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RESEARCH ARTICLE

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Prokaryotic expression analysis of an NBS-type *PtDRG01* gene isolated from *Populus tomentosa* Carr.

Abstract In order to investigate the protein features of an NBS gene (*PtDRG01*, EF157840) isolated from *Populus tomentosa* Carr., the full-length open reading frame was fused into a prokaryotic expression vector pGEX-KG. PCR analysis and double endonuclease digestion showed that the recombinant vector was successfully constructed and transferred into an expression host *E. coli* strain XA_{90}. It was indicated by SDS-PAGE analysis that IPTG treatment successfully induced the expression of a fusion protein of about 79 kD, which was consistent with the predicted value. In addition, the prokaryotic expression system was also optimized. The result suggests that 1 mmol/L IPTG treatment for 4 h at 37ºC was most effective, and the product was predominately soluble and not extra-cellular secreting. Moreover, the fusion protein was purified with an affinity chromatography column using Glutathione Sepharose 4B. This work will lay a foundation for further studies on biological functions of the *PtDRG01* gene.

Keywords *Populus tomentosa* Carr, NBS gene, prokaryotic expression, protein purification

1 Introduction

Populus tomentosa has a number of good characteristics, such as rapid growth, excellent resistance to diseases, and superior wood quality; therefore, it is highly valued and considered as the best among the native poplar species in China (Zhu and Zhang, 1997). It is widely cultivated in north China. However, a number of diseases seriously affected its popularization, with the expansion of cultivation areas (Yuan, 1998). *Melampsora magnusiana* causes one of the most serious diseases as *Melampsora magnusiana* Wanger, among most pathogens. It infects leaves and buds of young plants and makes trees form tiny yellow spots, and thus reduces the efficiency of photosynthesis (Yu et al., 2004), and the affected leaves drop early. A severe infection can cause large necrosis and badly affect growth, with the result that the infected leaves and buds die, the dry weight is reduced by 29%–32%, the volume is reduced by 31%–42% and the increment growth is reduced by 65% (Pei et al., 2003). Further, it may result in serious decline of resistance to the autumn frost, *Xanthomonas populi*, *Valsa sordida* Nit. and so on (Dowkiw et al., 2003).

So far, studies on the disease resistance of *P. tomentosa* remains so weak that the study on screening of disease-resistant genetic resources, selecting resistant varieties, mapping and cloning of resistance gene are still a blank field, in sharp contrast to the urgent needs of disease control in the production of *P. tomentosa* (Zhang et al., 2005). This is far behind the study on the disease resistance of Tacamahaca and Aigeiros (Stirling et al., 2002; Zhu et al., 2002; Dowkiw et al., 2004; Lescot et al., 2004; Yin et al., 2004). Therefore, researches in this area are of great significance.

Using the hybrid clones of *Populus* with high disease resistance screened by inoculation of *Melampsora magnusiana* Wanger *in vivo* as the material in previous researches, we used NBS conservative domain on an R disease-resistant gene to design degenerate primers, and obtained an entire gene named *PtDRG01*. *PtDRG01* is 2324 bp, encodes 678 amino acids and has a molecular weight of 79 kD. For further testing of its expression characteristics and verification of its functions, we constructed the prokaryotic expression vector of *PtDRG01*, established an efficient expression system of

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the gene in *Escherichia coli* and detected the specific protein with target length by optimizing the induced conditions and analysis of the expression characteristics. After the fusion protein was purified with the affinity chromatography column glutathione sepharose 4B, we obtained the purified target protein, which thus provides material for further study on the functions of proteins. This work lays the foundation for further studies on biological functions of the *PtDRG01* gene and revelation of their transmission mechanism of the disease resistance signal.

2 Materials and methods

2.1 Materials

An NBS-type *PtDRG01* gene isolated from *P. tomentosa* was cloned in our lab. The *PtDRG01* gene was provided with the accession number EF157840 in GenBank (Zhang, 2007). Expression vector pGEX-KG was purchased from GE healthcare, the *E. coli* strain XA90 was preserved by our lab.

2.2 Methods

2.2.1 Construction and identification of the prokaryotic expression vector of *PtDRG01*

Gene specific primers were separately designed based on the homing sequence and the terminator sequence of the coding region on *PtDRG01*, and the nucleotide sequence of the restriction sites of *Nco*I and *Xba*I were added at both ends of the primers. The nucleotide sequences of specific primers of the *PtDRG01* gene is ProU (*Nco*I): 5'-CATGCCATGGGTATGCAGAAAGAAAAACGCAAG-CAA-3' and ProD (*Xho* I): 5'-CCGCTCGAGTCAGCC-CAAGATAAAATCAGATGG-3'. The primers were synthesized by Shanghai Sangon Biological Engineering Technology And Service Co, Ltd. PCR products were digested by restriction enzymes *Nco*I and *Xho*I (Promega) and coupled-reacted with the expression vector PGEX-KG digested by the same enzymes. Finally, the recombinant vector was transferred into an expression host *E. coli* strain XA90 and cultured in a LB liquid medium with ampicillin (Amp, 100 mg/L). The positive clones were screened by gene-specific PCR amplification.

2.2.2 *PtDRG01* induced gene expression and SDS-PAGE analysis

A single positive bacterial colony XA90 was inoculated in LB medium containing 100 mg/L Amp, shaken cultured at 180 r/min, 37°C until OD_{550} reached 0.6. Then IPTG was added to a final concentration of 1 mmol/L. The culture was incubated at 37°C for 3–4 h. Its final concentration was 1 mmol/L by adding IPTG, and then it was cultured for 3–4 h (Li et al., 2004). The expressed product was analyzed with SDS-PAGE on 10% linear polyacrylamide gradient gel (Low Molecular Weight Markers Proteins were provided by TaKaRa).

Referring to the methods in our lab (Lin et al., 2005a), 5% stacking gel with 10% separation gel 1 mL of bacteria solution was centrifuged at 13000 r/min for 5 min and the supernatant was discarded and the bacteria were harvested. The pellet was resuspended in 2 mL of ddH_2O and ultrasonically disrupted at 30% for 6 s at the interval of 3 s, for a total of 20 min. Then cell lysate was centrifuged at 13000 r/min, and 25 μL of supernatant was commixed with an equal volume of a 2 × SDS gel-loading buffer containing 1 mol/L Tris-HCl (pH 6.8), 4.0 mL of 10% SDS, 2.5 mL β-mercaptoethanol, 2.0 mL glycerol, 1.0 mL of 0.1% bromophenol blue and 9.5 mL of ddH_2O. Thirty-five μL of the expressed product was analyzed by SDS-PAGE, commixed with an equal volume of a 2× SDS gel-loading buffer and analyzed by SDS-PAGE. The electrophoresis lasted for 4 h, 80 V constant voltage in the stacking gel and 100 V constant voltage in the separation gel. After acrylamide gel was stripped, the gel was stained overnight with coomassie brilliant blue R250, and placed in a destaining solution; the destaining solution was replaced until the protein band was clear. The results were analyzed by a gel imaging system.

2.2.3 The most effective condition for induced protein expression

The activated bacteria solution was added using 1 mmol/L IPTG when OD_{550} reached 0.6, shaken cultured at 200 r/min, 37°C. One mL of the sample was ultrasonic disrupted at different times (1–5 h) and different temperatures (25, 30, 37°C) respectively. The expressed product was analyzed with SDS-PAGE. The percentage of target protein accounting for the total bacterial protein was analyzed by a gel imaging system.

2.2.4 Secretory analysis of the target protein of *PtDRG01*

Four mL of the bacteria solution in the most effective condition was centrifuged at high speed for 15 min at 4°C. The pellet and the supernatant were harvested respectively. The pellet was resuspended with a 2× SDS gel-loading buffer, boiled and centrifuged, and analyzed by SDS-PAGE. The supernatant was filtrated by a 0.25 μm filter membrane, placed on ice, added into PMSF and Dnase, and precipitated with $(NH_4)_2SO_4$ (68 g/100 mL). After the solution dissolved fully, it was centrifuged at 40000 r/min for 20 min. The supernatant was discarded and the pellet dissolved in 1 mL of ddH_2O, 20 μL of which was commixed with an equal volume of a 2× SDS gel-loading buffer and analyzed by SDS-PAGE.

2.2.5 Dissolubility analysis of the target protein of *PtDRG01* gene

Four mL of the bacteria solution in the most effective condition was centrifuged at high speed for 15 min at 4°C and the supernatant was discarded. The pellet was resuspended in protein buffer (pH = 8), lysozyme-cleaved for 20 min, and centrifuged at 10000 r/min for 15 min at 4°C. The pellet and the supernatant were harvested, respectively, commixed with an equal volume of a 2× SDS gel-loading buffer and analyzed by SDS-PAGE.

2.2.6 Purification of the target protein of *PtDRG01*with the affinity chromatography column

A single positive bacterial colony XA90 was inoculated into LB medium containing 100 mg/L Amp, and shaken cultured at 180 r/min, 37°C until OD_{550} reached 0.6. Then IPTG was added to a final concentration of 1 mmol/L. The culture was incubated for 3–4 h. Its final concentration was 1 mmol/L by adding IPTG, and then it was cultured for 3–4 h (Lin et al., 2005b). Two mL of impure protein samples, extracted from the cell lysate from ultrasonic disruption, was added into the GE healthcare with glutathione sepharose 4B, stood for 30 min and fully integrated. The impure protein was eluted by 30 mL of PBS buffer containing 140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 1.8 mmol/L KH_2PO_4; GST fusion protein was eluted with elution buffer (0.61 g of Tris and 0.31 g of glutathione reductase dissolved in 60 mL of dH_2O, pH was 8.0 by adding HCl 5 mol/L, and the final volume was 100 mL by adding dH_2O); the samples of the eluting peak were collected by an automatic collector and concentrated after dialysis with 50% glycerol. The result was analyzed with SDS-PAGE.

3 Results and analysis

3.1 Results of construction of prokaryotic expression vector of *PtDRG01* and identification of enzyme digestion

In this study, two gene-specific primers were added with the restriction sites for *Nco*I and *Xho*I respectively. The expression vector and PCR amplification products were digested by two incision enzymes (Faivre et al., 2006), and then the two enzyme products were linked to get a recombinant plasmid (Fig. 1). The recombinant plasmid was transferred into an expression host *E. coli* strain XA90, and was inoculated overnight at 37°C in LB medium containing 100 mg/L Amp. A single bacterial colony was selected to extract the plasmid DNA and verify the linkage effect. From Fig. 2, we can see that we obtained two specific fragments by digesting the recombinant plasmid

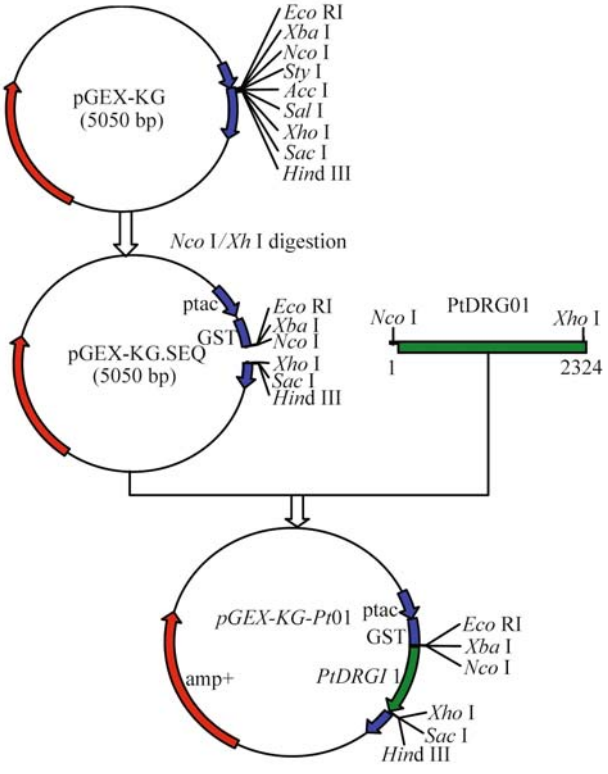

Fig. 1 Construction of prokaryotic expression vector *PtDRG01*

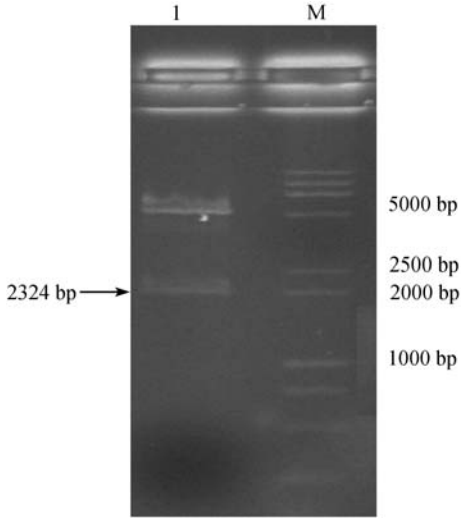

Fig. 2 Identification of recombinant *pGEX-KG-Pt01*
M: DNA marker; 1: production of *Nco* I/*Xho* I digestion

with the incision enzymes of *Nco*I and *Xho*I. According to the control Marker, we can see that the length of the target gene was about 2000 bp, indicating that the gene had been successfully integrated into the expression vector and the recombinant plasmid was correct.

3.2 Screening for the optimum conditions of induced expression

3.2.1 Screening for the optimum time of IPTG induction

When OD_{600} of the cell density of the recombinant strain, negative control, and positive control reached 0.6, 1 mmol/L of IPTG was added to induce the expression of exogenous protein, and the result was analyzed with SDS-PAGE after 1–5 h. It showed that *E. coli* with the recombinant plasmid was induced respectively using IPTG; the protein band with a molecular weight of about 80 kD markedly increased, and the expression amount of the protein increased with induction time, the maximum appearing at 4 h, followed by a downward trend (Fig. 3). Therefore, the specific proteins with a target length could be detected in the range of 1–5 h in IPTG induced time, showing that the *PtDRG01* gene had a complete encoding box, and the maximum expression amount of the protein appeared at 4 h. With the analysis software of the gel imaging system, we contrasted the results with the standard protein and got the curve of the expression (Fig. 4).

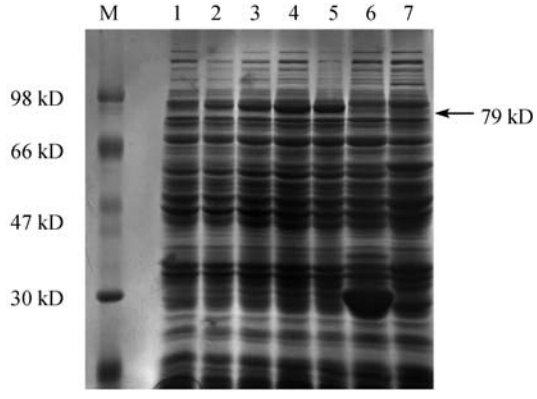

Fig. 3 Expression of recombinant in different induced time. M: protein marker; 1–5: expression of *pGEX-KG-Pt01* by IPTG induction after (1–5 h); 6: expression of XA_{90} cell with pGEX; 7: expression of XA_{90}.

3.2.2 Screening for the optimum temperature of IPTG induction

The induced temperature is another important factor for the target protein expression. When OD_{600} of the cell density of the recombinant strain, negative control and positive control reached 0.6, 1 mmol/L of IPTG was added to induce the expression of exogenous protein at 25, 30, and 37°C, respectively, and the result was analyzed with SDS-PAGE after 4 h. The results are presented in Fig. 5, showing that the expression amount of the protein increased with the increase in induced temperature, and the maximum appeared at 37°C. The 37°C temperature

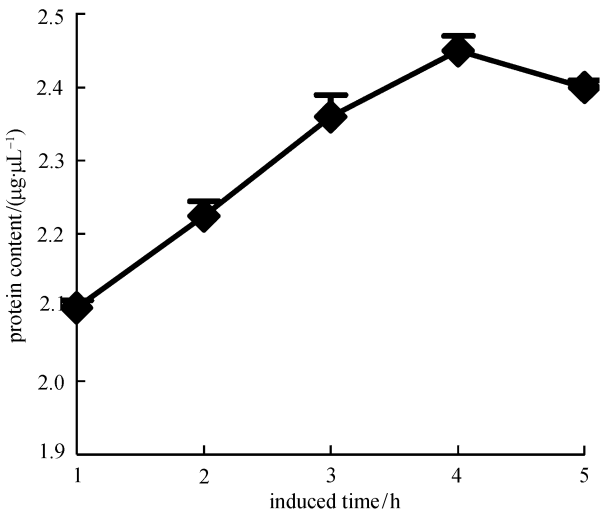

Fig. 4 Change in the expression of the recombinant in different induced time

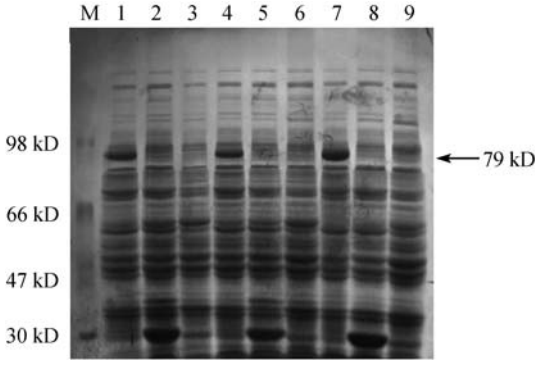

Fig. 5 Expression of recombinant at different induced temperatures. M: protein marker; 1, 4, 7: *pGEX-KG-Pt01* recombinant protein at 25, 30, 37°C; 2, 5, 8: expression of XA_{90} cell with pGEX; 3, 6, 9: expression of XA_{90}.

was optimum for prokaryotic expression of the *PtDRG01* gene. The result analyzed by the analysis software of the gel imaging system is shown in Fig. 6, which is consistent with the results above.

3.3 Extra-cellular secretion

If there is a signal sequence in the upper stream of the target gene, the fusion protein can be secreted out of the cell and we could directly collect a large number of medium to extract the fusion protein (Lin et al., 2005b). The extra-cellular medium of the recombinant strains and the intracellular proteins were collected respectively. The result was analyzed with SDS-PAGE. The expressed fusion protein was found in the intracellular extract but not in the medium of the recombinant strain, positive control or negative control (Fig. 7), indicating that the resulting protein was intra-cellular secreting.

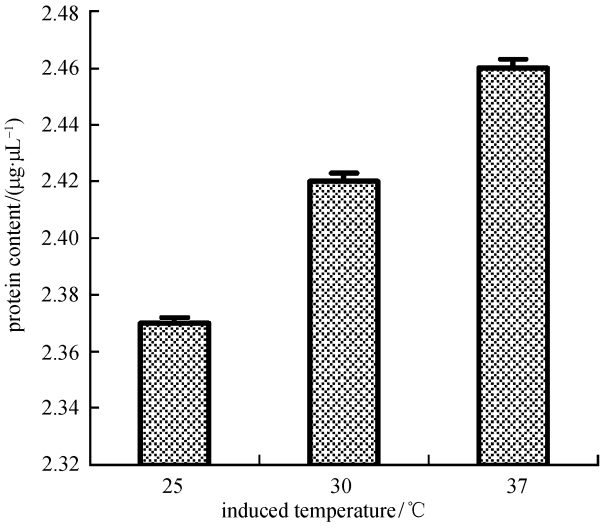

Fig. 6 Change in the expression of the recombinant at different induced temperatures

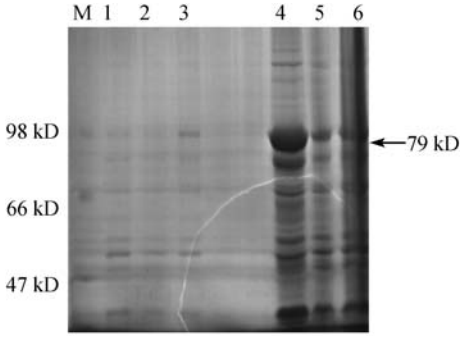

Fig. 7 Secreting analysis of fusion protein. M: protein marker; 1, 2, 3: the culture medium of *pGEX-KG-Pt01*, pGEX, XA_{90} cell ; 4, 5, 6: the extractant of *pGEX-KG-Pt01*, pGEX, XA_{90} cell.

3.4 Potential of solution

As the GST protein expressed by the vector pGEX was a water-soluble protein (Zhang et al., 2005), we could speculate that the fusion protein was also soluble. The recombinant and control cells were broken by lysozyme, and the supernatants and the pellet were analyzed by SDS-PAGE. The expressed fusion protein was found in the supernatants but not in the pellet (Fig. 8), indicating that the fusion protein was not in the inclusion bodies but was expressed in a soluble way.

3.5 Purification and Identification

The recombinant strains induced by IPTG were collected and broken ultrasonically in an ice bath. The fusion protein was extracted with ddH_2O and purified with the affinity chromatography column using glutathione sepharose 4B, concentrated after dialysis with 50% glycerol. The result

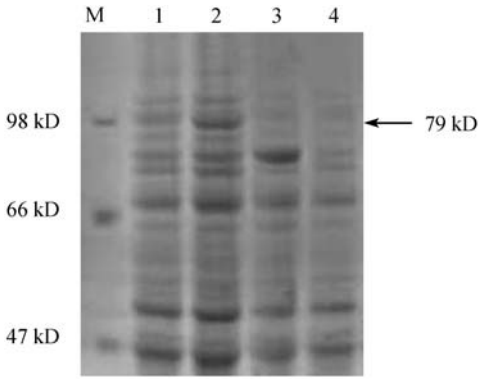

Fig. 8 Dissolving analysis of fusion protein. M: protein marker; 1, 2: the filtrate of XA_{90} cell and *pGEX-KG-Pt01* cell; 3, 4: the deposition of XA_{90} cell and *pGEX-KG-Pt01* cell.

was analyzed with SDS-PAGE (Fig. 9). The impure protein was eluted with elution buffer. The fusion protein of *PtDRG01* gene was purified and successfully induced the expression of a fusion protein of about 79 kD.

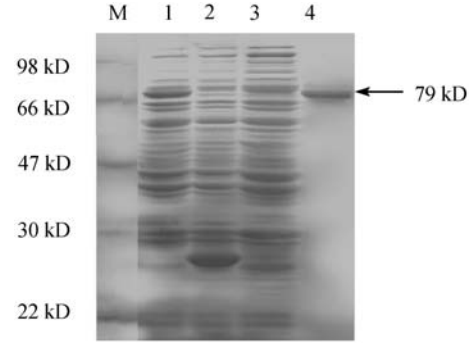

Fig. 9 Purification of recombinant protein. M: protein marker; 1,4: PtDRG01 protein before and after purified; 2: pGEX protein; 3: XA_{90} protein.

4 Discussion

The *PtDRG01* gene isolated from *P. tomentosa* had high homology with the NCBI gene, NBS-type genes of a large number of plants (including *P. trichocarpa*, *P. deltoides*, *P. balsamifera* subsp *trichocarpa*, as well as *P. tremula* and other poplars) logged in EST database, and the RGA gene (Zhang et al., 2001). Moreover, the *PtDRG01* protein had high homology with many well-known plant disease resistance proteins, in particular with the disease-resistant protein N of the tobacco mosaic virus (Liu et al., 2003); their amino acid sequences contained the complete structures of TIR and NBS. We found that the *PtDRG01* gene had ATP-binding sites in the NBS domain, using a large number of bioinformatics analysis; therefore, the *PtDRG01* gene might be associated with the ATP enzyme reaction and affect ATP activity inside the cell, which plays

a key role in the transmission of the disease resistance signal. We studied the prokaryotic expression to verify this.

At present, prokaryotic expression systems for different genes are quite different. The buckwheat trypsin inhibitor gene (BTI) was cloned into the expression vector pQE231, expressed in *E. coli* M15 host cells, and the recombinant BTI induced by IPTG, with the result showing that most of the recombinant protein found was soluble (Li et al., 2007). The *GlgC* gene was cloned to prokaryotic vector pET-28a-c (+) and then recombinant vector pET-glgC was transformed into the host cells of *E. coli* BL21, and the specific protein was produced by the host (Jia et al., 2006). The *B7.2 (lgV+ C)* gene was cloned to prokaryotic vector pGEX-4T-3, and the results indicated that the fusion protein reached the maximum level after 3 mmol/L IPTG treatment for 5 h (Yan et al., 2002). The prokaryotic vector pGEX-KG used in this study was an *E. coli* vector for expressing the fusion protein of glutathione S-transferase (GST), mainly due to the following: 1) pGEX-KG contains an inducible strong promoter tac, which is a mixed promoter with lac and trp. When the target gene is linked under the downstream of a strong promoter, the foreign gene *PtDRG01* would have a highly efficient expression in *E. coli*. 2) When the high-level expression of foreign genes inhibit the growth of the host cells, the repressor would protect the host so that the host bacteria could accumulate to a considerable number, and the expressed protein accumulates greatly in a short time and reduces the degradation of expression products by instantaneous derepression. IPTG combined with the repressor lac to induce the expression of the downstream (Sivaguru et al., 2003). PGEX-KG contained *lac*I gene, resulting in a large number of lac repressors and ensuring that the transcription of the downstream occurred only by IPTG induction. 3) The product of the expression vector was conducive to the separation and purification; the fusion protein was purified with the affinity chromatography column glutathione sepharose 4B in non-denaturing conditions, to maintain the integrity of the protein. 4) The coding region of the vector contained the foreign gene *PtDRG01* and an encoding gene of the vector, the fusion protein, contained a glutathione transferase of about 20 kD encoded by the vector. Thrombin restriction sites existed between the GST gene and the target gene, and the product could be digested by thrombin (Fan et al., 2007), so as to remove the GST part in the fusion protein and generate the *PtDRG01* protein.

One mmol/L IPTG treatment for 4 h at 37ºC was most effective for the expression, and the main reasons may be related to the host bacteria: *E. coli* is a kind of bacteria with a body temperature, and its optimum growth temperature was about 37°C; the growth can be divided into four phases: lag phase, exponential phase, stationary phase and decline phase. At first, the strains were activated overnight for about 12–16 h in our study, and then cultured in fresh culture medium for 2 h. The strains were exactly in the stationary phase after 1 mmol/L IPTG treatment for 4 h, when the copy of the foreign gene recombinant plasmid increased a great deal and the expression of a fusion protein increased.

In the bioinformatics analysis of the *PtDRG01* protein, we found that its isoelectric point was 8.165 and the hydrophilic amino acids accounted for the majority, indicating the protein was hydrophilic and alkaline-resistant. In addition, a large number of α-helix, β-sheet, curls and flexible zones existed in the secondary structure of the protein. Some foreign researches have shown that many exogenous proteins are expressed in *E. coli*, and the proteins cannot spontaneously fold to generate a certain spatial structure with a specific function, but exist in cells with inclusion bodies, a kind of insoluble precipitation, which is not conducive to the biological activity of foreign proteins (Elena et al., 2006). In this study, we extracted proteins from the pellet and the cell lysate respectively, and the result was analyzed with SDS-PAGE. The desired protein was only in the supernatant of the cell lysate, proving that the recombinant protein did not form inclusion bodies when expressed in cells, and was soluble. This work lays the foundation for further study on biological functions of the *PtDRG01* gene and the method of its realization.

After the purification of fusion proteins with the affinity chromatography column, we focused on the exact function of the protein and the impact and mode of action of the ATP activity, and revealed the significance of the function for the disease-resistant signal transmission pathway. On the other hand, we will take the *PtDRG01* fusion protein as the antigen to obtain specific antibodies, and prepare it for western blotting. It can also be used for molecular testing of the *PtDRG01* gene transforming into other plants, in order to identify the function of the *PtDRG01* gene in disease resistance and the signaling pathway.

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References

Dowkiw A and Bastien C (2004). Characterization of two major genetic factors controlling quantitative resistance to *Melampsora larici-populina* leaf rust in hybrid poplars: strain specificity, field expression, combined effects, and relationship with defeated qualitative resistance gene. Phytopathol, 94: 1358–1367

Dowkiw A, Husson C, Frey P, Bastien C (2003). Partial resistance to *Melampsora larici-populina* leaf rust in hybrid poplars: genetic variability in inoculated excised leaf disk bioassay and relationship with complete resistance. Phytopathol, 93: 421–427

Elena V, Doukhanina E V, Chen S, van der Zalm E, Godzik A, Reed J, Dickman M B (2006). Identification and functional characterization of the BAG protein family in *Arabidopsis thaliana*. Biol Chem, 24: 10–74

Faivre R F, Bataille L, Bresson A, Boudet N, Chalhoub B, Jorge V, Dowkiw A, Guerin V, Masle J P, Villar M, Bastien C (2006). International Poplar Symposium IV Abstracts. Nanjing: Nanjing Forestry University Press, 26–27

Fan B Y, Lu H, Jiang X N (2007). Immunofluorescent localization of 4-coumarate coenzyme a ligase of *Nicotiana tobacum*. Acta Bot-Occident Sin, 27(6): 1073–1077 (in Chinese)

Jia X Y, Zhang J W, Wang D (2006). Cloning and prokaryotic expression of *glgc* gene of *E.coli*. Boreal Acta Bot-Occident Sin, 26 (4): 0667–0671 (in Chinese)

Lescot M, Rombauts S, Zhang J, Aubourg S, Mathe C, Jansson S, Rouze P, Boerjan W (2004). Annotation of a 95-kb *Populus deltoides* genomic sequence reveals a disease resistance gene cluster and novel class I and II transposable elements. Theor Appl Genet, 109(1): 10–22

Li G L, Guo A G, Fan S H (2004). Cloning and expressing of *brazzein* gene. Acta Bot Boreal-Occident Sin, 24(7): 1271–1274 (in Chinese)

Li Y Y, Zhang Z, Li C, Wang Z H (2007). Prokaryotic expression and purification of Buckwheat Trypsin inhibitor. Acta Bot Boreal-Occident Sin, 2007, 27(1): 0016–0020 (in Chinese)

Lin Y Z, Lin S Z, Zhang Q, Wang X, Zhang Z Y (2005a). High level expression of glucose-6-phosphate dehydrogenase gene PsG6PDH from *Populus suaveolens* in *E. coli*. For Stud China, 7(3): 35–38

Lin Y Z, Lin S Z, Zhang W, Zhang Q, Zhang Z Y, Guo H (2005b). Cloning and sequence analysis of a glucose-6-phosphate dehydrogenase gene *PsG6PDH* from freeing-tolerant *Populus suaveolens*. For Stud China, 7(1): 1–6

Liu J J, Ekramoddoullah A K (2003). Isolation, genetic variation and expression of TIR-NBS-LRR resistance gene analogs from western white pine (*Pinus monticola* Dougl. ex. D. Don.). Mol Genet Genom, 270(5): 432–41

Pei M H, Ruiz C, Harris J, Hunter T (2003). Quantitative inoculation of poplars with *Melampsora larici-populina*. Euro Plant Path, 109: 269–276

Sivaguru M, Ezaki B, He Z H, Tong H Y, Osawa H, Baluka F, Volkmann D, Matsumoto H (2003). Aluminum-induced gene expression and protein localization of a cell wall-associated receptor kinase in *Arabidopsis*. Plant Physiol, 132: 2256–2266

Stirling B, Newcombe G, Vrebalov J, Bosdet I, Bradshaw H D (2002). Suppressed recombination around the *MXC3* locus, a major gene for resistance to poplar leaf rust. Theor Appl Genet, 103: 1129–1137

Yan X C, Lai B C, Zheng J, Ma J, Han J H, Wang Y L, Si L S (2002). The eukaryotic expression of human perforin protein and the establishment of hybridomas producing anti-human perforin protein. J Xi'an Med Univ, 23(1): 4–7 (in Chinese)

Yin T M, DiFazio S P, Gunter L E, Jawdy S S, Boerjan W, Tuskan A (2004). Genetic and physical mapping of *Melampsora* rust resistance genes in *Populus* and characterization of linkage disequilibrium and flanking genomic sequence. New Phytol, 164: 95–105

Yu Z D, Gao A Q, Cao Z M (2004). Poplar rust resistance study. Acta Bot Boreal-Occident Sin, 24(6): 1160–1164 (in Chinese)

Yuan S L (1998). Diseases of Trees and Shrubs in China. Beijing: China Forestry Publishing House, 110–112 (in Chinese)

Zhang J, Steenackers M, Storme V, Neyrinck S, van Montagu M, Gerats T, Boerjan W (2001). Fine mapping and identification of nucleotide binding site /leucine-rich repeat sequences at the *MER* locus in *Populus deltoides* 'S9-2'. Phytopathol, 91: 1069–1073

Zhang Q (2007). Screening for genes involved in resistance against leaf rust in *Populus tomentosa* Carr. and characterization of NBS type of disease resistance genes. Dissertation for the Doctoral Degree. Beijing: Beijing Forestry University (in Chinese)

Zhang Q, Lin S Z, Lin Y Z, Zhang Z Y, Wu Z L, Yang J J (2005). Research advances of resistant genes in forest trees. Acta Bot Boreal-Occident Sin, 25(9): 1921–1930 (in Chinese)

Zhang Q, Zhang Z Y, Lin S Z, Lin Y Z (2005). Resistance of transgenic hybrid triploids in *Populus tomentosa* Carr. against 3 species of lepidopterans following two winter dormancies conferred by high level expression of cowpea trypsin inhibitor gene. Silv Genet, 54(3): 108–116

Zhu H Y, Cannon S B, Young N D, Cook D R (2002). Phylogeny and genomic organization of the TIR and non-TIR-NBS-LRR resistance gene family in *Medicago truncatula*. Mol Plant-Microbe Interact, 15: 529–539

Zhu Z T, Zhang Z Y (1997). The status and advances of genetic improvement of *P. tomentosa* Carr. For Stud China, 6(1): 1–7