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Full Length Research Paper

Cloning of low-temperature induced gene from *Morus mongolica* C.K. Schn and its transformation into *Petunia hybrida* Vilm

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Mongol mulberry is one of the wild species of genus *Morus*, and mainly grows in cold regions. In this study, stem cuttings of Mongol mulberry were acclimated at 0°C for 48 h after germination. RNA was extracted form the stem and was reverse-transcribed into cDNA. Mulberry low-temperature induced gene WAP25 was then cloned by means of RT-PCR technique. The cloned gene has been submitted to GenBank (Accession N0. DQ104333). Analysis indicated that the WAP25 is 681 bp in length, encoding 226 amino acids. It is also confirmed that the product encoded by WAP25 is a member of late embryogenesis abundant protein (LEA) family. DNA fragment of WAP25 was cloned into plasmid pIG121-Hm to generate plant expression vector pIG121/*Wap*25. pIG121/*Wap*25 was then transformed into leaf discs of *Petunia hybrida* Vilm mediated by *Agrobacterium* to obtain 34 transformants, 11.48% of which are positive by the detection of PCR-Southern assay. The results provide theoretical basis for research of genetic improvement in cold resistance of mulberry and low temperature response mechanism of woody plants.

Key words: Mulberry, low-temperature-inducible gene, *Petunia hybrida* Vilm, transformation.

INTRODUCTION

Low-temperature resistance of plants originates form their intrinsic genetic basis via long-term genetic variation and natural selection. During the response process to low temperature, plants can adjust their physiological and biochemical processes such as the levels of carbohydrates, soluble proteins, free amino acids and endogenous hormones to alleviate the adverse effects of low-temperature stress. Therefore, elucidation of the mechanism of cold acclimation is a key point to understand the genetic basis of cold resistance in plants. Low-temperature induced proteins of plants were first discovered by Pomeroy (1971), who discovered 2 - 3 new proteins from over-wintering black locust trees. In the succeeding years, many researchers have devoted themselves to comparing proteins from different species under different temperatures, and they gradually realize that some proteins are relevant with low-temperature inducement and cold resistance.

Yoseida and Kato (1994) proposed that many plant species can increase their cold resistance by activating transcription of specific genes and synthesis of new proteins. Guy et al. (1985) first reported changes of gene expression and synthesis of some specific mRNA in spinach during cold acclimation. Thereafter, cloning of cold-regulated genes as well as their expression regulation has been hot spots in cold adversity of plants. In resent years, cold-regulated genes have been cloned from Arabidopsis, clover, wheat, barley, spinach, etc, and their structure and function were analyzed (Hughes and Dunn, 1996). The structure of most proteins encoded by these genes are similar to late embryogenesis abundant protein (LEA) (Thomashow, 1999). LEA protein was first detected from cotton embryos during maturation of seeds

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(Dure, 1981). LEA proteins were also found in the embryos of tomato, soybean, carrot, arabidopsis and other plant species, and is relative with resistance to dehydration of the seeds (Bewley and Black, 1994). Their expression, in response to osmotic stress and abscisic acid (ABA), could protect vegetative tissues from damage by dehydration (Ingram and Bartels, 1996). During cold acclimation, other proteins of LEA family also accumulate in many plant species, including herbaceous and woody plants (Arora and Wisniewski, 1994). While in the process of freezing around cells which can lead to cell dehydration, various protein families related with LEA will be generated, suggesting LEA family proteins may play role in resistance of plant cells under condition of freezing (Gilmour et al., 2000). Mongol mulberry is a considerably fri-gostable mulberry species (Zhang et al., 2004), thus it is very valuable to discover low-temperature resistance gene resources from it.

In present study, a gene related to low-temperature response of mulberry (*Wap*25) was cloned from Mongol mulberry, a type of wild species of genus *Morus* in cold regions. Then *Wap*25 gene was genetically transformed into leaf discs of *Petunia hybrida* Vilm mediated by *Agrobacterium*, and then was expressed in the plant. We expect to improve cold resistance of *P. hybrida* by means of genetic reformation, in order to make it suitable for growth in the northern part of the country.

MATERIALS AND METHODS

Mulberry resources with cold resistance

Totally thirty-two mulberry species were collected, one of which was selected for the present study. All the species were provided by the Institute of Sericulture of Heilongjiang Province, China.

Plasmids and bacteria strains

Plant expression vector pIG121-Hm was kindly presented by Pro. Mineo Kojima of Shinshu University, Japan; *P. Hybrida* Vilm was preserved in our laboratory.

Comparative experiment of cold resistant species

Annual shoots of 32 mulberry species were sliced into 12 cm stem cuttings, 5 stem cuttings for each species. The cuttings were planted into vermiculite about 5 cm deep, and then maintained at 25℃ and 12 h photoperiod to induce burgeon. When the winter buds grew to about 5 cm in length (8 d), these young shoots were transferred into a low temperature incubator under condition of 8℃ /1d; 3-4℃ /2d; 2℃ /1d; 0℃ /2d; -1℃ /2d; -3℃ /1d in order for the cold acclimation. Select species with living spires under -3℃ /1d.

Sample collection and preparation of total RNA

Graft selected species in bowls of 25 cm in diameter, three branches for each bowl. When the grafting was successful (the height of young plant was above 30 cm), transfer the plants into a low temperature incubator under condition of 8℃ /1d; 3-4℃ /2d; 3℃ /2d in order for the cold acclimation. Collect caulicle of grafting plant (about 2-3 cm) under cold acclimation of 3℃ for RNA extraction.

PCR amplification of target gene

Specific primers, P1 and P2, were designed based on the published literature (Norifumi et al., 2001). For construction of an expression vector, Xba 1 and Sac1 sites were introduced into 5' end of primer P1 and 3' end of primer P2, respectively.

Primer at 5' end (P1): actctagaatggcttgcaagcaacggc
Primer at 3' end (P2): atgagctctcagagctcctcatccctc

Primers were synthesized according to instruction manual for Superscript II, PCR amplification of target gene was performed using cDNA as the template, and amplification products were ligated with pGEM-T.

Sequencing of target fragment and its sequence analysis

Recombinant strains identified as positive by both enzyme digestion and PCR were sent to Shanghai Bioasia Biotech Co. Ltd. for sequencing. The target gene and its products were analyzed at websites such as http://www.ncbi.nlm..nih.gov/BLAST and http://www.ebi.ac.uk/clustalw/index.html.

Construction and identification of plant expression vector

Recombinant plasmids were double digested with *Xba* I and *Sac*I. Then 1% agarose gel electrophoresis was performed and the gel was cut to recover 700 bp fragments. Plant expression vector pIG121-Hm was also double digested with *Xba* I and *Sac* I, then 1% agarose gel electrophoresis was performed and the gel was cut to recover large fragments. These two recovered fragments were ligated using T4 ligase at 16℃ and the products were transformed into *E. coli* TG1 competent cells. The cells were plated on LB solid medium containing Kan (50 μg/mL) and Hm (25 μg/mL) at 37℃ overnight. Separate colony were pick into LB liquid medium containing Kan and Hm and shaken at 200 rpm/37℃ overnight. On the next day transformed DNA was extracted for identification by double digestion. Then *Agrobacterium* LBA4404 was transform with positive clones to obtain engineered Agrobacterium LBA4404/ pIG121/ *Wap*25.

Transformation of leaf discs of *P. hybrida* Vilm with plant expression vector pIG121/ Wap25

Minimal medium: MS + 2.0 μg/Ml, BA + 0.2 μg/mL NAA; Screening medium: MS + 2.0 μg/mL, BA + 0.2 μg/mL, NAA + 50 μg/mL, Kan + 15 μg/mL, Hm + 400 μg/mL Cef-5; Rooting medium: 1/2MS + 0.5 μg/mL NAA + 10 μg/mL Kan + 5 μg/mL Hm + 400 μg/mL Cef-5.

Seeds of *P. hybrida* Vilm were sowed into flower pot. Before blooming, leaves growing well at third to fifth level were selected and cleaned with 1% Tween 20. Thereafter, they were washed gently with sterile water for 30 min, sterilized with 75% alcohol for 1 min and 0.1% corrosive sublimate for 8 min, and washed with sterile water for 5 min. Then half of the leaves were cut near the basis of leaf stalk (0.5 × 0.5 cm) as the recipient materials.

Preparation of agrobacterium infective liquid

Engineered agrobacterium LBA4404/ pIG121/ *Wap*25 was inoculated into YEB liquid medium containing four antibiotics (Kan + Hm + Str + Rif) for shake cultivation at 28℃/220 rpm overnight. On the

Figure 1a. Mulberry buds acclimated at 3℃ for 24 h.

Figure 1b. Mulberry buds acclimated at - 1℃ for 24 h.

next day, the liquid was transferred into YEB liquid medium without antibiotics at the ratio of 1:10 and cultured at 28℃/220 rpm until its OD reached 0.5 (about 6 - 8 h). The culture was centrifuged at 4000 rpm/25℃, and the supernatant discarded. The cells were washed with equal volume of liquid MS and centrifuged once again. The precipitate was suspended with equal volume of liquid MS to obtain *Agrobacterium* infective liquid.

Infection and culture of leaf discs

Explant were soaked with infective liquid for 10 min, then the infective liquid was blotted on the explant surface with sterile filter paper and planted in minimal medium for 48 h at 22℃/dark. After 24 - 96 h of culture, the explant was washed with sterile water containing 500 μg/mL Cef-5 four times. Water drops were bloted and the explant transferred into screening medium at 25 ± 1℃/16 h light/d. When adventitious buds grew to 2 - 4 cm, they were cut from explant and transferred into rooting medium.

PCR detection of Wap25 gene from transformants

Transformants spires were collected to extract their genomic DNA using CTAB method. PCR and Southern identification were performed on genomic DNA from transformants. Wap25 probe preparation and southern detection were conducted according to instruction manual for DIG-High Prime DNA Labeling and Detection Starter Kit II.

RESULTS

Comparison of cold resistance of 32 mulberry species

After accelerating germination of the 32 mulberry species for three days at 25℃, winter buds on the stem cuttings began to sprout. On the ninth day, 3 - 4 leaves developed from each sprouting bud. Stem cuttings of different species after budding grew well at 3℃ (Figure 1a); however five species (Mongol mulberry, Zhaqi 18, Yilan 16, Dragon mulberry 1, Zoo 10) showed resistance advantages at cold stress of 0℃ to some extent, while cold injury occurred in leaves of other twenty-seven species (Figure 1b). Thereafter, the temperature was lowered to -1 ± 1℃ to acclimatize the stem cuttings for 24 h, and buds on cuttings of Mongol mulberry kept well. The temperature was lowered to -3 ± 1℃ to acclimatize the stem cuttings for 12 h; spires of Mongol mulberry began to shrink, with only minority of tassels keeping normal. After 24 h, flower buds also died. Such cold acclimatization tests indicate that cold resistance of Mongol mulberry is stronger than other species.

Acquirement and sequence analysis of gene coding region

A fragment, about 700 bp in length, from cDNA of Mongol mulberry caulicle after low temperature treatment was amplified using RT-PCR method and was designated as RT-700. After purification, this fragment was cloned into pGEM-T vector, and identification by double digestion of restriction enzyme *Xba* I and *Sac* I showed that recombinant plasmid contained 700 bp fragment, indicating WAP25 fragment has been inserted into T-vector. The recombinant plasmid was designated as pGEM/RT-700. Sequencing results of recombinant plasmid pGEM/RT-700 indicate: The cloning fragment is 681 bp in length, with an initiation codon ATG upstream and a termination codon TGA at 3' end, forming a complete open-reading frame (ORF) coding for 226 amino acids (Figure 2). Boldface letters underlined indicate primer sequences, and the arrowhead indicates the putative signal peptide cleavage sites. Boxed sequences indicate 11-mer amino acid motifs common in group 3 LEA proteins (12 repeats).

It is predicted that PI of the protein is 5.32 and its molecular weight is 25.3 kDa based on the amino acid sequence of the deduced protein. Hydrophobic amino acid residues comprise large portion of all the 226 amino acids of the deduced protein, including 35 alanines (A) (15.5%); 31 lysines (K) (13.7%); 29 glutamic acids(E) (12.8%). These three kinds of amino acids comprise 42% of total amino acids (226). The amino acid sequence con-

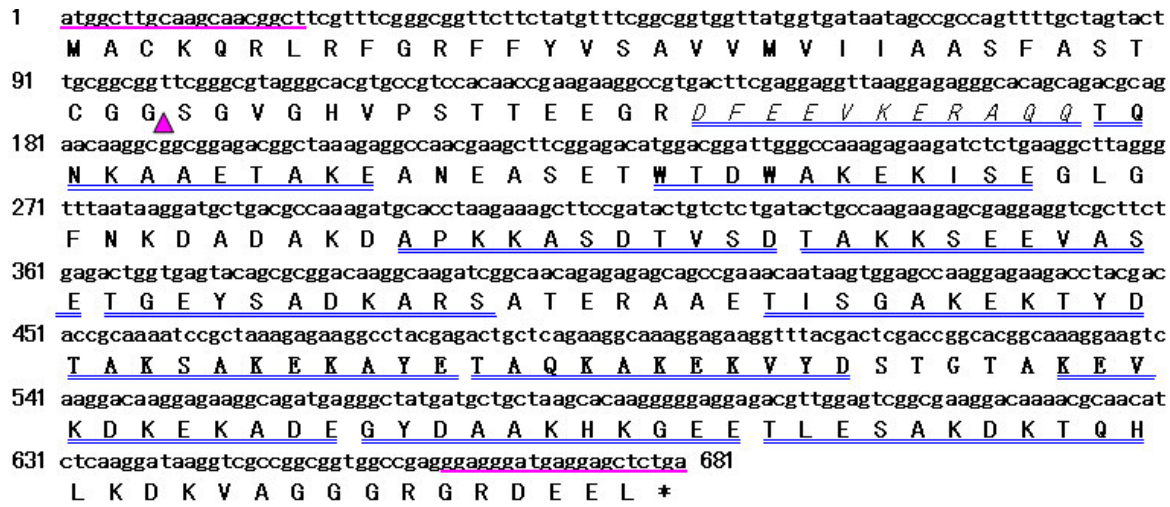

Figure 2. cDNA sequence of mulberry WAP25 gene and its deduced protein. Red letters underlined indicate primer sequences, and the red arrowhead indicates the putative signal peptide cleavage sites. Blue double underlined sequences indicate 11-mer amino acid motifs common in group 3 LEA proteins (12 repeats).

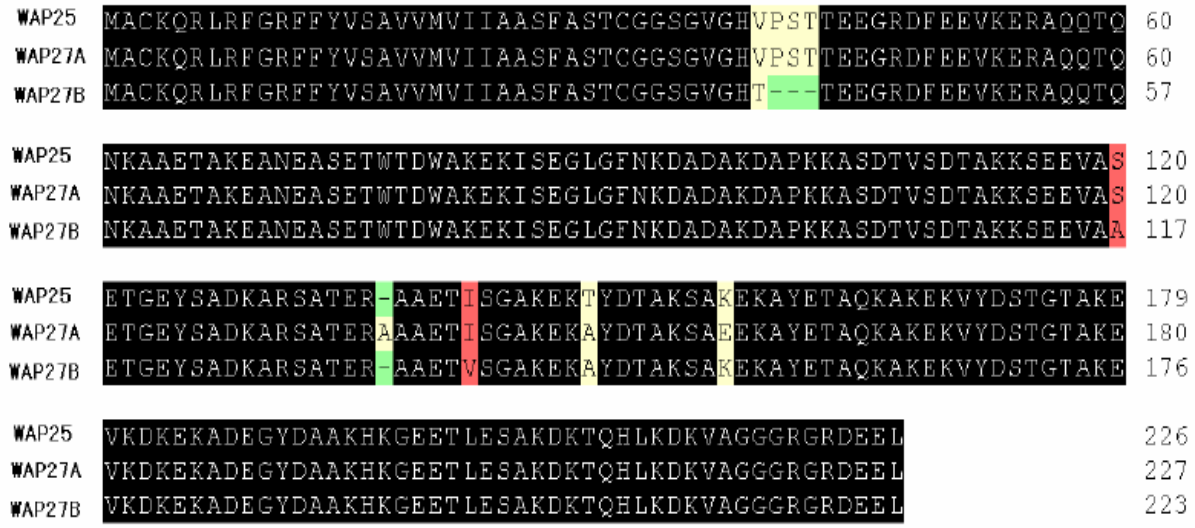

Figure 3. Amino acid sequence comparison between WAP25 and WAP27.

tains 12 repeats of a kind of 11-amino acid motif (T***AKEKA*D/E) which is the common feature of group 3 late-embryogenesis-abundant proteins (LEA3) (Dure et al., 1989; Dure, 1993), implying that expression product of the gene acquired is a member of LEA3 family. Because the protein is likely to be relevant with cold acclimation in winter and its molecular weight is about 25.3 kDa, the protein is designated as WAP25.

Similarity between WAP25 and WAP27

By comparing gene sequence and amino acid sequence of WAP25 and WAP27A we have found: WAP25 gene lacks three bases from position 411 to 413, leading to lack of alanine (A), however with no frame shift in the subsequent sequence; GCC in position 432 is substituted with ACC, leading to substitution of alanine (A) with threonine (T); GAA in position 465 is substituted with AAA, leading to substitution of glutamic acid (E) with lysine (K) (Figure 3). *Wap*25 gene has nine additional continuous bases from position 115 to 123(GTG CCG TCC), leading to addition of proline (P), serine (S) and threonine (T) in the protein. Moreover, bases in six positions has changed, but leading to changes of amino acids in only three positions, because changes in the other three bases belong to same sense mutation (Figure 3). Hence we can propose that *Wap*25 is neither the

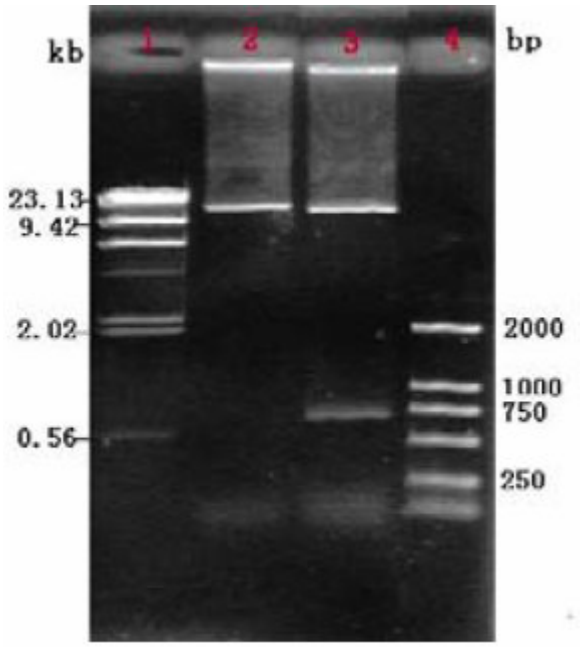

Figure 4. Restriction analysis of plant expression recombinant plasmid pIG121/*Wap*25. Lane 1. Marker (λDNA/*Hin*d III); 2. double-digestion with *Xba* I/*Sac* I; 3. single-digestion with *Xba*I; 4. Marker (DL2000).

same as *WAP27A* nor *WAP27B*. This polymorphism of homologous genes and proteins may be resultant from difference among mulberry species.

Construction and identification of plant expression vector

Recombinant plasmid consisting of plant expression vector (pIG121-Hm) and target fragment (RT-700) was double digested by *Xba* I and *Sac* I, and two bands were seen upon electrophoresis of digestion products, whose lengths were identical with target fragment, while only one band was seen upon single digestion of *Xba* I (Figure 4), indicating *Wap*25 fragment has been inserted into pIG121-Hm in the correct direction. This plasmid was designated as pIG121/*Wap*25.

Transformation of *Agrobacterium* with pIG121/*Wap25*

Plasmid pAL4404 from agrobacterium LBA4404 contains Rif and Str resistance gene, while expression vector pIG121/*Wap*25 contains Kam and Hm resistance gene; so only strains with both pAL4404 and pIG121/*Wap*25 plasmids can grow on plate with Kam/Str/Rif/Hm. Colony on plate with four kinds of the antibiotics (Kan, Hm, Str and Rif) is shown in Figure 5. Thee recombinant plasmid pIG121/*Wap*25 was introduced into *Agrobacterium* LBA4404, which was designated as engineered *Agrobacterium* LBA4404/pIG121/*Wap*25.

Transformation of leaf discs of *P. hybrida* Vilm with Wap25 gene

Leaf discs of *P. hybrida* Vilm infected with engineering *Agrobacterium* LBA4404/pIG121/*Wap*25 began to differentiate buds and generate adventitious buds when cultured on screening medium for 10 - 14 days. While the control region (infected with MS medium) did not differentiate and it began to etiolate and die (Figure 5). Batch, number and regenerating young plants of transformed leaf discs are shown in Table 1. New adventitious buds were continuously screened by transferring them into new screening medium in order to obtain young plants with resistance, while escaping adventitious buds began to etiolate (or go to ablation) and die.

PCR-Southern identification of regenerating plants of transgenic *P. hybrida* Vilm

Totally, 148 leaf discs were treated in two batches of transformation experiments (without CK^-), and 34 young plants with resistance were obtained. Eight of the plants were selected at random and their genomic DNA extracted for PCR identification. The results showed that four of them were positive, with the transformation efficiency of 11.48%.

Electrophoresis and membrane blotting of PCR products was performed, and PCR-Southern detection was carried out with the results shown in Figures 6 and 7. Except for lanes 4, 10, 14 and 15, hybridization signals could be seen in all the other lanes, indicating that PCR products are complementary DNA of *Wap*25. The results also indicate that exogenous gene *Wap*25 has been introduced into *P. hybrida* Vilm.

DISCUSSION

Dure (1993) proposed that LEA3 proteins contain multi-copies of eleven amino acid motif. This unique motif may provide us a clue to understand the functions of WAP25. We compared 12 repeats of the eleven amino acid motif in WAP27 with those in WAP25. It was found that amino acids in position 1, 2, 4, 6 and 8 are usually hydrophobic amino acids, among which high conserved arginine (K) residues with positive charge in positions 4, 6 and 8 can form the hydrophobic surface of an α-helix. Moreover, polar amino acids (E) in positions 7 and 11 can form the hydrophilic surface of the helix. The hydrophobic surface of WAP25 is close to lipid bilayer of cytoplasmic membrane, while its hydrophilic surface is close to the cytoplasm or apoplast. Such a structure is in favor of binding water molecules and some polar ions in cells, reducing the cellular osmotic pressure, thus lowering or

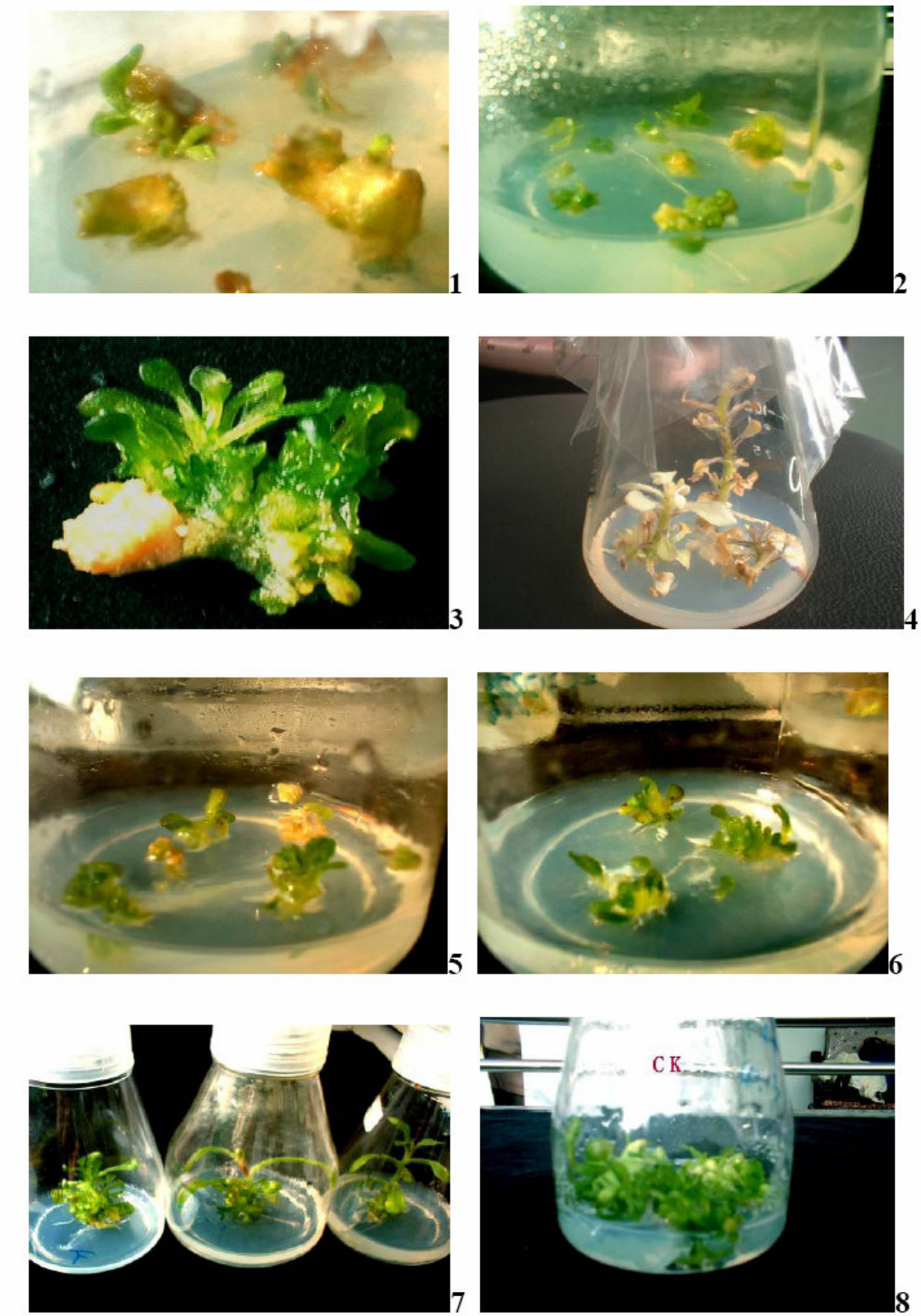

Figure 5. Transformant regeneration of Petunia; 1. Resistance and adventitious buds occurred on the edge of the leaves; 2. Resistance callus formed; 3. Blisk resistance buds differentiate; 4. Non-regeneration yellowing phenomenon; 5. Adventitious buds differentiated from resistance callus; 6. Adventitious buds proliferation in the medium with antibiotics; 7. Growth of the resistance Seedlings; 8. Blisk differentiation in the medium without antibiotics (CK).

Table 1. Transformant of growth and differentiation.

Batch	Preculture time (h)	Explant number (strain)	Green leaf disc number at 30 d (piece)	Differentiation of Leaf disc	Young plants with resistance at 60d (strain)
1	0 (CK$^-$)	8	0	Non	0
	48	52	35	Calli and adventitious buds were generated	19
2	24	32	15	Green islands remained on leaf discs	10
	48	25	10	Green buds were generated on the edge	4
	72	18	9	Green buds which didn't exist on the edge	1
	96	21	0	Non	0

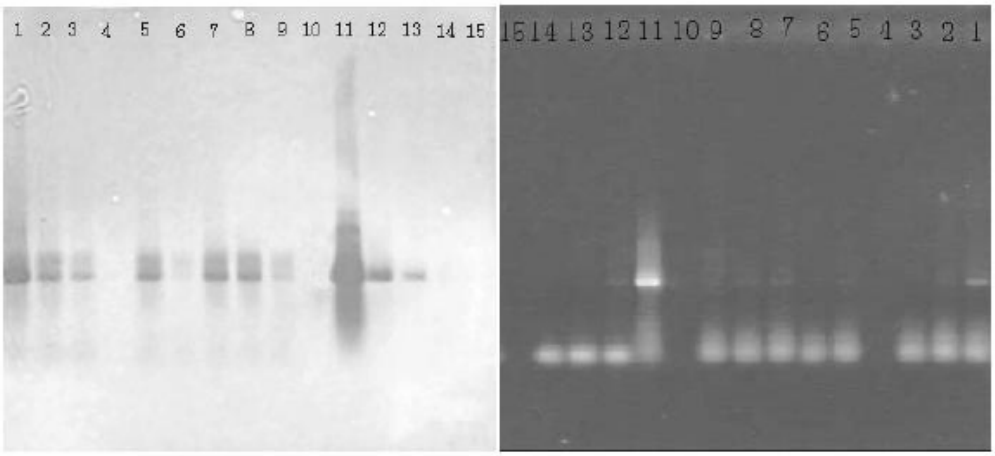

Figure 6. PCR-Southern blotting. Lanes 1 – 3, transformant No 1, 4, 10, 15 Vacant; lanes 5 - 6, transformant No 2; lanes 7 - 9, transformant No 3; lane 11, pIG1/*Wap*25(CK-); lanes 12 - 13, transformant No 4; lane 14, control PCR products (CK-).

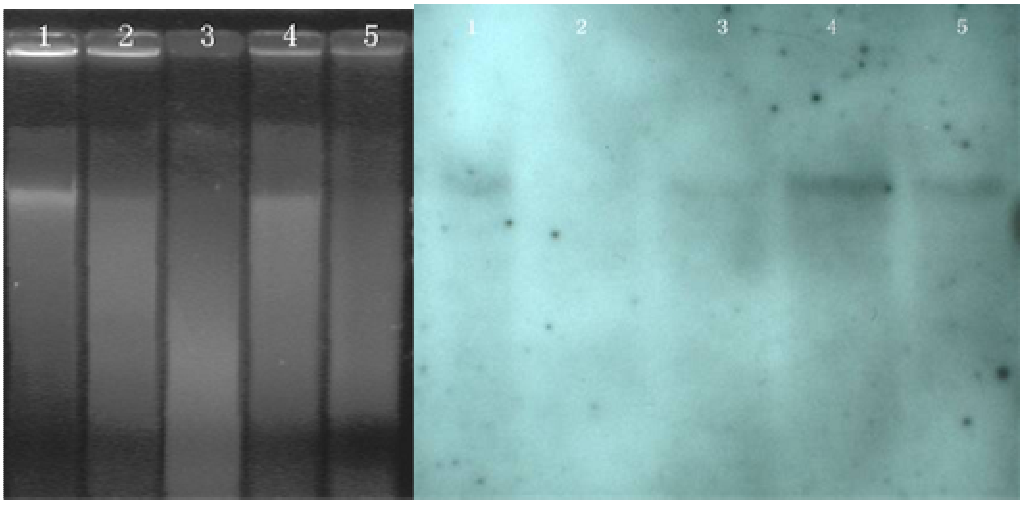

Figure 7. Southern blotting. The figure on the left shows a photograph of a 0.7% agarose gel DNA digested with *Eco*RI and on the right lane is the results of analysis. Lane 1, Transformant No 1; lane 2, control PCR products (CK-); lane 3, transformant No 2; lane 4, transformant No 3; and lane 5, transformant No 4.

clearing concentrated ions caused by cell dehydration when plants experience freezing stress (Ukaji, 2001).

For screening, plant leaf discs infected with engineering bacteria were grown for 24 - 96 h in screening medium. The medium was changed at interval of 4 - 7 days. Green buds appeared from wounds on the edge of leaf discs after 10 - 14 days, and these green buds differentiated into adventitious buds after 28 - 35 days. Meanwhile, adventitious buds also appeared on the surface of leaf vein. The obtained buds with resistance kept green on screening medium, while untransformed leaf discs etiolated partially or totally. The green components remaining in leaf discs is beneficial to differentiation of green buds, that is because autotrophy abilities of transformed cells is relatively weak so it is very difficult for them to differentiate into transformants, while accumulated products of green components by photosynthesis are able to nourish transformed cells. The green buds were cut and transferred into new screening medium, and some kept green while others etiolated or died. Hence the former is likely to be the individual which has been transformed with pIG121/ *Wap*25, while the latter may be the individual escaping from Kan and Hm in the earlier period. In new screening medium, Kan tends to inhibit the function of 70S ribosome in untransformed cells, prohibit normal development of chloroplasts or block translation process, thus interrupting protein synthesis, ultimately leading to death of adventitious buds. In addition, Kan is able to be transported in short distance, such that cells far from the medium are usually out of the effect of selection pressure, whose consequent adventitious buds can also display ablation after secondary culture. For this reason, leaf discs should not be planted upright but laid on the medium as flat as possible in the early period of culture in order to ensure their sufficient contact with antibiotics, and prevent occurrence of false positive individuals. Rooting of young plants with resistance shows that it is very difficult for them to root. Young plants with resistance obtained after 60 d of transformation grew well in screening medium and retained differentiation ability to some extent. However it is very difficult for them to root in the rooting medium, and they gradually went dead over time, leading to failure of obtaining complete plant in the present experiment. We presumably attribute this phenomenon to the inhibition of antibiotics on young plants rooting of *P. hybrida* Vilm group. Resistance of *P. hybrida* Vilm transformed with *Wap*25 to low temperature treatment and their changes in physiological parameters still need further study.

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