·Basic Research·

Construction of adenovirus vectors encoding the *lumican* gene by gateway recombinant cloning technology

Gui-Fang Wang, Bing Qi, Lei-Lei Tu, Lian Liu, Guo-Cheng Yu, Jing-Xiang Zhong

Department of Ophthalmology, the First Affiliated Hospital of Jinan University, Guangzhou 510630, Guangdong Province, China

Correspondence to: Jing-Xiang Zhong. Department of Ophthalmology, the First Affiliated Hospital of Jinan University, 613 Huangpu Road, Guangzhou 510630, Guangdong Province, China. lafangme@126.com

Received: 2015-09-08 Accepted: 2016-02-14

Abstract

• **AIM: To construct adenovirus vectors of *lumican* gene by gateway recombinant cloning technology to further understand the role of *lumican* gene in myopia.**

• **METHODS: Gateway recombinant cloning technology was used to construct adenovirus vectors. The wild-type (wt) and mutant (mut) forms of the *lumican* gene were synthesized and amplified by polymerase chain reaction (PCR). The *lumican* cDNA fragments were purified and ligated into the adenovirus shuttle vector pDown-multiple cloning site (MCS)-/internal ribozyme entry site (IRES)/enhanced green fluorescent protein (EGFP). Then the desired DNA fragments were integrated into the destination vector pAV.Des1d yielding the final expression constructs pAV.Ex1d-CMV>wt-lumican/IRES/EGFP and pAV.Ex1d -cytomegalovirus (CMV) >mut -lumican/IRES/EGFP, respectively.**

• **RESULTS: The adenovirus plasmids pAV.Ex1d-CMV>wt -lumican/IRES/EGFP and pAV.Ex1d -CMV >mut -lumican/IRES/EGFP were successfully constructed by gateway recombinant cloning technology. Positive clones identified by PCR and sequencing were selected and packaged into recombinant adenovirus in HEK293 cells.**

• **CONCLUSION: We construct adenovirus vectors containing the *lumican* gene by gateway recombinant cloning technology, which provides a basis for investigating the role of *lumican* gene in the pathogenesis of high myopia.**

• **KEYWORDS:** myopia; lumican; adenovirus vector; gateway recombinant cloning technology

DOI:10.18240/ijo.2016.09.06

Wang GF, Qi B, Tu LL, Liu L, Yu GC, Zhong JX. Construction of adenovirus vectors encoding the *lumican* gene by gateway recombinant cloning technology. *Int J Ophthalmol* 2016;9(9):1271-1275

INTRODUCTION

Myopia is a major cause of visual impairment, occurring at 20%-40% averagely [1-2] in the worldwide and 80% in Beijing [3]. Pathological myopia may lead to retinal degeneration, retinal hemorrhage and rhegmatogenous retinal detachment, ranking as one of the leading causes of irreversible blindness in young adults. The prevention and treatment of myopia has became a significant public health problem. *Lumican*, an important components of the sclera, regulates collagen fibril formation and organization [4-6], and scleral growth and metabolism. Chakravarti *et al* [7] found that the eyes of *lumican* and fibromodulin-null mice showed certain features of high myopia: increased axial length, thin sclera and retinal detachment. Increasing studies identified single nucleotide polymorphism (SNP) of *lumican* associated with high myopia susceptibility [8-12]. In our previous study, we revealed that *lumican* c.507 polymorphism might be a risk factor for the pathogenesis of high myopia. In order to further investigate the correlation between *lumican* gene and high myopia, we explore the construction of adenovirus vectors expressing the *lumican* gene.

The gateway recombinant cloning technology, an accurate cloning method based on the site-specific recombination properties of bacteriophage lambda, provides a highly efficient way to transfer DNA fragments between cloning vectors [13-15]. This powerful method allows precise cloning of DNA fragments of interest in a single step, yielding destination vectors rapidly, and it also contributes to the expression and purification of proteins with different tags. Recently, gateway recombination technology has been used to construct bacterial, viral, chloroplast and plant vectors[16-19].

MATERIALS AND METHODS

The experiment was authorized by the First Affiliated Hospital of Jinan University Ethics Committee.

Scheme of Gateway Recombinant Cloning Technology Here, we introduced the detailed protocol to construct vectors using gateway recombinant cloning technology (Figure 1A). In the BP reaction (attB×attP→attL×attR), the polymerase chain reaction (PCR) product flanked by the attB sites was inserted into the donor vector with the attP sites yielding the entry clone. The fragment containing the gene of interest flanked by attB1 and attB2 can either be produced by PCR or attB expression clone (Figure 1B). In the LR reaction (attL×attR→attB×attP), the entry clone containing the

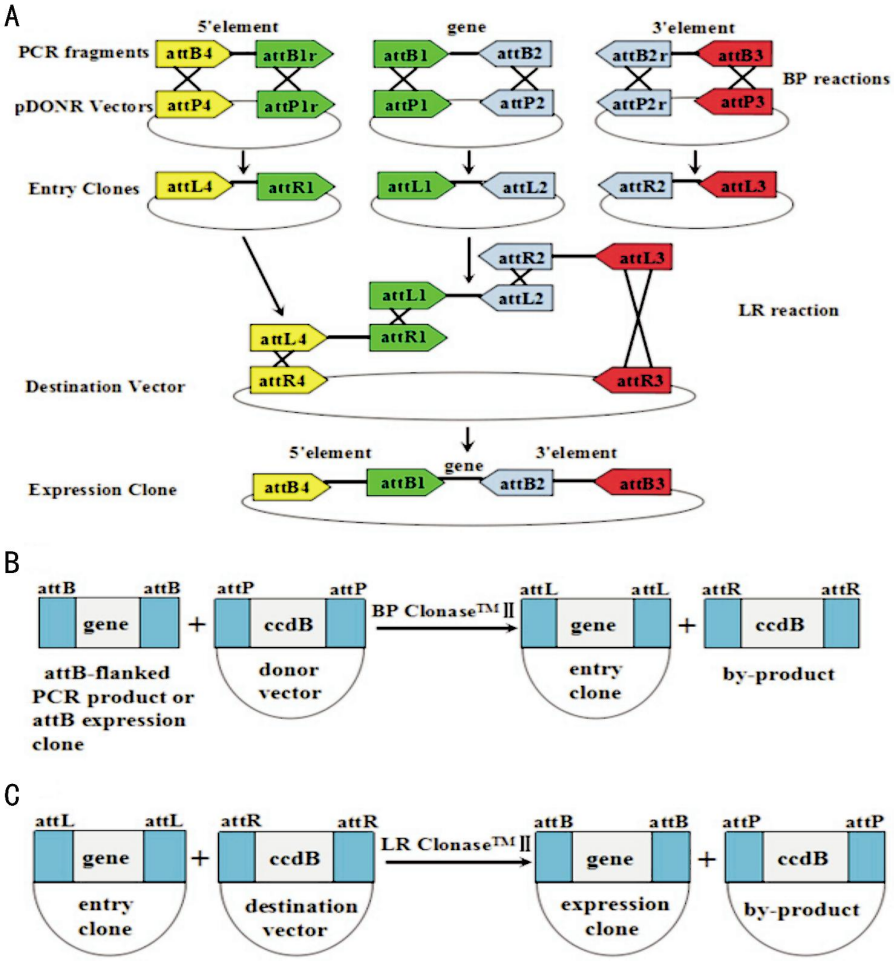

Figure 1 Schematic diagram of gateway recombinant cloning technology A: Flow chart depicting gateway cloning technology steps; B: In the BP reaction (attB×attP→attL×attR), PCR product was inserted into the donor vector by the BP Clonase™ II enzyme yielding the entry clone; C: In the LR reaction (attL×attR → attB×attP), the gene of interest was then integrated into the destination vector mediated by LR Clonase™ II enzyme yielding the final expression clone. attB, attP, attLand attR are gateway recombination sites.

Table 1 Primers used in this study

Primer name	Sequence (5'to 3')
EcoRI-SacI-lumican-F	CCGGAATTCGAGCTCATGAGTCTAAGTGCATTTACTCTC
lumican-XhoI-SpeI-R	CTAGACTAGTCTCGAG TTAATTAAGAGTGACTTCGTTAGCAA
lumican-507-F	CTTCATCCATCTCCAGCACAACCGGCTGAAAGAGGATGCTGT
lumican-507-R	ACAGCATCCTCTTTCAGCCGGTTGTGCTGGAGATGGATGAAG

gene of interest flanked the attL sites is then integrated into the destination vector flanked the attR sites yielding the final expression clone which is used for transformation and protein expression (Figure 1C). The att recombination sites in the donor and the destination vectors contains a *ccdB* gene (control of cell death) and a chloramphenicol-resistance gene[15], thus this vector can only be propagated in *ccdB* T1 or *ccdB* T2 resistant cells.

Primer Designing and Polymerase Chain Reaction Amplification of DNA Fragments Primer design is the vital step in constructing the vectors. Primers EcoRI-SacI-lumican-F, lumican-XhoI-SpeI-R, lumican-507-F and lumican-507-R (Table 1) were synthesized and the DNA fragments of wild-type and mutant forms of lumican gene were amplified respectively. The primers used in the amplification of EcoRI-wt-lumican-XhoI were EcoRI-SacI-lumican-F and lumican-XhoI-SpeI-R. Primers EcoRI-SacI-lumican-F and lumican-507-R were used to amplify the 5' segment of EcoRI-mut-lumican, and primers lumican-507-F and lumican-XhoI-SpeI-R for the 3' segment of mut-lumican-XhoI. Then primers EcoRI-SacI-lumican-F and lumican-XhoI-SpeI-R were used to amplify EcoRI-mut-lumican-XhoI. PCR amplification system included 5×primer STAR™ Buffer (Mg^{2+} 10 μL Plus), dNTP mixture 4 μL, forward primer 1 μL, reverse primer 1 μL, DNA 1 μL, primer STAR™ HS DNA Polymerase 0.5 μL, and H_2O 32.5 μL. PCR amplification conditions were: 98℃ 3min, 98℃ 30s, 60℃ 30s, and 72℃ 1min 30 cycles, and 72℃ 5min. In the meanwhile, the plasmid to be cloned was digested with appropriate restriction enzymes and the DNA fragment of *lumican*was excised from the agarose gel.

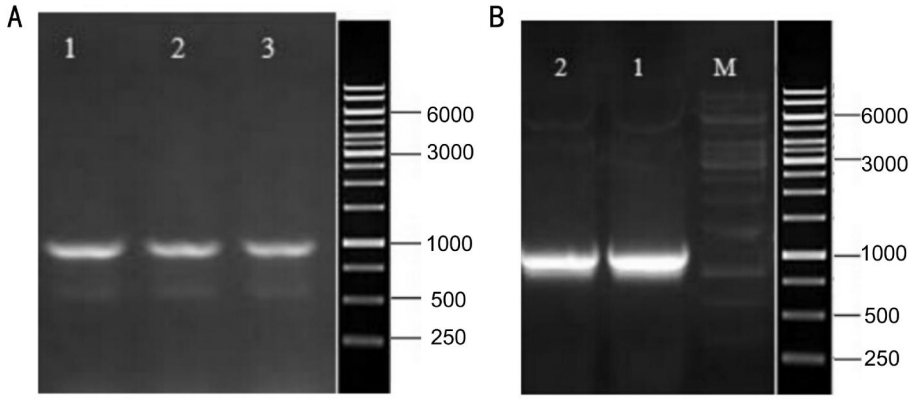

Figure 2 PCR amplification of the *lumican* gene A: The wt-lumican gene was amplified by PCR, the fragment in lane 1-3 (1000 bp) M: 1000 bp marker, lane 1-3: wt-lumican; B: The mut-lumican gene was amplified by PCR, the fragment in lane 1-2 (1000 bp) M: 1000 bp marker, lane 1-2: mut-lumican.

Construction of Adenovirus Vectors by Gateway Technology The DNA fragments of the *lumican* gene were purified and ligated with the adenovirus shuttle vector pDown-multiple cloning site (MCS)-/internal ribozyme entry site (IRES)/enhanced green fluorescent protein (EGFP). The *lumican* DNA fragments and shuttle vector pDown-MCS-IRES/EGFP were digested with XhoI and EcoRI and treated with T4 DNA ligase overnight, then the reaction products were transformed into competent *E.coli* strain stbl3. Positive clones were selected by PCR to obtain the entry clone pDown-lumican/ IRES/EGFP. Subsequently, the *lumican* DNA fragments were integrated into the destination vector pAV.Des1d mediated by integrase and excisionase yielding the final expression clone pAV.Ex1d-CMV>lumican/IRES/EGFP. Reaction system included: pDown-lumican/IRES/EGFP 10 fmol, pAV.Des1d 20 fmol, LR clonase 1 μL, and TE buffer up to 5 μL. An LR recombination reaction (between attL and attR sites) was carried out at 25℃ for 16h and the reaction products were transformed into competent *E. coli* Stbl3. Positive clones were selected by PCR to extract the recombinant adenoviral plasmids pAV.Ex1d-CMV> lumican/IRES/EGFP. The expression clones were identified by DNA sequencing. PCR amplification was carried out using 10×rTaq buffer 3 μL, dNTP 2 μL, forward primer 1 μL, reverse primer 1 μL, DNA 2 μL, rTaq 0.2 μL, and H_2O 20.8 μL. PCR amplification conditions included 30 cycles, each at 95℃ for 5min, 95℃ for 30s, 55℃ for 30s, 72℃ for 45s, and 72℃ for 5min. The primer sequences used in the PCR were shown in brackets (F: GAACCCACTGCTTACTGGCTT; R:TCGAGACCGAGGAGAGGGT).

Adenovirus Packaging and Amplification in HEK293 Cells The recombinant adenovirus plasmids pAV.Ex1d-CMV>lumican/IRES/EGFP were linearized by PacI and transfected into HEK293 cells for adenovirus packaging and amplification. Briefly, HEK293 cells were seeded at 2×10^5 cells per cm^2 into 6-well culture plates in Dulbecco modified Eagle's medium (DMEM) containing 10% FBS. Opti-MEM 500 μL, 1 μg adenoviral plasmid DNA, and 3 μL lipofectine were added into culture media after HEK293 cells adhered to dish, followed by incubation for 24h at 37℃. After incubation, the culture media were changed every 2-3d. The infected 293 cells showed significant cytopathic effect (CPE) at day 8. The cells were collected and cracked by repeated freezing and thawing between 37℃ and -80℃ for three times to release the viral particles. Then the virus were collected and stored at -80℃ for use.

RESULTS

Polymerase Chain Reaction Amplification of the *Lumican* Gene The DNA fragments of wt-lumican and mut-lumican gene were amplified by PCR. The expected band sizes are of 1000 bp (Figure 2).

Construction of Adenovirus Vectors The DNA fragments of *lumican* gene were inserted into the adenovirus shuttle vector pDown-MCS-/IRES/EGFP. Subsequently, the desired DNA fragments were transferred to the destination vector pAV.Des1d yielding the expression vectors pAV.Ex1d-CMV>wt-lumican/IRES/EGFP and pAV.Ex1d-CMV>mut-lumican/IRES/EGFP (Figure 3). The positive clones were identified by PCR and sequencing (Figure 4).

Recombinant Adenovirus Packaging The recombinant adenovirus plasmids identified by PCR and DNA sequencing were selected and packaged into pAV.Ex1d-CMV>wt-lumican/IRES/EGFP and pAV.Ex1d-CMV>mut-lumican/IRES/EGFP adenoviruses in HEK293 cells, respectively. Following conventional culture for 8d, infected HEK293 cells showed significant cytopathic effect and clear fluorescence (Figure 5).

DISCUSSION

Myopia is a common eye disease with complex causes, and both genetics and environment must play roles[20]. Heredity is an important factor associated with the pathogenesis of myopia. We selected *lumican* as a candidate gene to study the genetic predisposition of high myopia. This study for the first time constructed adenovirus vectors encoding the *lumican* gene by gateway recombinant cloning technology,

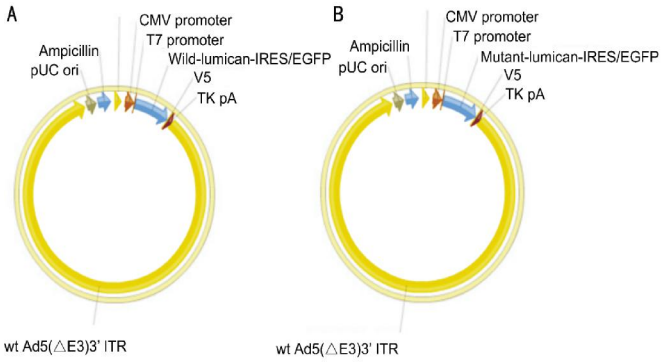

Figure 3 Construction of recombinant adenovirus vectors containing the *lumican* gene A: pAV.Ex1d-CMV>wt-lumican/IRES/EGFP; B: pAV.Ex1d-CMV>mut-lumican/IRES/EGFP.

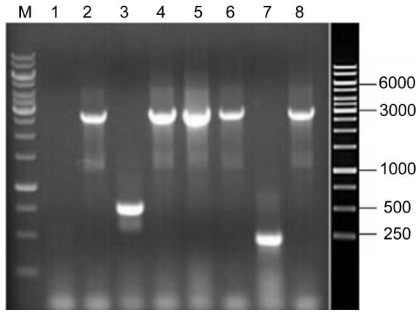

Figure 4 The positive clones were selected by PCR Lane 1-4: pAV.Ex1d-CMV>wt-lumican/IRES/EGFP clone 1-4, the fragments in lane 2 and 4 are the positive clones (2600 bp); Lane 5-8: pAV.Ex1d-CMV>mut-lumican/IRES/EGFP clone 5-8, the fragments in lane 5, 6 and 8 are the positive clones (2400 bp).

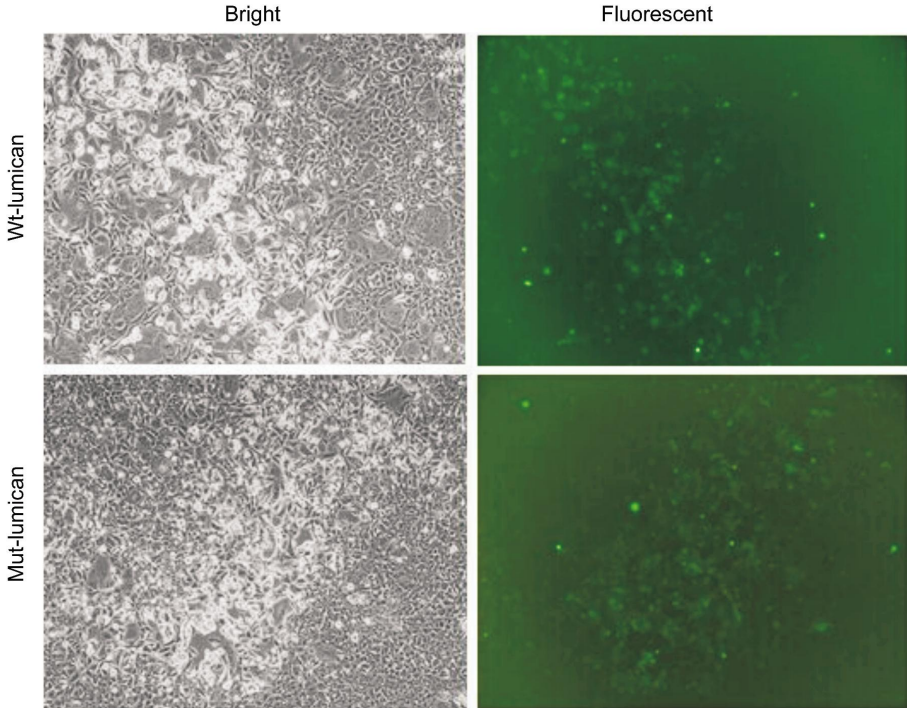

Figure 5 HEK293 cells infected by the adenovirus vector under an ordinary light microscope and a fluorescent microscope Cells showing significant CPE and clear fluorescence. Original magnification: ×100.

which provides a basis for further understanding the role of *lumican* gene in myopia.

We introduce gateway recombinant technology as a powerful method of constructing adenovirus vectors. This method offers several advantages over conventional cloning. 1) It allows efficient cloning, as many DNA fragments can be used in various combination, which also enables the rapid exchange of genes, promoters and dominant selectable marker cassettes; 2) It exploits specific recombination sites and recombinase. The gene of interest flanked by the attB sites was inserted into the donor vector containing the attP sites using the BP Clonase™ II enzyme mix. Then the entry clone containing the attL sites and the destination vector flanked by the attR sites were exchanged between the att sites mediated by LR Clonase™ II enzyme, yielding the final expression clone; 3) It allows precise cloning without alteration of the coding sequence. The presence of the *ccdB* gene and the chloramphenicol resistance gene in both donor and destination vector provides a unique system for negative selection to eliminate all unwanted byproduct plasmids after recombination, resulting in maximum cloning efficiency[21]. The EGFP coding sequence was inserted into the expression vector and its protein expression was monitored by confocal laser scanning microscopy. The conventional cloning methods are time consuming and laborious, and need to be dependent on the specific restriction sites. Compared with other current adenovirus expression system, the gateway technology offers higher recombination efficiency without the need for restriction enzymes and ligases. However, this method also has some limitations. The gateway site-specific recombination kits is more expensive, and it introduces additional amino acids that could affect the modification of the primary sequence[22].

In this study, we successfully constructed the adenovirus

vectors of the *lumican* gene by gateway recombinant technology, which provides a basis for investigating the biological function of *lumican* and its potential for gene therapy. In conclusion, the gateway recombinant technology is an efficient way to construct adenovirus vectors.

ACKNOWLEDGEMENTS

Foundations: Supported by the Natural Science Foundation of Guangdong Province (No.2015A030310158; No. 2014A030313359); the Fundamental Research Funds for the Central Universities (No.21611446); the Scientific and Cultivation Foundation of the First Affiliated Hospital of Jinan University (No.2015201).

Conflicts of Interest: Wang GF, None; **Qi B,** None; **Tu LL,** None; **Liu L,** None; **Yu GC,** None; **Zhong JX,** None.

REFERENCES

1 Williams KM, Bertelsen G, Cumberland P, *et al*. Increasing prevalence of myopia in Europe and the impact of education. *Ophthalmology* 2015;122 (7):1489-1497.

2 Parssinen O. The increased prevalence of myopia in Finland. *Acta Ophthalmol* 2012;90(6):497-502.

3 Wu LJ, You QS, Duan JL, Luo YX, Liu LJ, Li X, Gao Q, Zhu HP, He Y, Xu L, Jonas JB, Wang W, Guo XH. Prevalence and associated factors of myopia in high-school students in Beijing. *PLoS One* 2015;10 (3): e0120764.

4 Saika S, Miyamoto T, Tanaka S, Tanaka T, Ishida I, Ohnishi Y, Ooshima A, Ishiwata T, Asano G, Chikama T, Shiraishi A, Liu CY, Kao CW, Kao WW. Response of lens epithelial cells to injury: role of lumican in epithelial-mesenchymal transition. *Invest Ophthalmol Vis Sci* 2003;44(5): 2094-2102.

5 Chakravarti S, Magnuson T, Lass JH, Jepsen KJ, LaMantia C, Carroll H. Lumican regulates collagen fibril assembly: skin fragility and corneal opacity in the absence of lumican. *J Cell Biol* 1998;141(5):1277-1286.

6 Nikitovic D, Chalkiadaki G, Berdiaki A, Aggelidakis J, Katonis P, Karamanos NK, Tzanakakis GN. Lumican regulates osteosarcoma cell adhesion by modulating TGF β 2 activity. *Int J Biochem Cell Biol* 2011;43 (6):928-935.

7 Chakravarti S, Paul J, Roberts L, Chervoneva I, Oldberg A, Birk DE. Ocular and scleral alterations in gene-targeted lumican-fibromodulin double-null mice. *Invest Ophthalmol Vis Sci* 2003;44(6):2422-2432.

8 Lin HJ, Kung YJ, Lin YJ, Sheu JJ, Chen BH, Lan YC, Lai CH, Hsu YA, Wan L, Tsai FJ. Association of the lumican gene functional 3'-UTR polymorphism with high myopia. *Invest Ophthalmol Vis Sci* 2010;51 (1): 96-102.

9 Chen ZT, Wang IJ, Shih YF, Lin LL. The association of haplotype at the lumican gene with high myopia susceptibility in Taiwanese patients. *Ophthalmology* 2009;116(10):1920-1927.

10 Majava M, Bishop PN, Hagg P, Scott PG, Rice A, Inglehearn C, Hammond CJ, Spector TD, Ala-Kokko L, Mannikko M. Novel mutations in the small leucine-rich repeat protein/proteoglycan (SLRP) genes in high myopia. *Hum Mutat* 2007;28(4):336-344.

11 Lin HJ, Wan L, Tsai Y, Chen WC, Tsai SW, Tsai FJ. The association between lumican gene polymorphisms and high myopia. *Eye (Lond)* 2010; 24(6):1093-1101.

12 He M, Wang W, Ragoonundun D, Huang W. Meta-analysis of the association between lumican gene polymorphisms and susceptibility to high myopia. *PLoS One* 2014;9(6):e98748.

13 Hartley JL, Temple GF, Brasch MA. DNA cloning using in vitro site-specific recombination. *Genome Res* 2000;10(11):1788-1795.

14 Oey M, Ross IL, Hankamer B. Gateway-assisted vector construction to facilitate expression of foreign proteins in the chloroplast of single celled algae. *PLoS One* 2014;9(2):e86841.

15 Leclercq J, Szabolcs T, Martin F, Montoro P. Development of a new pCAMBIA binary vector using Gateway® technology. *Plasmid* 2015;81: 50-54.

16 Gottschamel J, Waheed MT, Clarke JL, Lossl AG. A novel chloroplast transformation vector compatible with the Gateway (®) recombination cloning technology. *Transgenic Res* 2013;22(6):1273-1278.

17 Ouellette SP, Gauliard E, Antosova Z, Ladant D. A Gateway (®)-compatible bacterial adenylate cyclase-based two-hybrid system. *Environ Microbiol Rep* 2014;6(3):259-267.

18 Liu YK, Huang LF, Ho SL, Liao CY, Liu HY, Lai YH, Yu SM, Lu CA. Production of mouse granulocyte-macrophage colony-stimulating factor by gateway technology and transgenic rice cell culture. *Biotechnol Bioeng* 2012;109(5):1239-1247.

19 Kagale S, Uzuhashi S, Wigness M, Bender T, Yang W, Borhan MH, Rozwadowski K. TMV-Gate vectors: gateway compatible tobacco mosaic virus based expression vectors for functional analysis of proteins. *Sci Rep* 2012;2:874.

20 Feldkamper M, Schaeffel F. Interactions of genes and environment in myopia. *Dev Ophthalmol* 2003;37:34-49.

21 Bernard P. New ccdB positive-selection cloning vectors with kanamycin or chloramphenicol selectable markers. *Gene* 1995;162(1):159-160.

22 Kilaru S, Steinberg G. Yeast recombination-based cloning as an efficient way of constructing vectors for Zymoseptoria tritici. *Fungal Genet Biol* 2015;79:76-83.