Development and Verification of PCR based Assay to Detect and Quantify Garden Pea *lec* Gene

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**Abstract**


Genetically modified organisms (GMOs) entering the food chain have become its part, which is necessary to monitor. GMO analyses are used as a control mechanism according to valid acquis communautaire for traceability and labeling of GMOs. Generally, approved PCR based protocols are used and they require stepwise procedures that use amplification of species specific gene as initial point. This study aims to develop and verify PCR based assay for amplification of garden pea lectin gene (*Pisum sativum* L.) as reference one. Lectin gene was analysed in silico, selected region was amplified and sequenced and new set of species specific primers for identification of garden pea was designed. Conditions of conventional PCR as well as real-time PCR were optimised and specificity of new primer set on DNA extracted from garden pea cultivars as well as DNA extracted from other selected species from *Fabaceae* family was tested. Quantification of garden pea lectin gene using real-time PCR based on SYBR Green I was optimised and performance characteristics recorded. The characteristics fit to method acceptance criteria range. Plasmid with garden pea lectin sequence was developed and plasmid is available as a positive control.

**Keywords:** GMO; lectin; PCR detection; real-time PCR

There are three fundamental types of agriculture in Europe considered. Conventional agriculture, ecological agriculture and GM (genetic modified) plants cultivation. Coexistence measures must be applied for these three types of agricultural.

Basic rules are carried out by technical and agronomical arrangements, e.g. use of buffer zones with specific variety structure, registry of GM plants cultivation, establish of GM plants cultivation methods and accidental admixtures of GM and non-GM crops definition. Regulation are in place for GM derived food and feed (e.g. EU Regulations – EC 1829/2003 and EC 1830/2003). Methods are in place that allows efficient control of in EU approved GMO.

The most used methods for GMO detection in food and feed are based on Polymerase Chain Reaction (PCR). Principles of these methods are described in the international standards (e.g. EN ISO 21571:2005) and validated protocols are avail-
able on the JRC (Joint Research Centre) website covering mostly GM soybean, maize, cotton, rapeseed or rice (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm – accessed 27. 7. 2011).

DNA extraction from representative samples constitutes the first step of PCR based GMO analysis. The DNA should meet certain criteria for further analysis. Several procedures have been described that allow efficient DNA extraction (EN ISO 21571:2005; ENGL 2008). Among them extraction exploiting selective precipitation in cetyltrimethylammonium bromide (CTAB) (Murray & Thompson 1980) was found to be an appropriate method, which is in line with preceding recommendation for DNA isolation from plants containing higher content of polysaccharides, lipids and polyphenols (Porebski et al. 1997; Ovesna et al. 2010).

After DNAs are isolated, they are subjected either to conventional or real-time PCR. In both cases, DNA amplifiability is verified by plant/species specific gene(s) amplification. This procedure is used for verifying if the PCR ran through and for the control of false negatives results in further analyses (Hemmer 1997).

In real-time PCR, nucleic acid increase is measured and the standard curve is used as a reference standard for extrapolating quantitative information for starting amount of nucleic acid (Paoletti et al. 2006). The resulting percentage of GMO in the sample is then calculated from the proportion of species-specific (endogenous) DNA and the transgenic target.

For correct GMO quantification, the reaction has to be fully optimised and reaction efficiency should reach at least 90% (Querci et al. 2005). The PCR efficiency can be affected by several factors as e.g. primer design, composition of reaction mixture, reaction profile and presence of inhibitors/enhancers (Holst-Jensen et al. 2006; Hodek et al. 2009; Bergerová et al. 2011).

As increasing number of various GM plants has appeared in the environment released deliberately for research or commercial purposes, appropriate assays are required for efficient control.

Also garden pea (Pisum sativum L.) has been already transformed and the cultivars are undergoing field tests (Rakousky et al. 2004; Švábová et al. 2005).

Development of PCR based assay for detection and quantification of garden pea species specific gene, verification of method performance and specificity was the main aim of the study.

A gene coding for lectin was selected as multiple sequencing data were available in the databases (Chrispeels & Raikhel 1991; Lioi et al. 2005). Moreover, garden pea lectin is a one-copy gene (Gatehouse et al. 1987; Kaminski et al. 1987; Galasso et al. 2003) and amplification of part of pea lectin gene as a control element was already used in several studies (Brežná et al. 2006; Hrnčirová et al. 2008).

MATERIAL AND METHODS

Plant material. The 13 cultivars of commercially available pea (Pisum sativum L.) seeds (Raman, Alderman, Arvika, Bajka, Hero, Dalila, Havel, Oskar, Pegaz, Rondo, Kelvedon Wonder, Ambrosia and Delikata) and selected species of the pea family (Fabaceae) vetch (Vicia villosa ROTH), soybean (Glycine max L.), lentil (Lens culinaris Med.), mung bean (Vigna radiata L.) and bean (Phaseolus vulgaris L.) were used. All used seeds were stored at room temperature until processed.

DNA isolation. Seeds were ground in liquid nitrogen to obtain a homogenous powder. The CTAB (cetyltrimethyl ammonium bromide) method for DNA extraction was performed according ISO standard (EN ISO 21571:2005). DNA was extracted from 200 mg of powdered sample. Two independent extractions were performed from each sample. Extracted DNAs were eluted in 60 µl of ultrapure DNA and RNA free H2O.

The extraction of plasmid DNA was performed using High Pure Plasmid Isolation Kit (Roche, Basel, Switzerland).

Assessment of DNA quality. The quality of extracted DNA (its integrity and rough estimation of quantity) was evaluated using electrophoretic separation on 0.8% agarose gel with ethidium bromide staining. The spectrophotometer NanoPhotometer (Implen GmbH, Munich, Germany) was used for measurement of DNA quantity. Absorption at 260 and 280 nm was measured and the spectra profile (from 190 nm to 760 nm) was recorded. DNA quantity was calculated from the absorption at wavelength 260 nm and DNA purity calculation was based on A260/A280 ratios.

Primers design. Free-software Primer3Plus was used for oligonucleotide primer designing (Untergasser et al. 2007). First, primers for amplification of 658 bp long part of pea lectin gene (primers Lec658) were designed according to DNA sequence
of pea lectin (EU825771.1). Amplicons were used for cloning and for subsequent DNA sequencing.

Primers Lec101 were designed according to sequenced DNA of 658 bp long part of pea lectin gene. Primers Lec658 and Lec101 are shown in Table 1.

The specificity of primers Lec101 was analysed in silico in ClustalW2 free-software for the multiple sequence alignment and in free accessible database of the Center for Biotechnology Information (NCBI) with using Basic Local Alignment Search Tool (BLAST) (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Conventional Polymerase Chain Reaction (PCR). Amplification reactions (25 µl) were performed using AmpliTaq Gold PCR reagents (Applied Biosystems, Carlsbad, USA). For the verification of DNA amplifiability of all samples used in this study, primers (Pla primers) for amplification of specific region within gene coding plastid t-RNA were used (Taberlet et al. (1991)) (Table 1).

PCR for preparation of 658 bp long part of pea lectin gene (with Lec658 primers) was performed with DNA extracted from seeds of garden pea cv. Raman.

PCR with Lec101 primers was used for reaction specificity test with all samples used in this study (all garden pea cultivars as well as chosen species belonging to the family Fabaceae).

Final concentration of PCR’s components were as follow: Gold buffer 1x, MgCl₂ 1.5mM, dNTPs (Fermentas, Burlington, Canada) 0.2mM, AmpliTaq Gold polymerase 1U, and primers both of Pla primers 0.5µM, both of Lec658 primers 0.5µM or both of Lec101 primers 0.24µM. The volume was adjusted with water for PCR to 20µl. PCR was performed using 5µl of template DNA. Several controls were included in each reaction set – positive control of PCR run, control of extraction reagents (CTRL EX) and control of amplification reagents (CTRL MM and CTRL MM open).

All reactions were performed in an MJTB-96 PCR thermal cycler (MJ Research, Watertown, USA) and the thermal profile was: 12 min at 95°C; 40 cycles at 95°C for 30 s, at 60°C for 30 s (Pla primers and Lec658 Primers) or at 65°C for 30 s (Lec101 primers), at 72°C for 30 s and final extension was at 72°C for 10 minutes. Used primers were distributed by GENERI BIOTECH, s.r.o. (Hradec Králové, Czech Republic).

Results of PCR were visualised using electrophoretic separation on 2% agarose gel with ethidium bromide staining.

Cloning. Positive plasmid control (Plasmid658) was prepared by cloning of 658 bp long amplicon of pea lectin gene. Cloning was performed using TOPO® TA Cloning Kit (Invitrogen, Grand Island, USA). Plasmids were linearised with the restriction enzyme HindIII (Fermentas, Burlington, Canada) before using in PCR – circular form of plasmid is not appropriate for that purpose (Hou et al. 2010).

DNA sequencing. Plasmid658 was used for DNA sequencing 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, USA). The sequencing was performed using BigDye® Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, USA) according to the manufacturer’s protocol. The sequencing reaction was performed independently twice with forward primer Lec658 and independently twice with reverse primer Lec658.

Real-time PCR. Real-time PCR was performed with Lec101 primers in the 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, USA). All reactions were done in triplicates using SYBR® Green (Applied Biosystems, Carlsbad, USA) detection. Final concentration of real-time PCR’s components were as follow: SYBR® Green 1x, both of Lec101 primers 0.05µM, volume was adjusted with water for PCR to 45 µl. The thermal profile of real-time PCR consisted of 10 min at 95°C; 35 cycles at 95°C for 15 s, and at 60°C for 45 seconds.

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**Table 1. Primers used in PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5’→3’)</th>
<th>Product size (bp)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pla F</td>
<td>CGAAATCGGTTAGACCGTACG</td>
<td>ca 600</td>
<td>Taberlet et al. (1991)</td>
</tr>
<tr>
<td>Pla R</td>
<td>GGGGATAGGGGACTTTGAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lec658 F</td>
<td>CCGAAAACAACCTGAAGAAATAC</td>
<td>658</td>
<td>this paper</td>
</tr>
<tr>
<td>Lec658 R</td>
<td>ACTCTGCGCTATTGAAAACCTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lec101 F</td>
<td>CCCGACCAACAAAAACCTAAAT</td>
<td>101</td>
<td>this paper</td>
</tr>
<tr>
<td>Lec101 R</td>
<td>TAGAGGGCTCTGCCAACAGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Real-time PCR characterisation. The occurrence of inhibitions or enhancers was controlled using 10-fold dilution of template DNA, when the difference between Ct values in no inhibited reaction should be around 3.3 Ct.

The real-time PCR efficiency was determined for genomic DNA as a template using serial of dilutions. The starting amount of DNA in reaction was 200 ng (corresponding to approx. 41 000 DNA copies; BENNET & SMITH 1976), sample was diluted 4-times with ultrapure H₂O for PCR at ratio of 1:3. Limit of Detection (LOD) and Limit of Quantification (LOQ) were defined. Specificity of used primers Lec101 was tested in real-time PCR with all of used samples in this study (all garden pea cultivars and chosen species belonged to the family Fabaceae).

Efficiency of reaction was calculated according to the formula:

\[ \text{Efficiency} = 100 - \text{ABS} \left( 100 - \left( \frac{1}{10 - \text{slope}} - 1 \right) \times 100 \right) \]  

Data from 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, USA) were evaluated in Microsoft Office Excel.

Results and Discussion

The CTAB method was proved to be suitable for DNA extraction from seeds of the Fabaceae family. CTAB based method thus represents an adequate alternative to GeneSpin protocol exploited by e.g. BŘEŽNÁ et al. (2006). DNA amplifiability that serves an independent check of its quality was verified using a tRNA-leu specific PCR assay (TABERLET et al. 1991) and confirms required DNA purity.

Using cv. Raman DNA as a template in PCR with Lec658 primer pair we amplified 658 bp long fragments. Fragments were cloned into the plasmid vector to prepare Plasmid658 control DNA. Insert representing part of the lectin gene in the Plasmid658 was sequenced and DNA sequences were in silico analysed against NCBI database. Results confirm 100% homology for all of 658 nts with genomic DNA sequence of Pisum sativum L. lectin gene (EU825771.1, X66368.1), 98% homology (646/658 nts identity) was observed with genomic DNA sequence of Pisum sativum L. lectin gene

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EU825771.1</td>
<td>Pisum sativum lectin (psl) gene, complete cds</td>
</tr>
<tr>
<td>X66368.1</td>
<td>Pisum sativum psl gene for Ps1 lectin</td>
</tr>
<tr>
<td>Y00440.1</td>
<td>Pisum sativum lecA gene for lectin</td>
</tr>
<tr>
<td>M18160.1</td>
<td>Pea PSL1 gene encoding lectin, complete cds</td>
</tr>
<tr>
<td>J01254.1</td>
<td>pea lectin (alpha and beta subunits) mRNA</td>
</tr>
<tr>
<td>HQ337024.1</td>
<td>Pisum sativum lectin gene, 5’ upstream region</td>
</tr>
<tr>
<td>AY342212.1</td>
<td>Glycine max lectin (Le2) gene, complete cds</td>
</tr>
<tr>
<td>HM348715.1</td>
<td>Vigna radiata cv. Wileczek lectin gene, complete cds</td>
</tr>
<tr>
<td>AJ277588.1</td>
<td>Vigna unguiculata partial mRNA for lectin (lec gene)</td>
</tr>
<tr>
<td>AJ920065.1</td>
<td>Phaseolus vulgaris partial lec1 gene for lectin precursor</td>
</tr>
<tr>
<td>AJ318222.2</td>
<td>Lens odemensis lectin gene, clone Ode9-1</td>
</tr>
<tr>
<td>AJ318220.2</td>
<td>Lens ervoides lectin gene, clone Erv6-22</td>
</tr>
<tr>
<td>AJ419573.2</td>
<td>Lens lamottei lectin gene</td>
</tr>
<tr>
<td>AJ318221.3</td>
<td>Lens nigricans lectin gene, clone Nig7-42</td>
</tr>
<tr>
<td>AJ421799.2</td>
<td>Lens culinaris subsp. tomentosus lectin gene</td>
</tr>
<tr>
<td>AJ318219.2</td>
<td>Lens culinaris subsp. orientalis lectin gene, clone Ori4-31</td>
</tr>
<tr>
<td>AJ318218.2</td>
<td>Lens culinaris subsp. culinaris lectin gene, cv. Eston, clone Est1-17</td>
</tr>
<tr>
<td>AJ318217.2</td>
<td>Lens culinaris subsp. culinaris lectin gene, cv. Laird, clone Laird-71</td>
</tr>
<tr>
<td>DQ005103.1</td>
<td>Lens culinaris cv. L-4076 lectin mRNA, complete cds</td>
</tr>
<tr>
<td>AJ318216.1</td>
<td>Lens culinaris subsp. culinaris lectin gene, partial</td>
</tr>
<tr>
<td>AJ438490.2</td>
<td>Vicia faba lec2 gene for lectin</td>
</tr>
<tr>
<td>AJ438593.2</td>
<td>Vicia faba lec1 gene for lectin</td>
</tr>
</tbody>
</table>
Y00440.1 (recorded DNA sequence contained 12 nts deletion compared to our DNA sequence), 98% homology (651/661 nts identity) was observed with mRNA sequence of *Pisum sativum* L. lectin gene M18160.1 and the 98% homology (414/415 nts identity) was observed with mRNA sequence of *Pisum sativum* L. lectin gene J01254.1.

The results document that this sequence fragment corresponding to the garden pea (*Pisum sativum* L.) lectin gene is highly conservative and thus suited for designing of specific primers. Sequenced DNA includes border part of 5′-upstream region DNA (labeled black) with the exon part of lectin gene (Figure 1).

Primers Lec101 were designed to be specific to the exon part of lectin gene. Specificity of Lec101 primers was tested: (1) *in silico* by comparison with NCBI recorded DNA sequences corresponding to lectin gene in tested species of the family *Fabaceae* (Table 2); (2) experimentally in the conventional PCR after the assay had been optimised.
Figure 3. The alignment of reverse primer Lec101 with sequences of the lectin gene of selected species of the family Fabaceae. Target sequence for primer is underlined. Mismatches in target sequences are labelled in a black box.
Results of in silico analysis showed that both Lec101 primers show 100% homology only for the garden pea exon part of lectin gene (Y00440.1, J01254.1, M18160.1, EU825771.1, and X66368.1). However, both primers show certain homology to several clones of lentil, mung bean and vetch (HM348715.1, AJ318222.2, AJ318220.2, AJ419573.2, AJ318221.3, AJ421799.2, J318219.2, AJ318218.2, AJ318217.2, DQ005103.1, AJ318216.1, AJ438490.2, AJ438593.2). Mismatches were identified within available sequences corresponding to primer sites (Figures 2 and 3).

In experimental test, no PCR products were recorded when vetch, soya-bean, lentil and bean DNAs were used as template DNA, while amplification of all pea DNA isolates showed the expected amplification products (Figures 4 and 5).

To allow quantification, optimised protocol was used in real-time PCR with SYBR® Green detection system regardless its disadvantage, i.e. tendency to inhibit PCR at higher concentration (Karsai et al. 2002; Giglio et al. 2003; Gasparic et al. 2010).

SYBR® Green real-time PCR was specific also only for samples of garden pea – no amplification signal was recorded when the DNAs from the family Fabaceae were used as a template that confirmed previous results obtained by conventional PCR.

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SYBR® Green real-time PCR was specific also only for samples of garden pea – no amplification signal was recorded when the DNAs from the family Fabaceae were used as a template that confirmed previous results obtained by conventional PCR.

Obtained Ct values of reaction based on garden pea DNA (200 ng of DNA in reaction, corresponding to approx. 41 000 genome copies) reached on average 23.56 ± 0.36, which is an acceptable value for analytical assay (Figure 6).

Dissociation melting curves of final PCR products confirmed that reaction lead to a unique specific product amplification (Figure 7).

The specific product size of 101 bp was also checked by gel electrophoresis (data not shown). A difference of 3.23 Ct between the signal of pea samples at a DNA concentration of 40 ng/μl (23.28 Ct) and the same 10-fold diluted samples (26.51 Ct) exclude inhibition by template (Figure 8).

The results confirm that DNAs quality is fitting for the purpose.

Real-time PCR efficiency was calculated for genomic DNA as a template. The efficiency was 99% and the average value of $R^2$ was 0.997 (Figure 9).

On the base of dilution, limit of quantification was defined to be 160 copies of garden pea DNA. That is the lower limit of linear range, in which the reaction fulfils an acceptance criteria for real-time PCR based GMO quantification (ENGL 2008). The

Figure 6. Fluorescent signal of garden pea cultivars with the average value of 23.56 ± 0.36 Ct

Figure 7. Dissociation curves of real-time PCR, selected plants of the family Fabaceae and pea cultivars
Figure 8. Control of the reaction inhibition signal of pea samples at DNA concentration of 40 ng/µl (23.28 Ct) and the same 10-fold diluted samples (26.51 Ct)

Figure 9. Serial of dilution of genomic DNA for the calculation of real-time PCR efficiency
the limit of detection was revealed to be 40 copies of garden pea DNA.

As the reaction fulfill also criteria described by Ahmed (2002) and Anklam et al. (2002) we verified that the amplicon suits as a comparator for the quantification in real-time PCR.

In conclusion, we designed and optimised the real-time PCR based assay for specific detection and quantification of garden pea lectin gene (*P. sativum* L.). This reaction was developed to be used as a reference system for the quantification of GM pea varieties on the market. Although the SYBR Green assay is based on non-specific fluorescent detection, the characteristic of reaction meets requirements for GMO detection defined by the Community Reference Laboratory (CRL) for GM food and feed (ENGL 2008). The assay could be used as well for relative quantification of pathogens as it was shown for other species (Leisova et al. 2006) or allergens (Wensing et al. 2003). Moreover, positive plasmid control containing part of garden pea lectin gene is available and can be used according currently available standards.

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