Study of Extensin Gene Expression as a Candidate Responsible for Pea Pod Dehiscence

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Abstract: The aim of this work is the study of one of the key genetic principles in domestication traits in pea – pod dehiscence by comparative analysis of wild and domesticated pea genotypes. Pod dehiscence is one of various mechanisms of seed spreading into their surroundings for the purposes of species preservation. Wild pea after maturing pods suddenly opens and fruits are scattered far and wide. This undesirable characteristic is for humans problematic because collection pea pod when touched if bursts is difficult and may cause yield loss in legumes before or during harvest. Pea is an example of crops that have undergone a process of domestication. Non-pod dehiscence, non-dormant seeds or bigger seeds are domestication traits that differentiate cultural kinds from wild species peas. There was evaluated a group of several RILs formed by crossbreeding of contrast parent genotypes of wild JI 64 Pisum sativum ssp. elatius L. and cultural pea JI92 Pisum sativum ssp. sativum L. This work was preceded by the method called MACE (Massive Analysis of cDNA Ends) to identify differences in gene expression during pod maturation. The expression of a selected gene was determined using qRT-PCR for pod dehiscence.

Key Words: pea, pod dehiscence, qRT-PCR

Introduction

Domestication is a phenomenon which in wild-type plants causes genetic changes through selection controlled by man. It is an evolutionary process in which a result of the selection are plants changed genetic, morphological and physiological characteristics. The result of the domestication is a plant adapted to survive in culture conditions, and having characteristics that prefer producers and consumers. Domesticated plants are different from their wild ancestor in several morphophysiological characters, most of which are associated with seed retention and germination, growth habit, size and coloration (Ladizinsky 1998, Sakuma 2011).

Pea is one of the oldest legumes on the world. The legume seeds are contained in the pod, which is composed of a single seed-bearing carpel. Pea pod have a seam that runs along both sides that can split open. When matures, splits open along two seams, a process called pod dehiscence. Purpose is to propel seed from pot away from the plant (Gao and Zhu 2013, Ambrose et al. 2008).

The genetics of the dehiscence was first reported by Marx (Marx 1971) where he described pods as being tough and leathery and prone to dehiscence (Ambrose et al. 2008). Genetic nature of the loss of pod dehiscence during domestication of a legume was illustrated for example in soybeans (Dong et al. 2014). In this case SHAT1-5 gene was identified as domain NAC transcription factor. It is believed that this gene affects the process of the secondary cell wall thickening. For pea it is probably a similar mechanism of loss of pod dehiscence but with a different genetic nature. Wild pea exhibits full pod dehiscence upon maturity, while cultivated peas have indehiscent pods that allow all the seeds to be retained at maturity. Despite its long history as a genetic model system, dating to Mendel’s famous early studies, surprisingly little is known about pea domestication genes. Previously, it was a character of pod dehiscence located on LGIII in Dpo1 (Weeden 2007, Bordat et al. 2011), but the gene has not yet been published.
MATERIAL AND METHODS

Characterization of plant material and DNA extraction

Two lines of wild and cultivated pea and their 134 RILs were used for this research. Evaluated plants of pea (*Pisum L.*) were grown in the greenhouse conditions in Mendel University in Brno. As a parental lines were used lines obtained from the John Innes Centre, Norwich Research Park, Norwich, United Kingdom: JI64 (*Pisum sativum ssp. elatius L.*) wild pea with dehiscent pods and dormant seeds collected in Turkey and JI92 (*Pisum sativum ssp. sativum L.*), with indehiscent pods and non-dormant seeds and which we rank among the cultural pea. It is a landrace from Afghanistan. Recombinant inbred lines (RILs) were obtained by reciprocal crosses of both parental lines (North et al. 1989). The evaluation of pod dehiscence phenotype (indehiscent / dehiscent) is not clearly visible and is thus difficult.

For our study RNA of both parents as well as several selected RILs (indehiscent / dehiscent) of F6 were isolated from tissues directly related to the pod dehiscence – dorsal and ventral pod suture.

The RNA was isolated from pod sutures of three developmental stages: 10, 15 and 20 days after flowering using NucleoSpin RNA Plant kit (Machery-Nagel, Düren, Germany). RNA isolated from dehiscent/indehiscent RILs and their parents was used for Massive analysis of cDNA Ends (MACE) to identify differences in gene expression during pod maturation. The cDNA for qRT-PCR was obtained from isolated RNA using Promega (Madison, USA) chemicals.

PCR amplification

Expression of candidate gene for pod dehiscence was assigned using Real-Time qRT-PCR by using of LC 480 SYBR Green I Master kit (Roche, Basel, Switzerland). The forward primer sequences used: 5’- CACCGTCATCTACTACCCA -3’; reverse: 5’- ACCGACCAAAATTGTAATGGA -3’ and as a reference published specific primers for β-tubulin were used (Die et al. 2010). Based on qRT-PCR results the ΔCt values were calculated.

For detailed study of the candidate gene also genomic DNA of parents and RILs were used. Genomic DNA was isolated from young leaves by Invisorb Spin Plant Mini Kit (STRATEC Biomedical AG, Birkenfeld, Germany). Differences in candidate gene DNA sequence were tested by sequencing (Macrogen, Amsterdam, The Netherlands) of parent lines by PCR (MyTaq DNA Polymerase kit, Bioline, Taunton, Massachusetts, USA) with primers designed based on GenBank (National Center for Biotechnology Information) sequence JI917454 using Primer-BLAST tool.

RESULTS AND DISCUSSION

This work was focused on the evaluation of one candidate gene for pod dehiscence of pea. This candidate gene was predicted by the method MACE (Massive Analysis of cDNA Ends) which was used to identify differences in gene expression during pod maturation between parental lines and bulks of indehiscent / dehiscent RILs. Based on MACE results we identified 77 genes with different expression between indehiscent and dehiscent genotypes (minimal difference in expression fold log2>3). The gene with the highest difference in expression (our main candidate gene) was identified as an extensin-like protein (GenBank accession No.: JI917454.1). In parental lines we can see differences in the expression of the candidate gene, corresponding with contrasting phenotypes of pod dehiscence. Genotype with dehiscent pods (JI64) showed higher expression of the candidate gene and genotype with indehiscent pods (JI92) lower than the reference gene (Figure 1). However, it can be seen that differences in expression between the developmental stages are not significant as well as there is no significant difference between the expression of the dorsal and ventral seam. Therefore, RNA from RILs was isolated from the mixture of both seams.

Evaluated lines were divided based on phenotype into two groups (Table 1): indehiscent (Figure 2) and dehiscent (Figure 3).
Figure 1 Evaluation of qRT-PCR: expression of candidate gene for pod dehiscence in parental pea lines

Table 1 RILs divided into groups according to phenotype

<table>
<thead>
<tr>
<th>Indehiscent lines</th>
<th>Dehiscent lines</th>
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<tr>
<td>16 64x92</td>
<td>23 64x92</td>
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<tr>
<td>45 64x92</td>
<td>42a 64x92</td>
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<td>61 64x92</td>
<td>60 64x92</td>
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<td>63 64x92</td>
<td>93 64x92</td>
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<td>99 64x92</td>
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In Figure 4 expression of the candidate gene in evaluated RILs can be seen. It is obvious that indehiscent samples tested for expression of the candidate gene have lower expression than the reference gene. The opposite was observed in dehiscent samples where gene expression of the candidate gene was higher compared to the reference gene. Between the values of indehiscent and dehiscent test samples is thus a significant difference. Except for one line (60 64x92) all others correspond in phenotypic evaluation of pod dehiscence with results of gene expression (Table 1). The evaluation of electrophoresis of the line 60 64x92 revealed its heterozygous state. It may explain the differences in phenotypic and genotypic evaluation. In dehiscent RILs the expression is slightly higher than the reference gene, except of line 23 62x92, which has a very high value. All indehiscent lines have values lower than the reference gene.

Identification of new markers for domestication traits is in the interests of breeders for many years. The reason is the possible use of the potential of landraces, which contain in their genome a variety of resistance genes. But landrace crops often have traits not suitable for cultivation. In the case of legumes one of the most undesirable is dehiscence of matured pods causing a yield loss.
For example, in *Arabidopsis thaliana* has already been identified the gene *SHATTERPROOF (SHAT)*, which controls the deposition of lignin during maturation of the fruit. Also another gene *INDEHISCENT (IDEH)* was identified, which belongs to regulators of formation of lignified layer of the fruit (Fourquin et al. 2013).

*Figure 4 Evaluation of qRT-PCR: expression of the candidate gene for pod dehiscence in tested pea samples (ΔCt)*

The genetic basis of pod dehiscence loss during domestication was recently explained in soybeans, where the identified gene *SHATTERING 1-5* (Dong et al. 2014) functionally activates creation of the secondary cell wall.

Funatsuki et al. (2014) identified a gene *Pdh1* controlling dehiscence of the soybean pod, which supports deposition of lignin to the cells of endocarp. These genes are the only identified genes associated with a domestication of legumes. In case of peas is in cultural forms probably similar mechanism of pod dehiscence loss but with a different genetic basis. In the studies of Weeden et al. (2009) and Weeden (2007) the authors claim that pod dehiscence of pea is controlled by two loci, semidominant and monogenic *Dpo1* locus (binding group LGIII) and by *Dpo2* locus (found only in some crossbreeds) (Bordat et al. 2011) but the specific gene is not yet published. Based on comparison of our candidate gene position in *Medicago truncatula* genome and SNPs map which was created by Tayeh et al. (2015) we recognized that our candidate gene is present on pea LGIII in the estimated region of *Dpo1* locus. This fact corresponds with Weeden (2007) and Bordat et al. (2011).

**CONCLUSION**

The expression of our candidate gene for dehiscent pea pod by qRT-PCR in JI64 (*Pisum sativum ssp. elatius* L.), JI92 (Afghan cultural pea *Pisum sativum ssp. sativum* L.) and their recombinant inbred lines (RILs) created from reciprocal crossing was evaluated. Gene expression in lines with dehiscent and indehiscent pea pods were detected. Lines with dehiscent pods showed expression higher than the reference gene and conversely, lines with indehiscent pods showed lower expression.

This candidate gene is present on pea LGIII in the estimated region of *Dpo1* locus and could therefore be responsible for pod dehiscence in peas.

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