypes
Seed condition

5 days

Inoculation & Co-cultivation



$10\%B_5$ salts
$100\%B_5$ vitamin
pH5.4
L-cysteine
DTT
Na-sulfate
24^0C
Wounding

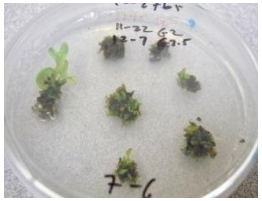
5 days

Shoot

B_5/B_5
pH5.7
BAP1.7mg/L
Selection

4 weeks

Shoot elongation



MS/B_5
pH5.7
Zeatin 1mg/L
IAA 1mg/L
GA 0.5mg/L
Selection

6-12 weeks

Rooting

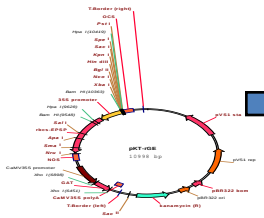
MS/B_5
pH5.7
IBA 1mg/ml

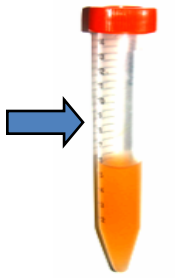
2 weeks

Acclimatization

2-4 weeks

Greenhouse





4-6 months

Exotic gene

1. *PEPC* Gene Cloning

Planting sorghum seeds, growing 10d in natural light.

Extracting sorghum genomic DNA by CTAB method.

Amplifying sorghum C4 type *PEPC* gene.

Chinese Science Bulletin 2003, Zhang F. et al

1.1 Primer Design

Sorghum *PEPC* gene (published by Lepiniec et.al (1993),GenBank:X63756) is very big one with full length of 7071bp. Therefore, we designed two sets of primers to amplify the gene from both sides. The primers are showed as follows:

5’-end primers：（*PEPC*5）

pl5: 5’-TTA AGA TCT TTA CAG CAG CAA AGC CAA GCC-3’
Bgl II
pl3: 5’-GCA GCC ATC ATT CTA GAC AGC AAG-3’
Xba I

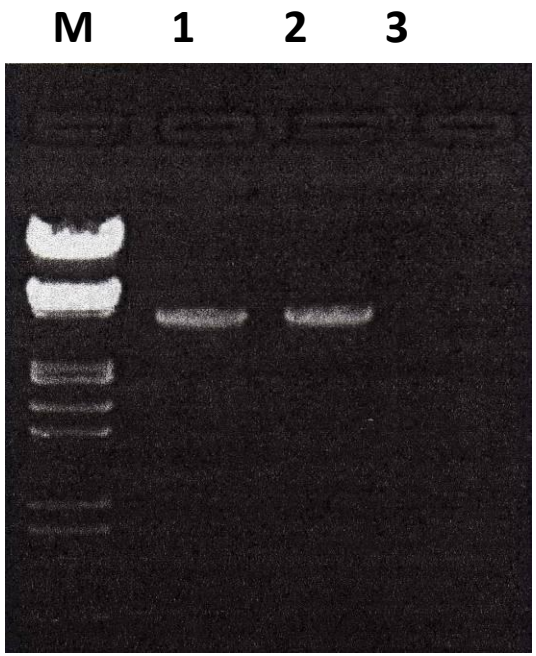
3’-end primers：（*PEPC*3）

pr5: 5’-CTT GCT GTC TAG AAT GAT GGC TGC-3’
Xba I
pr3: 5’-TTA AAG CTT CAC TAT AGC GCC GTT GGA TCG-3’
Hind III

1.2 PCR

Reaction conditions

94℃	1min	
94℃	30sec	
55℃	30sec	30 cycles
68℃	4min	
72℃	7min	



M. λDNA/EcoR Ⅰ+HindⅢ
1-2.transformation colony；3.check

—3.6Kb
—3.4Kb

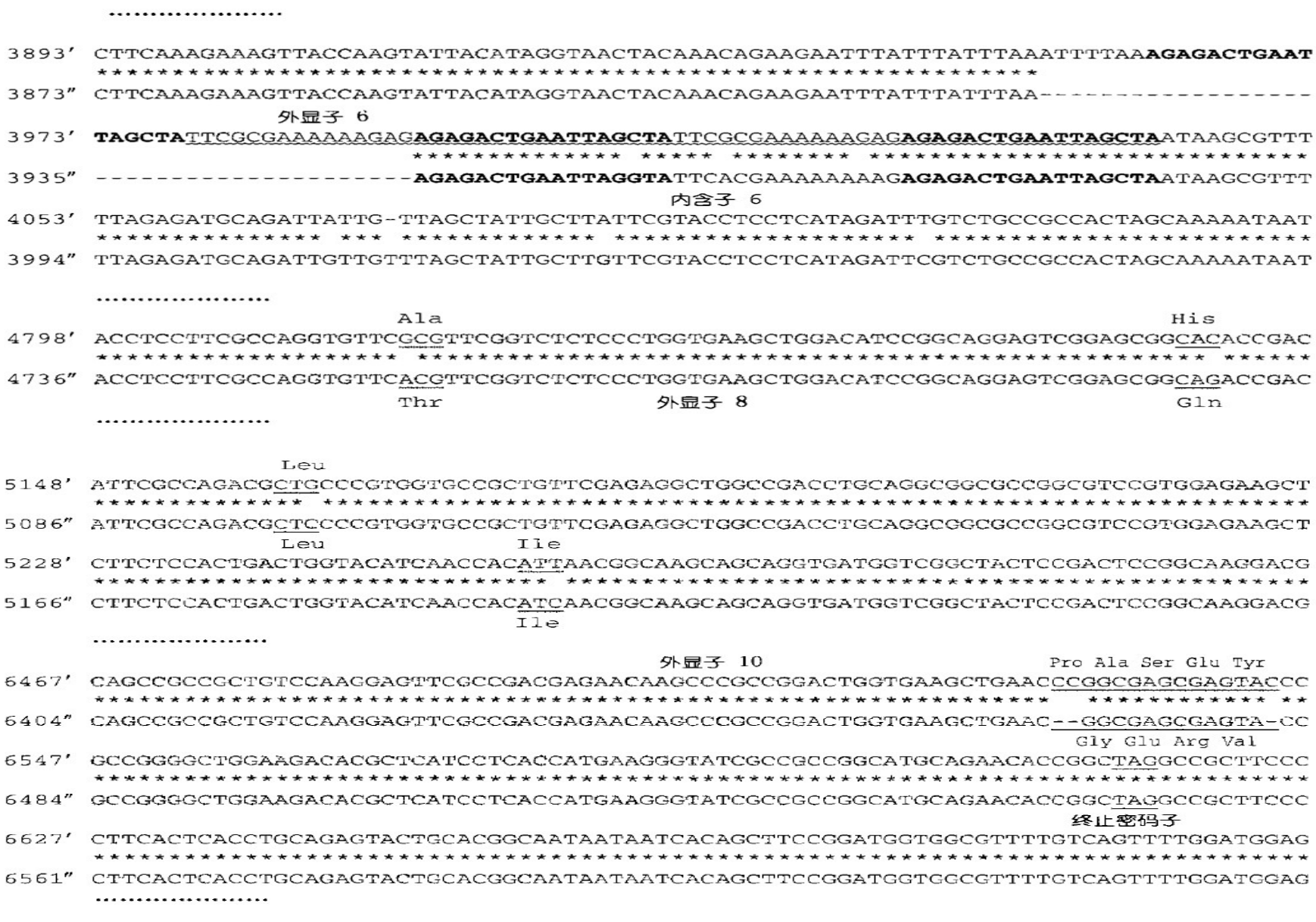
Fig. PCR amplifying of DNA in plasmid

The amplified fragment from 5’ end is 3.6kb.

The amplified fragment from 3’ end is 3.4kb.

The two fragments were combined to the whole gene sequence.

1.3 Sequencing

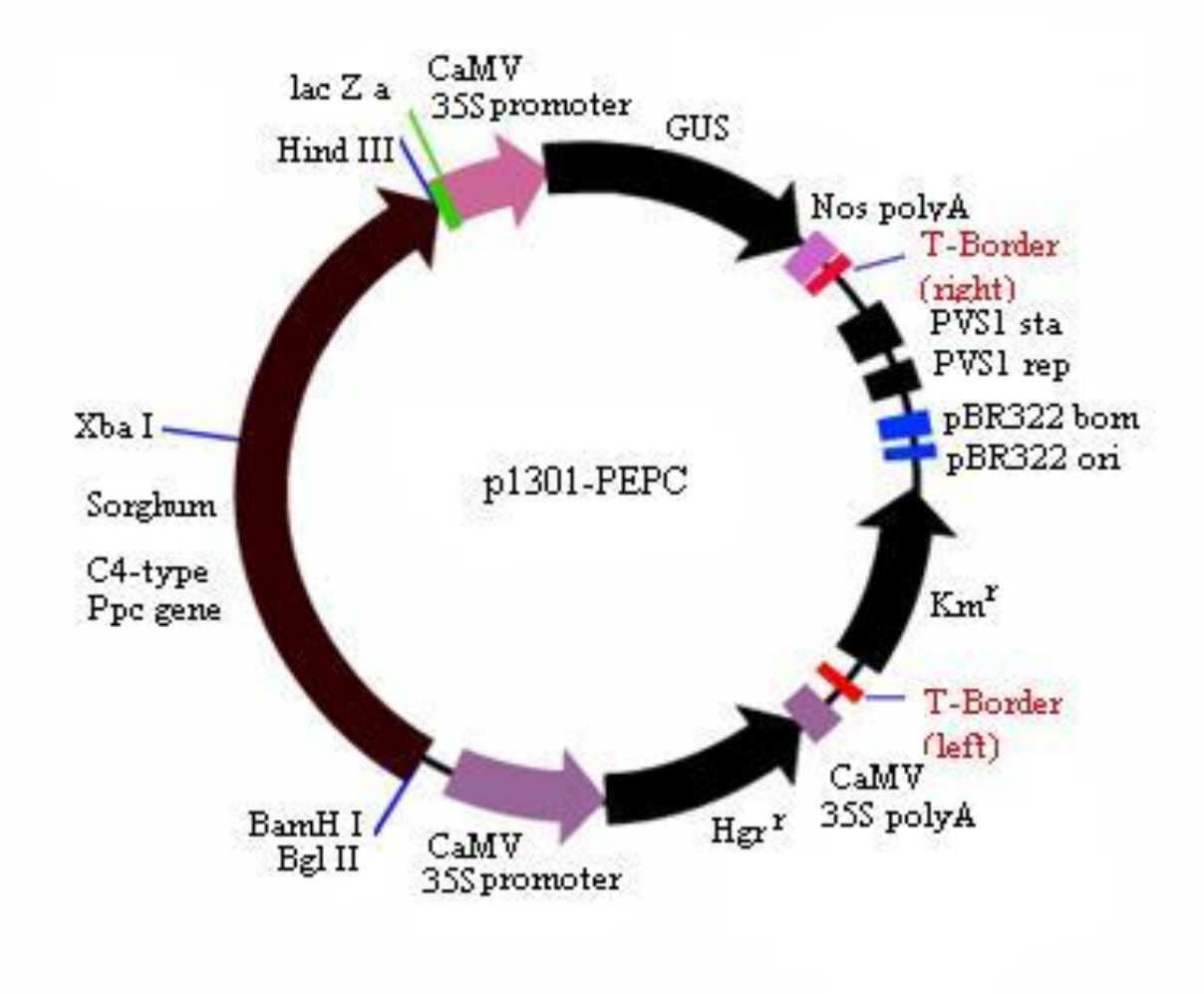


Restriction enzyme with digestion sites combine the two parts into a complete *PEPC* gene, then sequencing. We made a comparison of sequence homology which sequence of sorghum C4 type *PEPC* gene was published in Gene Bank.

Nucleotide homology compared to the reported one achieved 97.9%, and amino acid homology reached 99.2%.

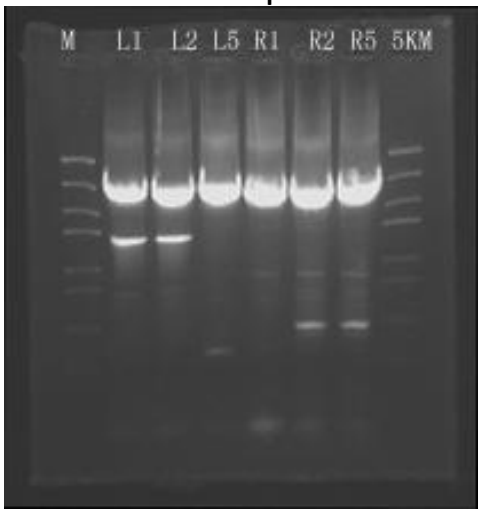
There are exactly the same sequence of nucleotide sequences which exon are 1-7, there are 8 bp difference in exon 8, but it doesn't affect amino acids encoding, and there are several bp difference in starting zone.

1.4 Vector Construction



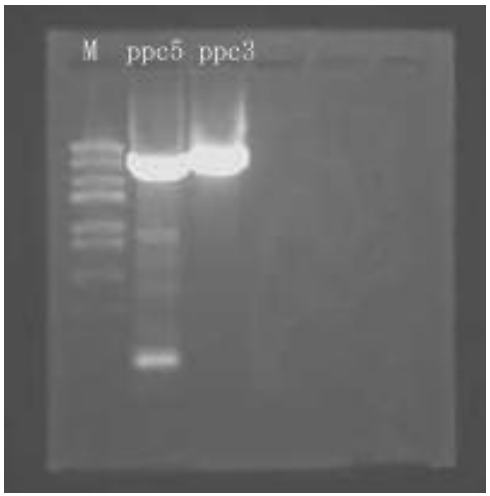
1.5 Expression Vector Detection in *Agrobacterium-tumefacien* Strain by PCR

Transforming the expression vector into Agrobacterium strain EHA105 by CaCl2 method. The extracted plasmid was digested by restriction enzyme and the transduction of plasmid was confirmed by PCR.



Transforming identification of *Agrobacterium*

L1,L2,L5 amplifying 3.4kb
R1,R2,R5 amplifying 3.6kb



Transforming identification of *Escherichia coli*

1 Marker, 2 PEPC5 , 3 PEPC3

2. Establishment of High Efficient Genetic Transformation System of Soybean

2.1 Screening Genotype

Soybean cultivars (*Glycine max*) as donor: Heinong35, Heinong41, Heinong58

Basic medium : MSB

Explants: cotyledon node

1.0 mg·L-16-BA MSB growing 5~6d

Shoot tip :

Adding 3.5 mg·L-16-BA MSB growing with light 24 h

germinating rate = germinating cotyledon No./No. of cotyledon inoculated x100%
Elongation rate= bud elongation No./shoots No. x100%
Culturing temperature:(26±1)℃ ,
Light intensity: 40 μmol·m-2·s-1 ,
Light/dark cycle: 16 h /8 h.

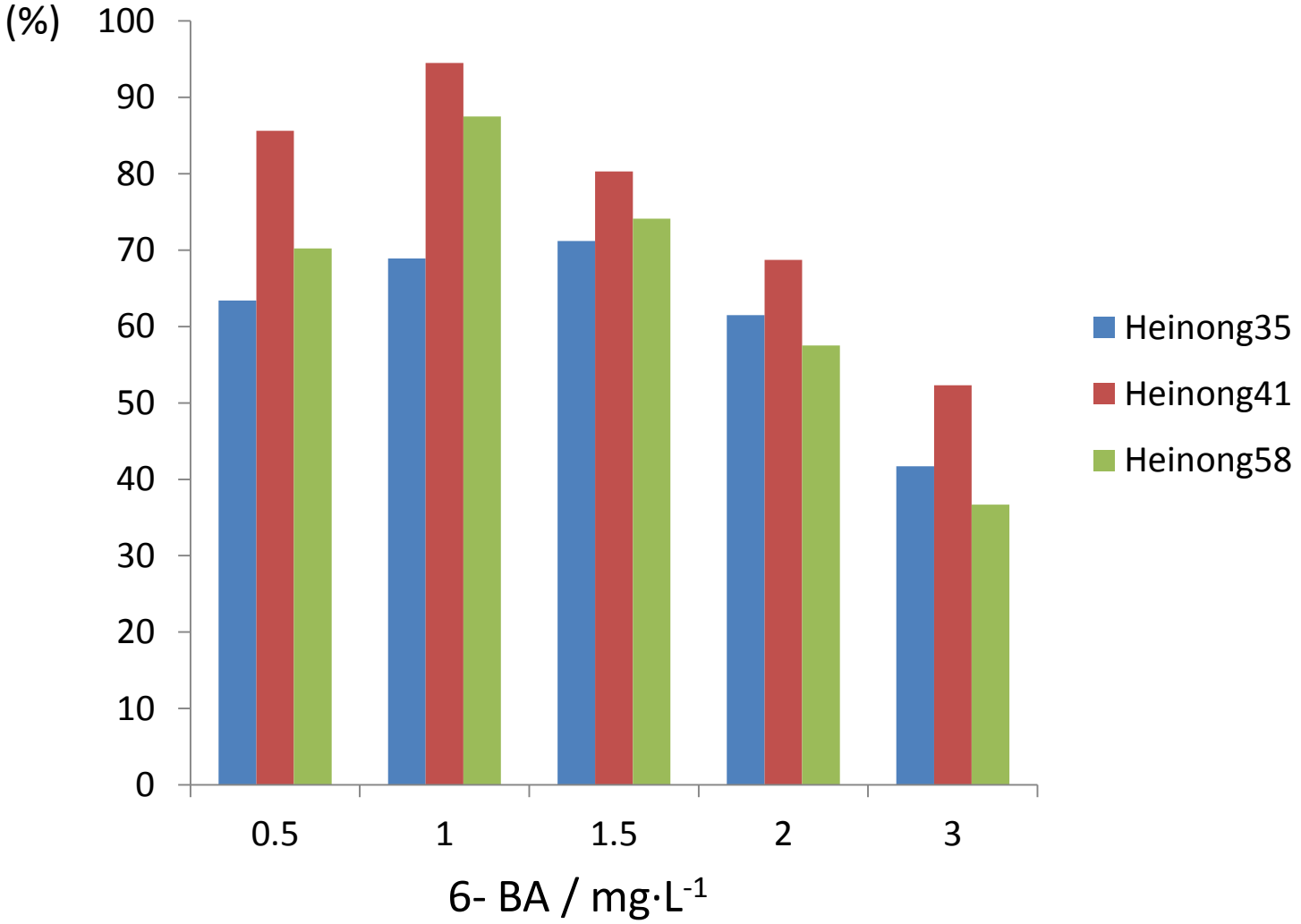


Fig.1 Effects of different concentrations of 6-BA on the shoot regeneration frequencies of soybean cotyledonary node

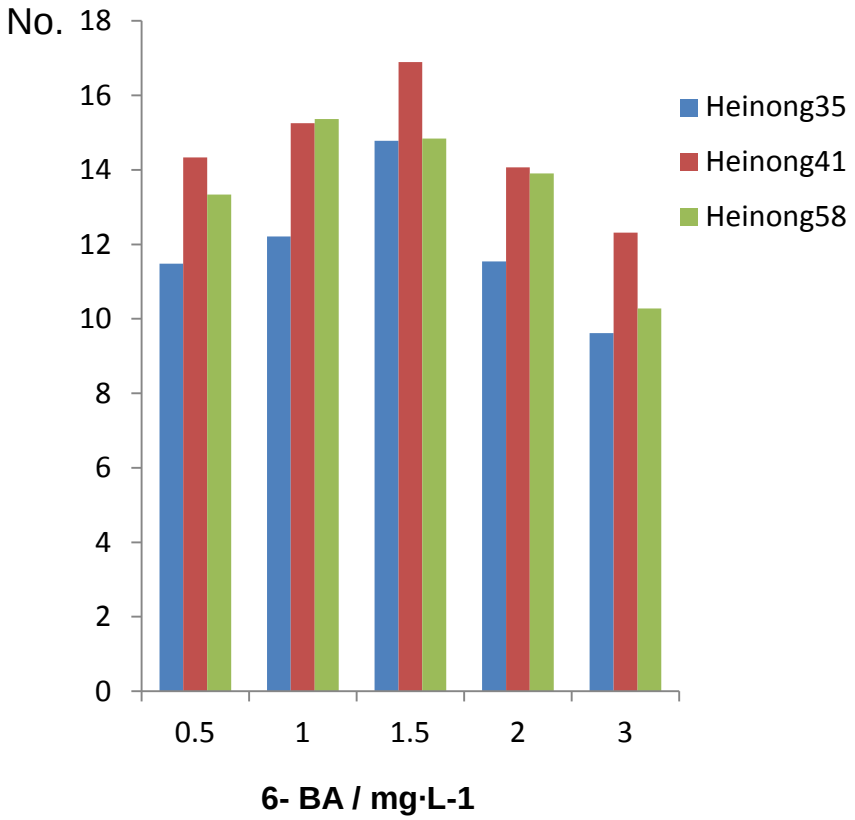


Fig.2 Effects on shoot regeneration No. of soybean cotyledonary node

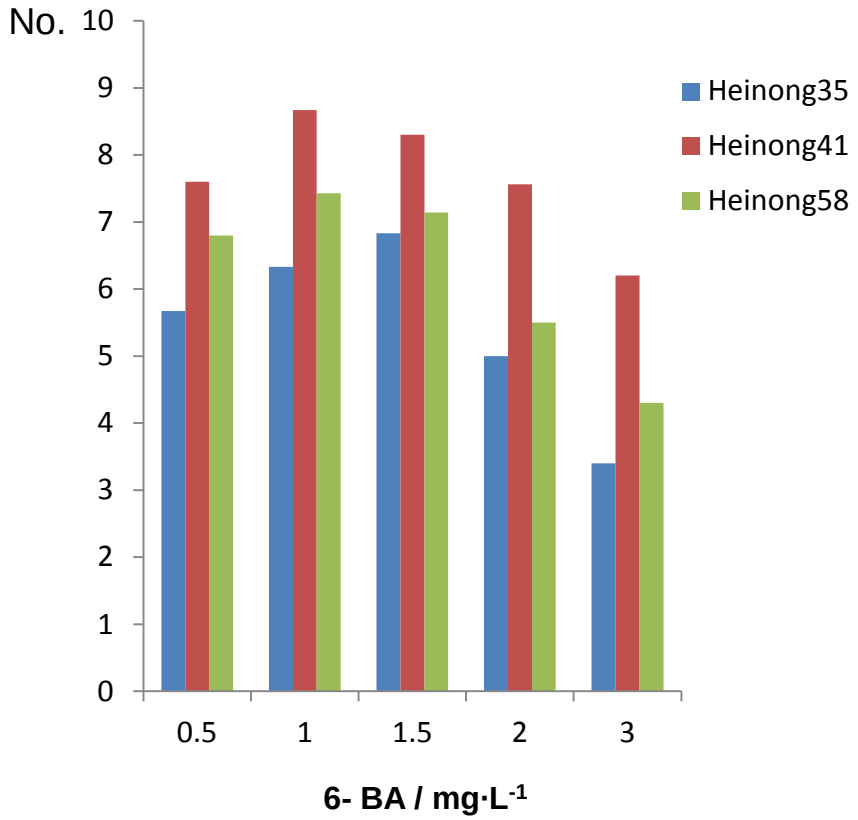
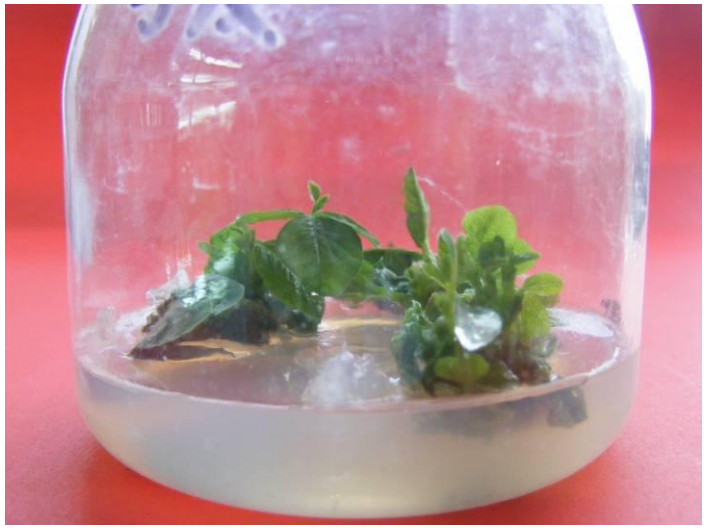


Fig.3 Effects on shoot elongation No. of soybean cotyledonary node



Cotyledon regeneration system of Heinong 41

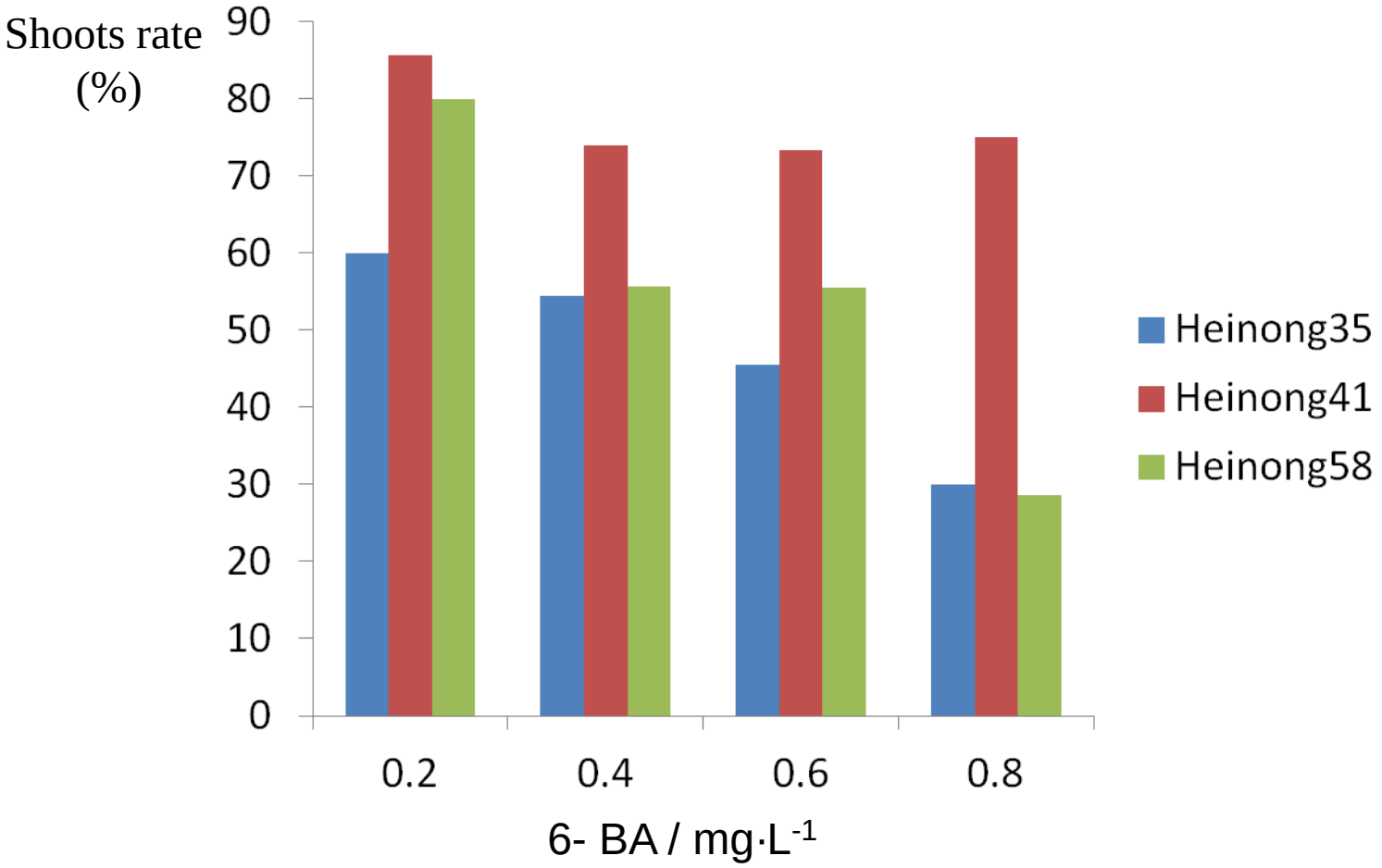


Fig.4 Effects of different concentrations of 6-BA on the shoot regeneration frequencies of soybean embryonic tip

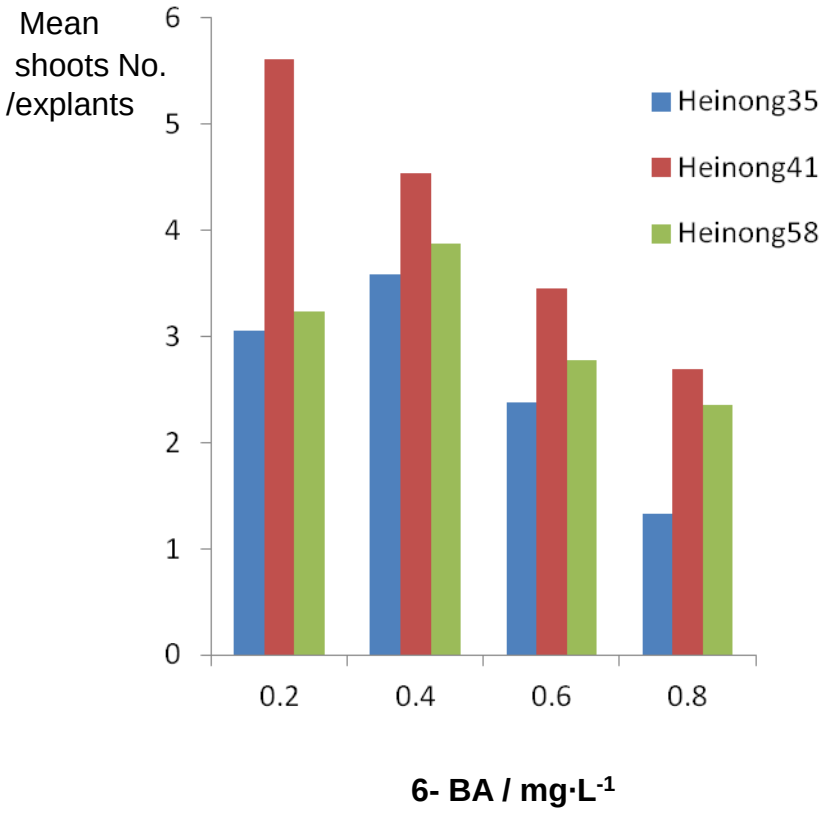


Fig.5 Effects on shoot regeneration No. per explants of soybean embryonic tip

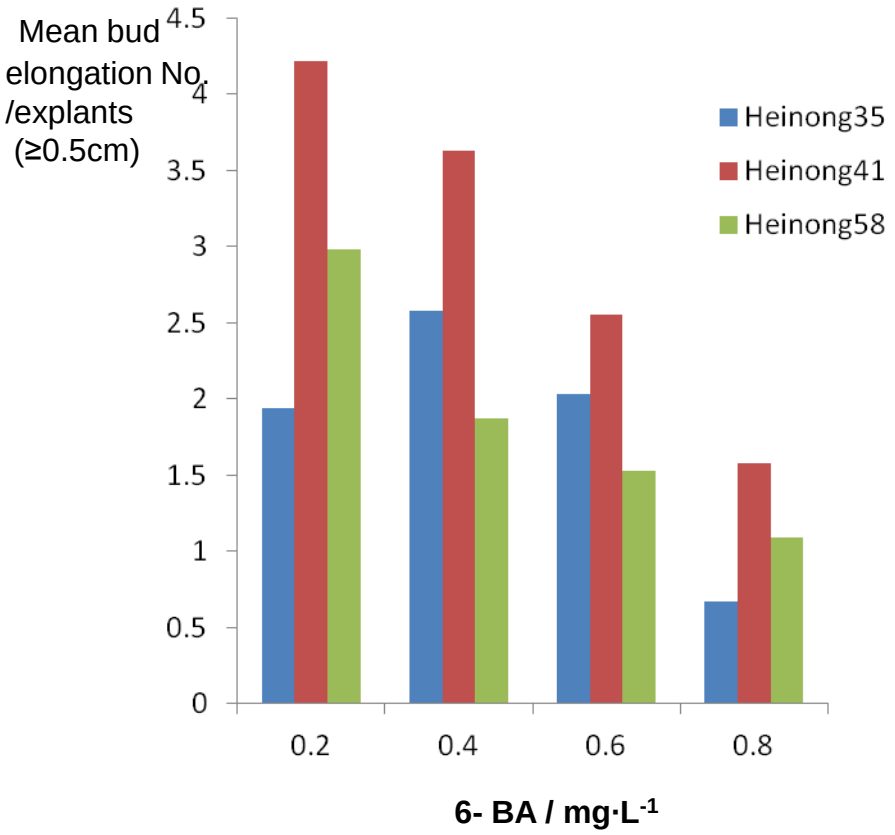


Fig.6 Effects on shoot elongation No. per explants of soybean embryonic tip

Embryonic tip regeneration system of Heinong 41

Table.1 Comparison on two kinds of regeneration system between cotyledon and embryonic tip of Heinong41

Explants	Shoots rate (%)	Mean shoots No. /explants	Mean bud elongation No. /explants (≥0.5cm)	Elongation rate（%）
Cotyledon	94.5	15.25	8.67	56.8
Embryonic tip	85.7	5.61	4.22	75.2

2.2 Explants Screening

Material:Heinong41
Explants: Mature cotyledonary node, immature cotyledonary node, Hypocotyl, Mature embryo tip.
Transformation: Agrobacterium infection

2.2.1 Mature cotyledonary node:

Taking the sterile seedling of planting 4-5d, removed the buds, made the cut in the cotyledons and cotyledon node , disseminated 1hr, co-cultured in 20mg/L.Hyg for 5d,

Plant Physiology Communications, 2010, Zhang Y et al

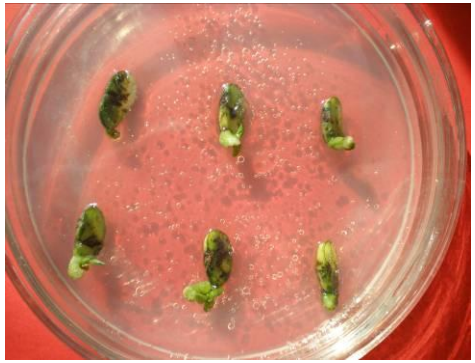
culture medium for selecting :MSB+1mg/LBA+0.2mg/L IBA,
that for rooting : 1/2MSB+1.5mg/LIBA




The traditional cotyledon node
15-20mg/L Hyg

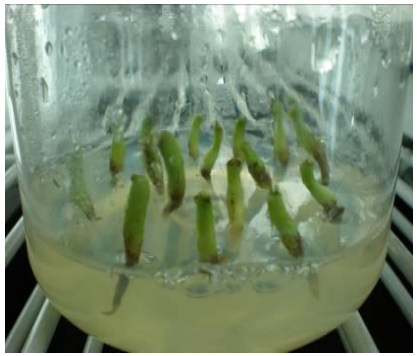
2.2.2 Immature cotyledonary node:

Taking immature pods, 75% alcohol disinfection 1min, mercuric chloride,20min, removed beans, peeling, and took a little part of immature cotyledon and Hypocotyl, removed the buds, disseminated 1hr, co-cultured in 20mg/L.Hyg for 5d, the basic medium for selecting was MSB+1.5mg/LBA, after 4 weeks selecting, continued selecting for one to two week in MSB+0.5-0.8mg/LBA, then turned into 1/2MSB+1.5mg/LIBA for rooting.

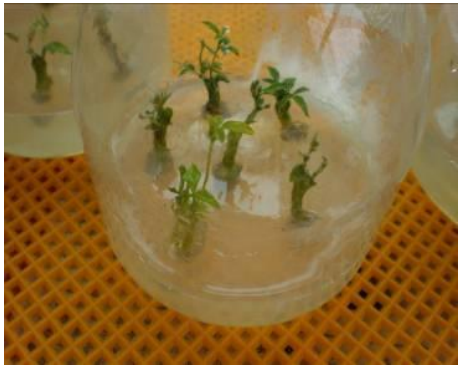


2.2.3 Mature Embryo Tip: taking embryonic tip upward, MSB+3.5mg/LBA light pre-culture 24hr, 1/2MSB+6mg/L6-BA+100μ M AS in 28 ℃ oscillating culture 20hr, 5d co-culture, turn into recovery medium MSB+0.2mg/LBA+0.2mg/LIBA, 20mg/LHyg for selecting, subculture one time every 10d.




The adventitious bud is very weak with this methods, it is relatively difficult to obtain strong seedlings, thus affecting the survival rate of transgenic plants.

2.2.4 Hypocotyl: 4-5d seedlings of aseptic seeding, removed two cotyledons and leaf buds, hypocotyl was cut into about 0.5cm, 1hr, infection, co-cultured for 5d, 20mg/L.Hyg for selecting, selecting medium: MSB+0.1mg/L TDZ+0.2mg/L IBA. 1 month later, turned into 1/2MSB+3.0mg/LIBA for rooting.



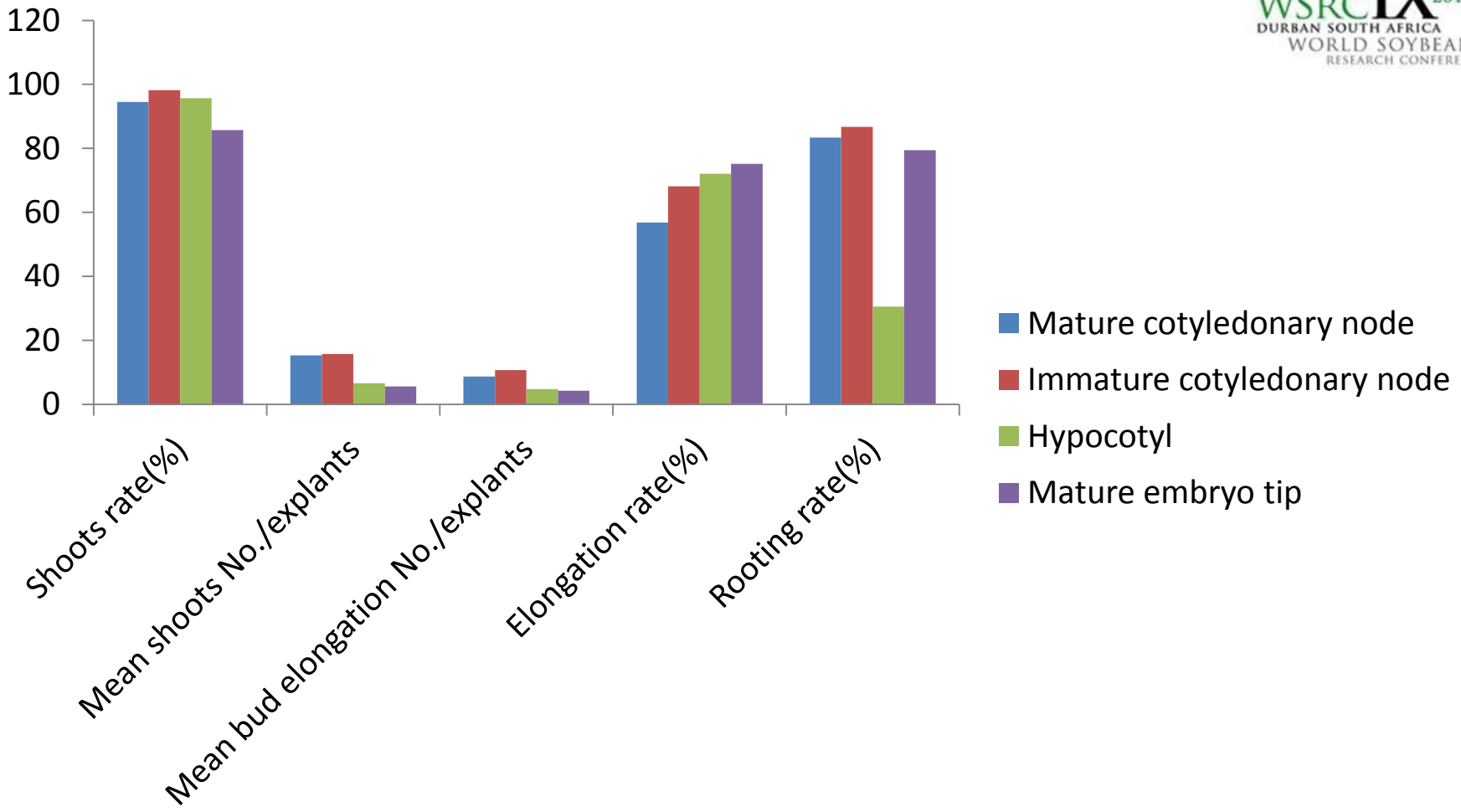


Fig.7 Comparison on regeneration systems in different explants of Heinong41

2.3 Transformation Method

Material: Immature cotyledonary node of Heinong41

Table.2 Effects of antagonistic buds induction in different transformation methods

Treat	Explants No.	No. of elongated bud with resistance	Resistant plant	Positive plant in PCR	Transformation rate(%)
Agrobacterium infection	300	253	25	3	1
Particle bambadment + Agrobacterium infection	300	249	27	5	1.7

2.4 Optimal Hygromycin Concentrations

Table.3-1 Effects of Hygrimycin concentrations on buds induction and the elongation of Heinong 41 shoots

Concentration	Hyg.(mg/L)					
	0	10	20	30	40	50
Induction bud(%)	15.73	3.76	1.80	0.5	0	0
Bud elongation(%)	10.71	0.66	0	0	0	0

Table.3-2 Effects of Hygrimycin concentrations on buds induction and the elongation of Heinong 41 shoots

Concentration	Hyg.(mg/L)						
	0	5	10	15	20	25	30
Induction bud(%)	15.73	8.76	3.76	2.54	1.87	1.02	0.5
Bud elongation(%)	10.71	2.08	0.66	0	0	0	0

Table.4 Effects of Hygromycin concentrations on rooting of Heinong 41 plants

	Hyg.(mg/L)										
Concentration	0	1	2	3	4	5	6	7	8	9	10
Main root No.	4	4	4	3	3	3	3	3	2	2	2
Root colour	white	white	white	light brown	light brown	light brown	light brown	brown	brown	brown	brown

Summary

1. **Cluster bud elongation rate**: mature cotyledonary node (56.8%) < immature cotyledonary node (68.1%)< hypocotyl (72.1%) < Embryonic Tip (75.2%).
This is because most of the buds of cotyledonary node are easy to become a termination although there are many buds, so bud elongation rate of cotyledonary node is only 56.8%-68.1%. on the contrary, although the budding number is not high for hypocotyl and embryonic tip, the elongation rate is very higher, reaching 72.1% and 75.2%, and grew more uniformly.
2. **Rooting rate**: immature cotyledonary node (86.7%) > mature cotyledonary node (83.4%) ,mature embryo tip (79.4%) > hypocotyl (30.5%).
Hypocotyl rooting is not good, need high IBA, root is easy to blackening, survival rate is very low after transplanting.
3. **Cluster bud occurring time**: cotyledonary node explants needs 5~6 d's growth after aseptic seeding, while embryonic tip and immature cotyledonary node need a short time.
4. **Transformation method**: particle bambadment + Agrobacterium infection
5. **Hyg concentration :** adventitious buds for 15-20mg/L, rooting for 3-5mg/L.
It can be summarized that immature cotyledonary node has a better potential than the other three methods for soybean transformation because of its basic conditions of highly efficient regeneration, so immature cotyledonary node can regard as the explants of effective genetic transformation in soybean.

3. Detection of Transgenic Generations

3.1 Conditions on Molecular Detection of Transgenic Generations

5×PCR Buffer	10μl
dNTPs	4μl
P1	1μl
P2	1μl
DNA(100ng/μl)	1μl
PrimeSTAR^{TM}HS(2.5u/μl)	0.5μl
ddH_2O	32.5μl
	50μl

Prime STAR HS kit

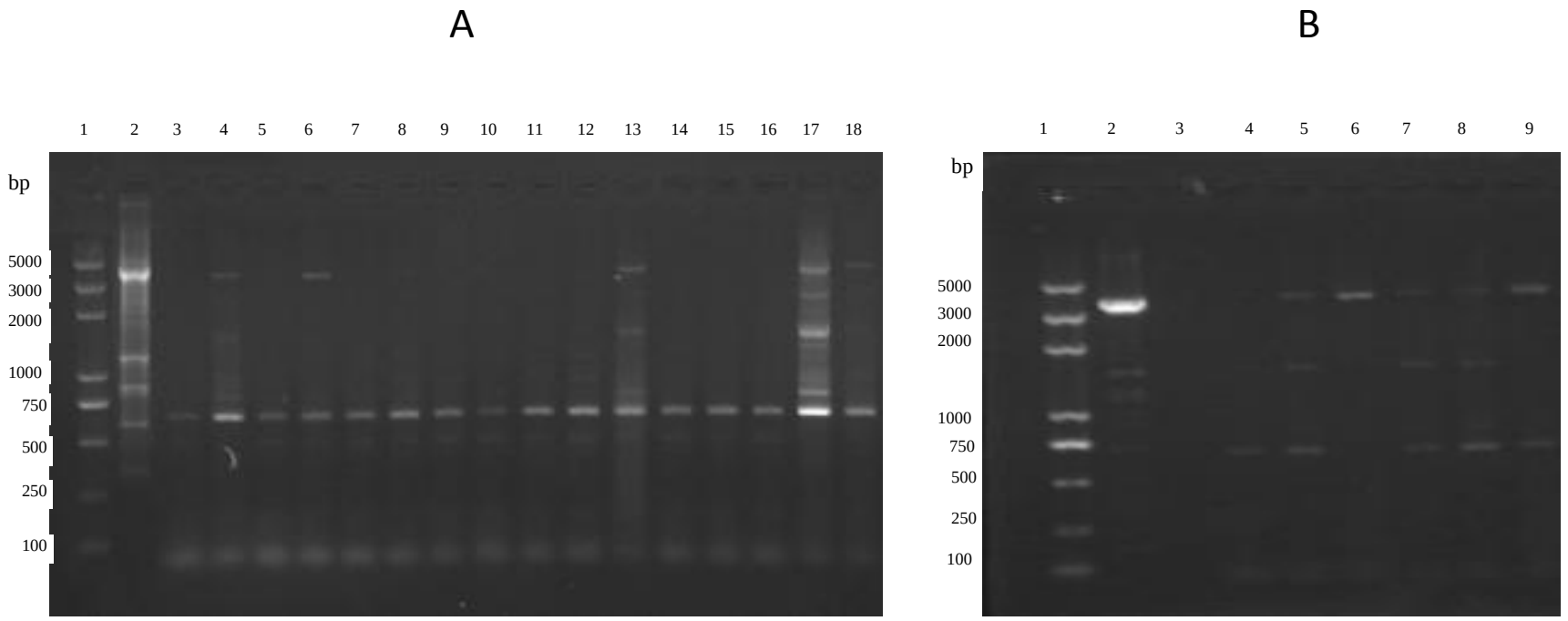
94℃ 1min

98℃ 10sec
68℃ 3min

72℃ 10min

35cycles

3.2 PCR Molecular Detection of T0 Transgenic Plant



Fig·8 PCR analysis of transgenic soybean

A: Result of PCR Amplifying with 5'-end primers of *PEPC* gene

marker; 2.positve check:(plasmid p1301-*PEPC*); 3.negative check(non-transgenic plants); 4,6,13,17,18. transgenic plants; 5,7~12,14~16. non-transgenic plants

B: Result of PCR Amplifying with 3'-end primers of *PEPC* gene

1.marker; 2.positve check:(plasmid p1301-PEPC); 3.H_2O 4.negative check(non-transgenic plants); 5~9 transgenic plants

3.3 Agronomic Traits and Physiological Parameters of Transgenic Soybean in T0 Generation

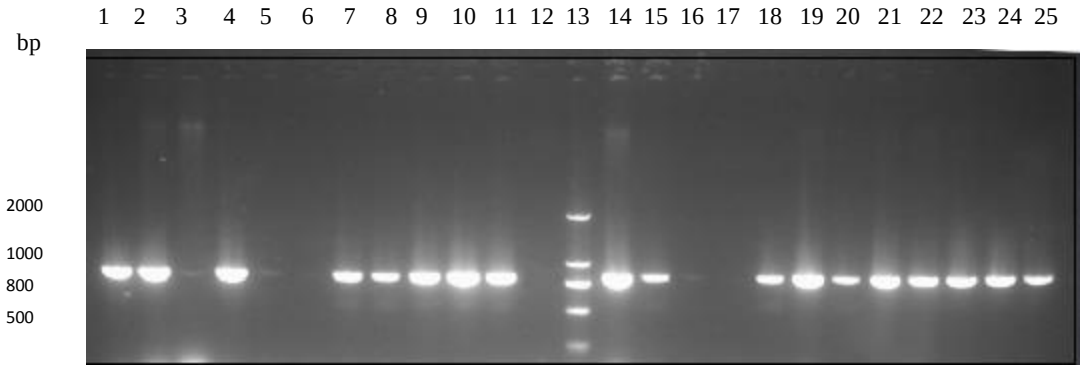
Table.5 Agronomic performance of transgenic plant in T0

Material	Pod number			Seed No.
	1-seed pod	2-seeds pod	3-seeds pod	
T0-1	1	2	2	11
T0-3	6	3	1	15
T0-10	2	2	0	6
T0-14	0	0	0	0
T0-15	7	1	1	12

Table.6 Photo-physiological parameters of T0 transgenic soybean

Material	Photo (μmol $CO_2.m^{-2}\cdot s^{-1}$)	Cond (μmol.$m^{-2}\cdot s^{-1}$)	Ci CO_2(ppm)	Tr ($g\cdot m^{-2}\cdot s^{-1}$)
T0-1	20.2	1.42	269	9.07
T0-3	24.8	1.51	260	9.28
T0-10	20.1	1.36	270	9.05
T0-14	25.7	1.58	257	9.38
T0-15	23.9	1.49	263	9.13
Control	18.7	0.93	275	8.91

3.4 PCR Detection of T1 Transgenic Soybean (5’-end primer)



Fig·2 PCR analysis of T1 transgenic soybean

13.marker; 11.positve check:(plasmid p1301-PEPC); 12.H_2O; others: transgenic plants

Amplified by PCR with 5’-end primers of 44 T1 plants, resulted as follow: 32 plants showed positive, 12 plants showed the negative, the proportion was near 3:1 which fitted for Mendel's laws of inheritance.

3.5 PCR Detection of T1 Transgenic Soybean (full length gene)

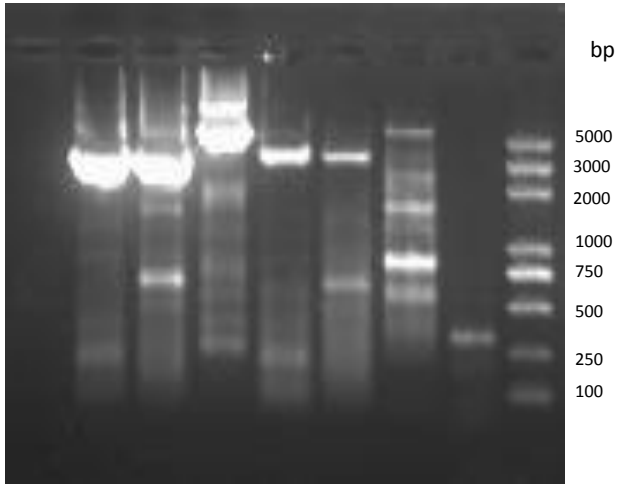


Fig.10 PCR analysis with full length gene of T1 transgenic soybean

1.positive check(PCR amplifying with 5'-end primers); 2. positive check(PCR amplifying with 3'-end primers); 3. positive check(PCR amplifying with full length gene); 4.transgenic plant(PCR amplifying with 5'-end primers); 5.transgenic plant(PCR amplifying with 3'-end primers); 6. transgenic plant(PCR amplifying with full length gene); 7.negative check(non-transgenic plant); 8.marker

3.6 PCR-Southern Blot Detection of T1 Transgenic Soybean

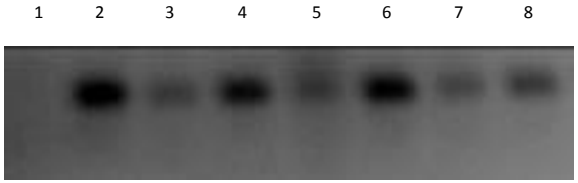


Fig.11 PCR southern blot of transgenic soybean in T1

1.negative check(non-transgenic plant); 2. positive check:(plasmid p1301-*PEPC*); 3~8. transgenic plants

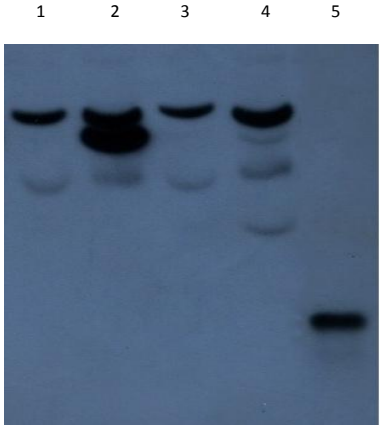


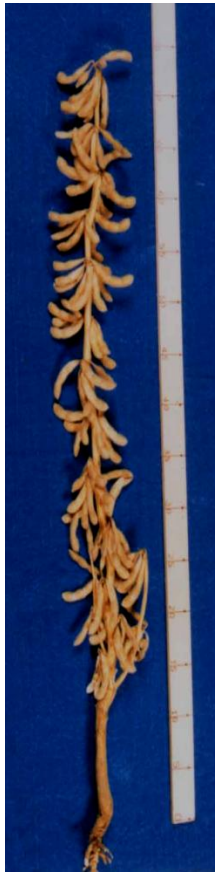
Fig.12 Southern blot of transgenic soybean in T1

1~4. transgenic plants T1-1-2、T1-3-3、T1-10-4、T1-15-11; 2. positive check:(plasmid p1301-*PEPC*)

3.7 Agronomic Traits and Physiological Parameters of Transgenic Soybean in T1 Generation

Table.7 Agronomic performance of T1 transgenic plant in mature stage

Material	Plant height (cm)	Bottom pod height (cm)	Branch No.	Node No.	Pod No./ plant	100seeds weight(g)	Yield/ plant(g)
T1-1-2	85	13	0.1	18	65	20.1	12.7
T1-3-3	86	13	0.2	18	69	20.3	14.0
T1-3-9	86	14	0.2	18	68	20.2	13.9
T1-10-4	84	12	0.1	18	60	20.1	12.1
T1-15-6	85	12	0.2	18	66	20.1	13.3
T1-15-11	86	14	0.2	18	68	20.2	13.8
Control	84	13	0	18	55	20.0	11.0

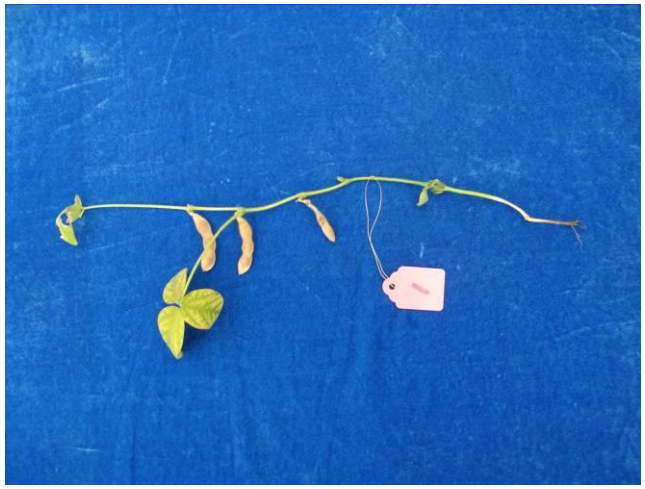


3.7 Agronomic Traits and Physiological Parameters of Transgenic Soybean in T1 Generation

Pn,LI-6400，time: 10:00, condition：CO2 400μmol·mol-1，25℃，light 1500μmol (light quantum)·m-2·s-1，stage:R5, section: the middle leaflet of trifoliolate of the second to last in main stem

Table.8 Photo-physiological parameters of T1 transgenic soybean

Material	Photo ($\mu mol\ CO_2.m^{-2}\cdot s^{-1}$)	Cond ($\mu mol.m^{-2}\cdot s^{-1}$)	Ci CO_2(ppm)	Tr ($g\cdot m^{-2}\cdot s^{-1}$)
T1-1-2	24.1	0.89	255	9.05
T1-3-3	27.4	1.09	248	9.32
T1-3-9	26.8	1.02	250	9.26
T1-10-4	24.2	1.00	260	9.07
T1-15-6	26.5	1.01	254	9.19
T1-15-11	26.7	1.05	252	9.29
Control	22.7	0.86	269	8.91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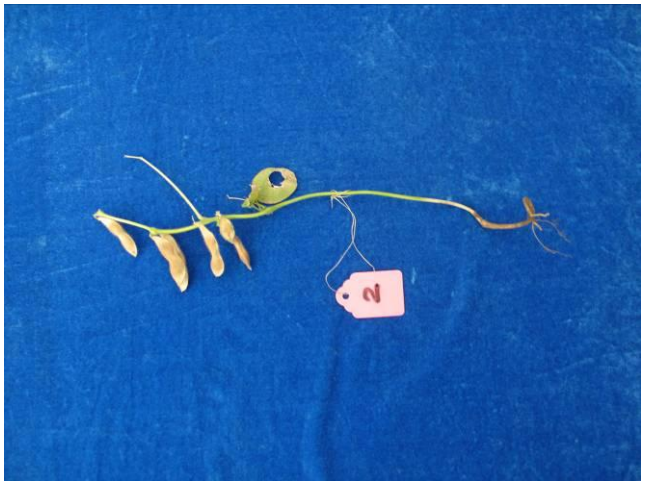
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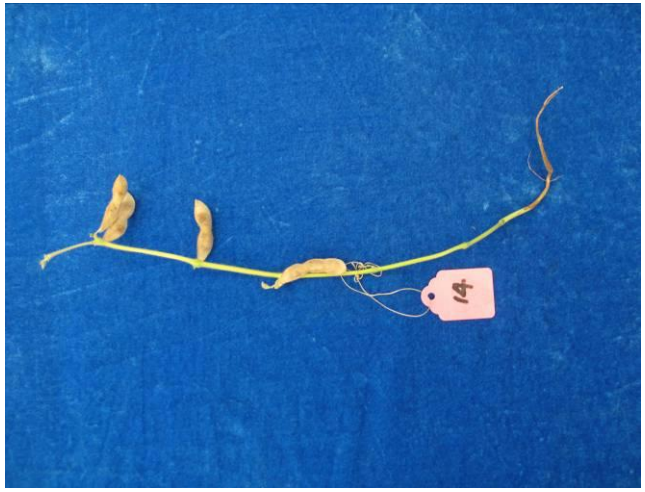
14


24

Summary

Trans-*PEPC* Gene

In the T0 generation of transgenic plants, photosynthetic rate increased in different degrees, from 7.5% to 37.4%. Among the transgenic plants, photosynthetic rate of T0-14 is the highest, probably because of its highly sterility. The photosynthetic rate of T0-3, T0-15 were increased by 32.6% and 27.8%. Other photosynthetic physiological parameters were also produced corresponding change.

Photosynthetic rate of T1 generation of transgenic soybean reached on average of 25.95 μmol $CO_2/m^2 \cdot s$ in seed filling stage, increased by 14.3% compared to the control, the range of the increase was from 6.2% to 20.7%. At the same time, stomatal conductance and transpiration rate increased, and intercellular CO2 concentration decreased.

Conclusion

1. Through screening of soybean genotypes, we determined that Heinong 41 is the best transgenic donor material.

2. The optimized transformation system was: particle bambadment + *Agrobacterium* infection by using immature cotyledonary node of Heinong 41 as explants.

3. During the elongation after adventitious bud induction, reducing the concentration of BA to 0.6 mg/l or less will be benifitial for enhancing rooting rate of transgenic plants.

4. Optimal Hyg concentration were identified, induction of adventitious buds is 15-20mg/L, while rooting is 3-5mg/L.

5. Photosynthetic efficiency of transgenic plant were improved compared to control plant. The range of increase was from 7.5% to 37.4%.

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