18 Induced Mutagenesis for Improvement of Bean (*Phaseolus vulgaris* L.) Production in Bulgaria

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Abstract

Although historically a surplus food producer, Bulgarian agriculture has faced a downturn in recent decades. Local legume cultivars have lost favour with farmers and the canning industry, due to their low productivity in comparison with imported ones. Diseases and abiotic sstresses are the most important factors limiting the production of edible legumes, costing farmers hundreds of euros in lost revenue each year. The overall objective of our ongoing bean mutation breeding programme was to enrich the gene pool of *Phaseolus vulgaris* L. and to develop genotypes resistant to *Xanthomonas axonopodis* pv. *phaseoli* (Smith) (*Xap*) and *Pseudomonas savastanoi* pv. *phaseolicola* (Burkh.) (*Psp*) using EMS. The two, an elite line and most common cultivar (an heirloom and a snap bean type) in Bulgaria, were selected as parents and the chemical mutagen EMS was used for generating mutations. In total, 1000 seeds were treated and the two generated M_1 populations were grown in the field. All M_2 mutant plants (1650 from initial line IP564 and 2420 from initial cultivar 'Mastien 11b') were grown in field conditions and a number of phenotypic changes were observed on these mutated plants. They were also screened for *Xap* disease resistance via leaf artificial inoculation under greenhouse conditions. Individual plant selection was performed for the putatively resistant M_2 plants. In the M_3 generation these lines were screened using artificial inoculation with *Xap* and *Psp* pathogens (leaves and pods) under field conditions. Selected M_3–M_4 lines with confirmed disease resistance were tested for fresh pod quality. Yield tests were started in M_4 and M_5 generations and, according to their yield, mutants were advanced to the M_6/M_7 generation for validation. The expression patterns of genes putatively involved in the resistance reactions towards two races of *Psp* were determined using qRT-PCR for the specific and reference genes. In conclusion, 50 plants with visible morphological changes and/or increased tolerance to the two targeted bacterial diseases were selected. A total of 20 advanced mutant bean lines are currently being evaluated for their competitiveness in multiple sites.

Keywords: common bean • induced mutagenesis • disease resistance • halo blight • common bacterial blight

1 Introduction

Common bean is a very important component of the diets of people in several continents, representing an important source of minerals and proteins (Gepts *et al.*, 2008). Consumption of dry and vegetable (snap) beans in Bulgaria has its traditions long back in the country's history and it continues to rise in response to consumer demand and scientific recognition of beans as a major health food. In addition to being high in fibre and protein, beans serve as an important natural

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source of folate and other B-vitamins, minerals and antioxidants. Snap beans are produced in almost every region of Bulgaria chiefly on small and medium-size farms, and dry beans are produced mainly in Southern Dobrudja, a hilly region with an average altitude of about 200–300 m and a continental climate; these beans are part of crop rotations and intercropping systems.

Common bean is exposed to a large number of diverse yield constraints during its growth. Diseases and abiotic stresses (drought, soil compaction, low soil fertility, high temperatures, etc.) are the most important factors limiting the production of beans and thus contributing to the large gap between actual and potential yield, costing farmers hundreds of euros in lost revenue each year. Sustainable agriculture requires reduced pesticide use, so cultivars with better pathogen tolerance are not only environmentally friendly but they also lower costs and raise product quality. Genetic resistance provides the best means of controlling common bacterial blight, a devastating seed-borne disease caused by *Xanthomonas axonopodis* pv. *phaseoli*, which plagues bean production in South-east Europe (Sofkova *et al.*, 2009; Singh and Miklas, 2015). Resistant cultivars can be selected from naturally occurring variation or induced mutants.

Common bean possesses a high level of natural mutations (Rukmanski, 2005). Induced mutagenesis was applied as a method for generating novel genetic diversity and obtaining new bean lines or cultivars in Bulgaria (Svetleva *et al.*, 1999a,b). Chemical mutagenesis in common bean is efficient and reliable. Although different agents have been used, mutagenesis with ethyl methanesulfonate (EMS) has been the most successful for different breeding traits, including increased yield and nodulation (Park and Buttery, 1988; Svetleva, 2004; Ramandeep *et al.*, 2018). EMS alkylates primarily guanine (G), leading to mispairing; alkylated G pairs to thymine (T) instead of cytosine (C). EMS mutations can be useful in obtaining genotypes with higher yield potential, or with altered plant architecture so that they are suitable for mechanical harvesting or resistant to lodging, or those that are early ripening, have increased levels of protein and beta-carotene, are disease-, pest- and drought-resistant, or that have ecological plasticity (Tomlekova *et al.*, 2014a,b).

For the past few years our breeding efforts have been directed towards developing bean cultivars with improved disease resistance. The overall objective of our common bean programme is to enrich the gene pool of *P. vulg*aris L. through developing new genotypes for breeding by using induced mutagenesis. One of the sub-objectives is to develop and identify mutant bean genotypes resistant to *Pseudomonas savastanoi* pv. *phaseolicola* (*Psp*) and *X. a.* pv. *phaseoli* (*Xap*) bacterial diseases. Moreover, we aimed to study the expression patterns of genes putatively involved in the resistance reaction to *Psp* by using quantitative reverse transcription polymerase chain reaction (qRT-PCR). The qRT-PCR has allowed for the calculation of differential gene expression in different organs or tissues under several treatments using cDNA molecules synthesized from mRNA (Heid *et al.*, 1996; Bustin, 2002). qRT-PCR is noted for its accuracy, precision and relative ease of use due to its speed, sensitivity and specificity (Reece, 2004; Bustin *et al.*, 2009). Given these features, we decided to use this method in our study.

2 Materials and Methods

2.1 Plant material

The line IP564 was developed in the Maritsa Vegetable Crops Research Institute (VCRI), Plovdiv, by a phased sexual hybridization between multiple parental components. Plants are characterized by a determinate type of growth with height of 50–55 cm, with a strong and erect stem and medium-size shrub. It has cylindrical, long (13–15 cm), straight green beans. The vegetation period is on average 48–50 days from germination to the occurrence of technological maturity of the pods. The seeds are white, kidney- shaped and of medium size (30.5 g/100 seeds). Plants of line IP564 have resistance to anthracnose (*Colletotrichum lindemuthianum* races 6 and 81), rust (*Uromyces appendiculatus* races 20-0, 20-2 and 20-3) and bean common mosaic virus (BCMV), but are moderately susceptible to halo blight (*P. savastanoi* pv. *phaseolicola* (Burkh.) Gardan *et al.* races 1 and 6) (*Psp*) and sensitive to the agent of bacterial blight (*Xanthomonas axanopodis* pv. *phaseoli*) (*Xap*).

Cultivar 'Mastilen 11b' was developed in the VCRI from the local Mastilen population. The plant

has a determinate growth habit and average height about 40–45 cm, with good foliage distribution. The stem is erect with 6–8 branches, each typically approximately 18–20 cm in length. Pods are set in the lower part of the plant and some of them rest on the ground. The leaves are dark green and large. Lamina is ovate, with a rather pointed tip, 10–12 cm long and 7–10 cm wide. The flowers are purple and of medium size. Pods are flat, broad, slightly curved; the main colour is green with anthocyanin patterns. Dimensions of pods are: length 11–14 cm, width 13–16 mm. One peduncle bears on average two to three pods. At harvest pods are fragile, juicy, with small cavities without fibres and without a sclerenchyma layer and they have an average of four to five seeds per pod. Seeds are of medium size, ellipsoidal, convex, with a main colour of beige-violet and anthocyanin patterns, and weigh about 35.5 g/100 seeds. The hilum is white with a light brown halo. Days to maturity are 45–50 on average. 'Mastilen 11b' is sensitive to bacterial blight *Xap* and halo blight *Psp*.

2.2 Mutation breeding towards tolerance to bacterial pathogens

Initially, 100 seeds (M_0) from parent genotypes (line IP564 and cv. 'Mastilen 11b') were treated with five different concentrations of EMS. All plants from the M_1 generation were grown in the field, and then half of the M_2 seeds obtained from each M_1 plant were used for disease screening in the greenhouse; the other half were grown for seed reproduction.

In the M_2 generation, mutant lines with differential disease reaction were selected and further screened for disease resistance in the field. Identified disease-resistant M_3–M_4 lines were tested for grain quality. Yield tests were started in the M_4 and M_5 generations, according to quality results.

Our molecular breeding approach was based on the expression analysis of plant pathogenesis-related proteins (PRP) in M_6 advanced mutant lines.

EMS-treatment assay

The mutagenesis protocol was first established in the molecular biology laboratory of the Maritsa VCRI with the assistance and guidance of Professors Christov and Svetleva. One hundred seeds for every treatment from two snap bean genotypes were treated with five different EMS concentrations (1.55, 3.1, 6.2, 12.4 and 24.8 mM) for 6 h at room temperature with slow shaking, adding 1% methanol as an intermediary solvent. As a result of an assay conducted before treatment, the time of absorption of water by the seeds was assessed and the protocol was modified; the treatment with EMS was conducted for 6 h. Before the present study, the reported results for the number of grown seedlings, survived plants and plants with formed seeds helped to establish protocols for the treatment of bean seeds with EMS (Svetleva, 2004; Christov *et al.*, 2014). A 30-min washing with running tap water was performed after treatment. The untreated seeds were pre-soaked in distilled water and were used as control. Seeds were rinsed and sown in the field in a complete randomized-block design (CRBD) with two replicates, to the first mutant (M_1) generation. Each plot had two rows 3 m long with a row-to-row and plant-to-plant distance of 70 cm and 10 cm, respectively.

Inoculum preparation

Bacterial cultures of *X. axanopodis* pv. *phaseoli* (*Xap*) and races 1 and 6 of *P. syringae* pv. *phaseolicola* (*Psp*) were provided from the collection of Dobrudja Agricultural Institute General Toshevo, and propagation potato dextrose agar medium at the Laboratory of Phytopathology of Institute of Plant Genetic Recourses 'Konstatin Malkov', Sadovo. For the inoculation treatment, a 2-days-old bacterial suspension of 10^8 cfu/ml, further adjusted on optical spectrophotometer on wavelength 600 nm to OD = 0.3, was used to inoculate the fully expanded first leaf of each plant using multiple needle technique as described by Aggour *et al*. (1989).

Mutants in M_2–M_4 progenies were screened for *Xap* and *Psp* disease resistance by field inoculation of fully developed trifoliate leaves and green immature pods by pricking with a syringe according to the method of Aggour *et al*. (1989).

Bioassays

M_6 plants from four mutant lines were inoculated with *Psp* bacteria under greenhouse conditions using the same methods as described above. Parental line IP564 was used as a positive control due to its resistance to race 1 and moderate suitability to

race 2 of *Psp* (unpublished data). Ten plants were planted in potting mix under controlled conditions at 22/16°C (day/night) and ca. 80% humidity.

Symptoms were recorded in the inoculated area 10 days after inoculation by using a 1–9 severity scale where 1 = no visible symptoms and 9 = very severe symptoms (Aggour *et al.*, 1989). Leaves from five plants from untreated control and inoculated treatments were separately collected at different periods: 6, 24, 48 and 74 h post-inoculation (hpi). Finally, each sample represented a bulk from five individual plants. In addition, the experiment was repeated twice and another total RNA bulk was obtained, resulting in two biologically replicated experimental bulks. Plant materials were immediately frozen in liquid nitrogen and maintained at –80°C until RNA isolation.

2.3 Gene selection and primer design

The following target PRP genes were chosen for the experiment: *FLS2*, *FLS2.1*, *FLS2.3*, *RPG1-B* (AAR19097.1), *PTO* (AAK52036.1) and *PTO1* (Table 18.1). The gene sequences used in this study were obtained through bibliographical reviews of studies involving biotic stresses in common bean (Trabanco *et al.*, 2014). All of the target genes contained typical protein domains involved in resistance responses, such as leucine-rich repeat (LRR) domains. Genes that encode typical R proteins co-localized with the major resistance genes *Pse-3* and *Pse-1* (at the end of LGs Pv02 and Pv10).

Reference genes actin (*act*11) and ß-tubulin (*tub*8) were selected according to Borges *et al.* (2011).

The gene-specific primers were designed by Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) using default parameters and were ordered from EUROGENTEC (Belgium). Primer specificity was checked with end-point polymerase chain reaction using bean cDNA or gDNA as template (Table 18.2).

2.4 Quantitative RT-PCR

Total RNA extraction

For each sample, 100 mg of plant material was crushed in 1 ml of TRI REAGENT® (MRC) and the total RNA was extracted following the manufacturer's recommendations. DNAse I (Life Technologies) treatment was applied to remove any residual DNA contamination. RNA quantity was evaluated spectrophotometrically (IMPLEN) at 260 nm and purity was determined by evaluating the absorbance ratio at 260/280 nm. RNA quality was verified on 1.0 % agarose gel for the presence of the two predominant intact bands corresponding to 28S and 18S rRNA.

First strand cDNA synthesis

For each sample, 2.5 µl (5 µg) total RNA was added to: 4 µl of 5× Reaction Buffer (Thermo Scientific®); 1 µl of RiboLock RNase Inhibitor (20 U/µl); 2 µl of 10 mM dNTP Mix and 1 µl (200 U/µl) of MuLV Reverse Transcriptase (Thermo Scientific® RevertAid H minus First Strand cDNA Synthesis Kit), following procedures recommended by the manufacturer. The reverse transcription reaction was performed at 42°C for 1 h. The reverse transcription reaction was either used directly in further PCR applications or stored at –20°C.

qRT-PCR

Quantitative reverse transcription polymerase chain reactions for the specific and reference genes were carried out on a CFX96 cycler (Bio-Rad) in one step using 2.5 µl of cDNA (100 ng), 12.5 µl 2X SYBR Go Taq® qPCR Mastermix (Promega) and 300 nM of each primer in final volume of 25 µl. Two technical replicates were performed. Melting curve analysis was applied in order to verify primer specificity. A primer efficiency test was performed using tenfold serial dilution.

2.5 Data analyses

Raw data of fluorescence levels was processed and C_T values were provided from Bio-Rad Manager software. This programme performs baseline correction and linear regression analysis on each amplification curve. Further on, this data was used to calculate the relative gene expression of the target and reference genes using the equation described by Livak and Schmittgen (2001) for the $2^{-\Delta\Delta C}{}_T$ method:

$$\Delta\Delta C_T = (C_{T,\ \text{Target gene}} - C_{T,\ \text{reference gene}})_{\text{Time X}} - (C_{T,\ \text{Target gene}} - C_{T,\ \text{reference gene}})_{\text{Time 0}}$$

Table 18.1. Genes involved in the resistance to *Pseudomonas savastanoi* pv. *phaseolicola*.

Psp resistance genes	Species of origin	Genebank Accession no.	Identified candidate gene	Annotated function in *P. vulgaris* genome	Identity %	E value	Physical location	LG	QTL mapped
FLS2	*A. thaliana*	NP_199445.1a	Phvul.004G136500.1	LRR receptor serine/ threonine protein kinase	31.1	$5.4e^{-102}$	Chr04: 41556561–41560351	4	*Psp*4^{812XC}
FLS2.1	*A. thaliana*	NP_199445.1a	Phvul.004G175700.1	LRR receptor Serine/ threonine protein kinase	30.0	$3.4e^{-103}$	Chr04: 45622442–45626397	4	*Psp*4^{812XC}
FLS2.3	*A. thaliana*	NP_199445.1a	Phvul.004G175900.1	LRR receptor Serine/ threonine protein kinase	30.5	$3.6e^{-96}$	Chr04: 45637544–45641336	4	*Psp*4^{812XC}
Pto	*G. max*	NP_001241285.1a	Phvul.004G164000.1	Serine/threonine protein kinase	45.9	$8.5e^{-65}$	Chr04: 44601151–44605163	4	*Psp*4^{812XC}
Pto1	*P. vulgaris*	AAK52036.1	Phvul.006G080500.1	Serine/threonine protein kinase	46.3	$4.7e^{-50}$	Chr06: 19953718–19967156	6	*Psp*6^{812XC}
RPG1-B	*G. max*	AAR19097.1	Phvul.006G066800.1	LRR-containing protein ADP binding	57.7	0	Chr06: 18542599–18546221	6	*Psp*6812XC

[a] Sequences obtained from RefSeq database

Table 18.2. Primers designed for qRT-PCR analyses.

Gene	Expected size (bp)	Specific primer sequence
PvFLS2 Phvul.004G136500.1 LRR receptor Serine/ threonine protein kinase	102	*PvFLS2*-D CAACCTCATCCCTGGTGACT *PvFLS2*-R TCAAGAGGAACCTTGCCATC
PvFLS2.1 Phvul.004G175700.1 LRR receptor serine/ threonine protein kinase	119	*PvFLS2.1*-D GCATGCTTTCTCAAACCACA *PvFLS2.1*-R TTTCTGGGATTTCCCCTACC
PvFLS2.3 Phvul.004G175900.1 LRR receptor serine/ threonine protein kinase	120	*PvFLS2.3*-D TCCTTCCAAGTTCCCATCAG *PvFLS2.3*-R TTCTCCAAAGCCACCATTTC
PvPto Phvul.004g164000.1 Serine/threonine protein kinase	115	*PvPTO*-D TGGTGAACCACTTCCTCCTC *PvPTO*-R AAGGAGGTTCGCATCAGAGA
PvPto.1 Phvul.006G080500.1 Serine/threonine protein kinase	108	*PvPTO.1*-D GATCGGCTTCAATCTTTTGG *PvPTO.1*-R CAAGCAATTGCCCCTACAAT
RPG1-B Phvul.006G066800.1 LRR –containing protein ADP binding	109	*PvRPG1-B*-D CAAGCCAAAGGGGTGATCTA *PvRPG1-B*-R TCAGATCCAACAACCAACGA
Actin-1 (reference gene 1)	190	*Act11*-D TGCATACGTTGGTGATGAGG *Act11*-R AGCCTTGGGGTTAAGAGGAG
Tubulin beta-8 (reference gene 2)	163	*Tub8*-D AATGTGAAGTCCAGCGTGTG *Tub8*-R CTTCCCCAGTGTACCAATGC

3 Results and discussion

3.1 Mutagenesis, plant maintenance and phenotype screening

The highest number of changes in the morphology of the plant was recorded at 6.2 mM mutagen concentration.

Initial treatment by EMS of M_0 seeds was followed by propagation from M_1 to M_6 generation.

In the M_2 generation, 218 mutants derived from the line IP564 and cultivar 'Mastilen 11b' were selected for their morphological changes and/or better tolerance to bacterial blight (Fig. 18.1). After artificial infection with isolate *Xap,* 124 mutagenized plants of IP564 and 22 plants of 'Mastilen 11b' were selected in which morphological changes were observed in combination with a better response of tolerance to bacterial blight compared with the initial line.

Morphological description of elite plants was performed and seeds were collected from all of them. EMS treatment resulted in a number of phenotypic changes in the progeny (Fig. 18.2).

The following phenotypic changes compared with the initial genotype were observed.

- type of growth: from determinate to indeterminate and transformation of the apical bud from an inflorescence meristem to a vegetative meristem in mutant plants originating from IP564;
- length and number of internodes, fruit stems and branches;
- colour of flowers from magenta to white in mutants from the initial cultivar 'Mastilen 11b';
- colour and shape of the pods (from green to dark green, green with purple stripes and/or spots on the branches of the initial IP564 and vice versa) in mutants of the initial cultivar 'Mastilen 11b'; and
- colour, size and shape of the seeds.

As a result of the field screening conducted with *Xap* inoculation, 1000 M_3 plants from 216 M_2

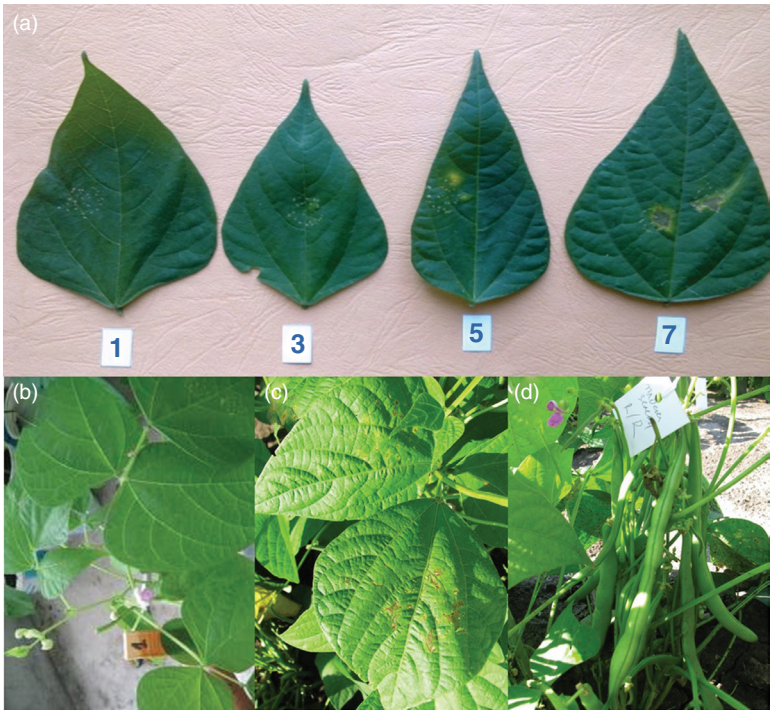

Fig. 18.1. (a) Descriptive assessment scale for a bean plant infected with *Xanthomonas axonopodis* pv. *phaseoli (Xap*). **(b, c, d)** Differential reaction on leaves and pods of the mutant bean lines 14 days after inoculation with *Xap*.

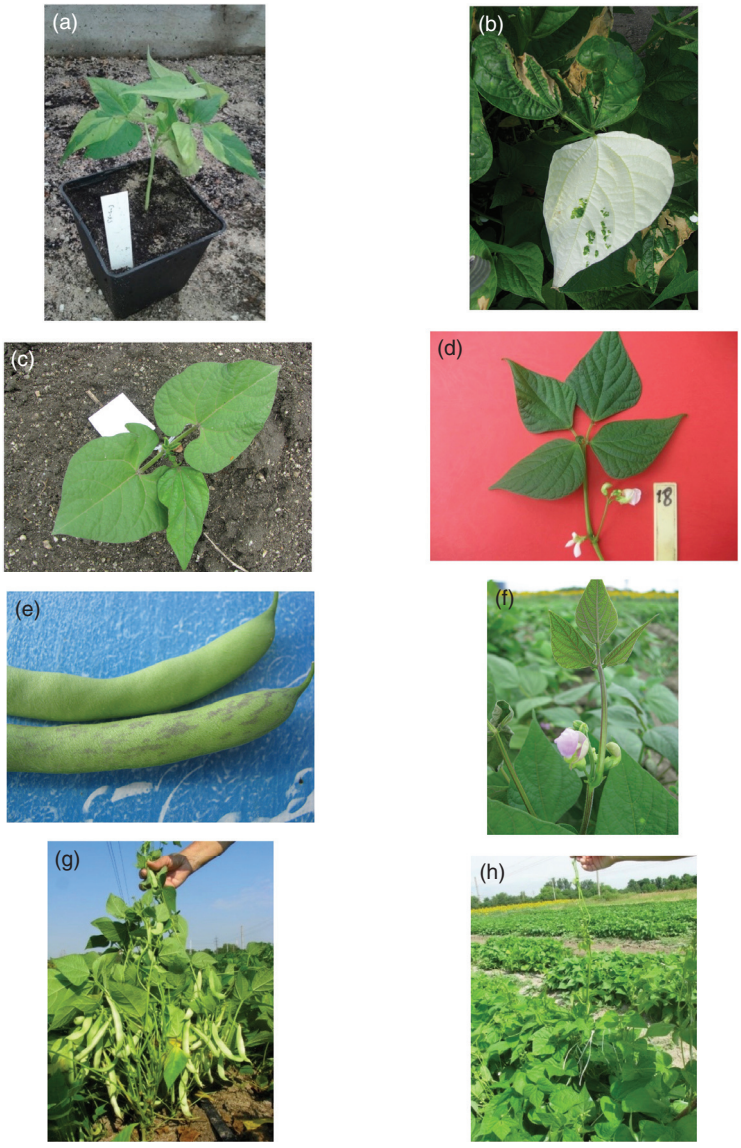

Fig. 18.2. Observed phenotypic changes in mutant bean plants of M_2 and M_3 generations obtained by treatment with EMS. **(a)** Leaf chlorophyll mutation. **(b)** Albino mutation. **(c)** Unifoliate seedling. **(d)** Flower coloration. **(e), (f)** Plant habit mutation. **(g)** Multi-pinnate leaf mutation. **(h)** Pod coloration.

mutant lines with phenotypic changes, confirmed in the M_3 generation, had in addition increased tolerance to bacterial blight compared with the initial lines (Table 18.3).

The results from the extended screens for pathogen resistance at Dobrudja Agricultural Institute's field facilities for disease screening are presented in Table 18.4. Seventeen lines have shown an increase in their degree of resistance against both pathogens. These results demonstrate that EMS mutagenesis is an efficient approach to generate a significant number of

Table 18.3. List of the bean (*P. vulgaris* L.) mutant lines identified with multiple resistance of leaves and pods to *Pseudomonas savastanoi* pv. *phaseolicola* (*Psp*) races 1 and 6, and two isolates of *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*) according to the inoculation assays.

	Pseudomonas savastanoi pv. *phaseolicola*				*Xanthomonas axonopodis* pv. *phaseoli*			
	Race 1		Race 6		XB96221		XB99132	
Ref # 2014	leaf	pods	leaf	pods	leaf	pods	leaf	pods
Artificial inoculation under field conditions								
34-1-1	R	R	R	R	R	MR	MR	MS
34-1-2	R	R	R	MR	R	MS	MR	S
43-1-1	R	R	R	MR	R	MR	MR	MS
46-1-1	R	MR	R	R	R	I	MR	R
175-3-1	R	R	R	R	R	MS	R	MS
175-3-3	R	I	R	R	R	R	MR	R
175-3-4	R	I	R	I	R	MS	R	MR
190-1-1	R	R	R	R	MR	MR	R	MR
190-3-6	R	R	R	MR	R	S	R	S
190-4-1	R	R	R	R	R	S	R	S
191-1-1	R	MR	R	R	R	MS	MR	MS
193-9-2	R	R	R	MS	R	S	MR	S
Artificial inoculation under greenhouse conditions								
564-5-1	R	R	R	R	R	MR	MR	MS
564-22-1	R	R	R	MR	R	MS	MR	S
564-32-1	R	R	R	MR	R	MR	MR	MS
564-42-2	R	MR	R	R	R	I	MR	R
564-60-1	R	R	R	R	R	MS	R	MS
564-69-1	R	MR	R	R	MR	MR	MR	MR
564-69-3	R	MR	R	R	R	MR	R	MR
564-69-4	R	MR	R	R	R	MR	R	MR
564-69-5	R		R	R	R	MR	R	MR
564-69-6	R	MR	R	R	R	MR	R	MR
564-69-7	R	MR	R	R	R	MR	R	MR
564-69-8	R	MR	R	R	R	MR	R	MR
564-69-12	R	MR	R	R	R	MR	R	MR
564-69-32	R	R	R	R	MR	MS	MS	MS
564-69-35	R	MR	R	R	MR	MS	MS	MS
564-71-1	R	I	R	R	R	R	MR	R
564-74-4	R	I	R	I	R	MS	R	MR
564-75-1	R	R	R	R	MR	MR	R	MR
564-85-1	R	R	R	MR	R	S	R	S
564-89-1	R	R	R	R	R	S	R	S
564-110-1	R	MR	R	R	R	MS	MR	MS
564-161-15	R	R	R	R	R	S	R	S
564-12-1	R	MR	R	R	R	MS	MR	MS

Abbreviations: I, immune; R, resistant; MR, moderately resistant; MS, moderately sensitive; S, sensitive

mutants with multiple resistances. Seeds from selected mutants were collected to study their agronomic and yield characteristics in a field trial. Screening for phenotype, grain quality and yield was performed in the M_4–M_5 generations.

3.2 Molecular assays

Halo blight is an economically significant disease of leguminous agricultural crops caused by the Gram-negative bacterial pathogen *Pseudomonas*

Table 18.4. List of bean (*P. vulgaris* L.) mutant lines identified with multiple resistance of leaves and seeds to *Pseudomonas savastanoi* pv. *phaseolicola* (*Psp*) races 1 and 6, and two isolates of *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*).

		Pseudomonas savastanoi pv. *phaseolicola*				*Xanthomonas axonopodis* pv. *phaseoli*			
Ref №	Ref №	Race 1		Race 6		XB96221		XB99132	
2014	2013	leaf	seed	leaf	seed	leaf	seed	leaf	seed
16-1	34-1-1	R	R	R	R	R	MR	MR	MS
16-2	34-1-2	R	R	R	MR	R	MS	MR	S
18	43-1-1	R	R	R	MR	R	MR	MR	MS
20	46-1-1	R	MR	R	R	R	I	MR	R
31-1	56-4-1	R	R	R	MS	R	HS	R	HS
31-2	56-4-2	R	R	R	R	R	HS	R	HS
31-3	56-4-3	R	R	R	MS	R	MR	R	MR
32	56-5-1	R	R	R	MR	R	HS	MR	S
33	56-12-1	R	R	R	MR	R	HS	MR	S
38	64-1-1	R	R	R	R	R	S	MR	S
49	70-9-1	R	R	R	MR	MR	HS	MR	HS
53	73-1-1	R	MS	R	S	R	HS	MR	HS
54-1	73-2-1	R	MS	R	HS	R	HS	MR	HS
54-2	73-2-2	R	MS	R	S	R	MS	MR	S
55-1	73-3-1	R	MS	R	MS	R	S	MR	S
55-2	73-3-2	R	MR	R	MS	R	HS	MR	HS
55-3	73-3-3	R	I	R	MR	R	MR	MR	MS
55-4	73-3-4	R	MS	R	MS	R	HS	MR	HS
55-5	73-3-5	R	R	R	MR	R	HS	MR	S
55-6	73-3-6	R	MR	R	MS	R	HS	MR	HS
55-7	73-3-7	R	R	R	S	R	HS	MR	HS
57	73-5-1	R	R	R	MS	R	HS	MR	HS
58-1	73-6-1	R	R	R	MR	R	HS	MR	S
58-2	73-6-2	R	R	R	MS	R	HS	MR	HS
58-3	73-6-3	R	MR	R	MS	R	HS	MR	S
61	74-3-1	R	R	R	MS	R	HS	MR	S
63	74-6-1	R	MR	R	MR	R	HS	MR	S
93	172-2-1	R	R	R	R	R	MS	MR	MS
98-1	175-3-1	R	R	R	R	R	MS	R	MS
98-2	175-3-2	R	I	R	MR	R	S	MR	S
98-3	175-3-3	R	I	R	R	R	R	MR	R
98-4	175-3-4	R	I	R	I	R	MS	R	MR
118	190-1-1	R	R	R	R	MR	MR	R	MR
120-1	190-3-1	R	R	R	R	R	MR	R	MS
120-2	190-3-2	R	R	R	R	R	MR	R	MR
120-3	190-3-3	R	I	R	R	R	MR	R	MS
120-4	190-3-4	R	R	R	MR	R	MR	R	MS
120-5	190-3-5	R	MR	R	R	R	MR	R	MS
120-6	190-3-6	R	R	R	MR	R	S	R	S
120-7	190-3-7	R	R	R	R	R	R	R	R
121	190-4-1	R	R	R	R	R	S	R	S
122-1	191-1-1	R	MR	R	R	R	MS	MR	MS
122-2	191-1-2	R	MR	R	MR	R	S	R	S
125-1	192-2-1	R	S	R	S	MR	HS	R	HS
125-2	192-2-2	R	MS	R	MS	R	S	R	S
126	192-3-1	R	R	R	R	R	MR	MR	MR
132-1	193-9-1	R	R	R	MS	R	MS	MR	MS
132-2	193-9-2	R	R	R	MS	R	S	MR	S

Abbreviations: I, immune; R, resistant; MR, moderately resistant; MS, moderately sensitive; S, sensitive; HS, highly sensitive

syringae pv. *phaseolicola* (*Psp*) (Romantschuk and Bamford, 1986).

An integral part of the research was to study: (i) the variation in the expression of selected common bean mutants and its relationship with resistance to *Psp*; (ii) the expression levels of six genes involved in the plant-pathogen resistance at different time points after inoculation with *Psp* (races 1 and 6); and (iii) the differences in gene expression between races 1 and 6 of *Psp* with different levels of aggressiveness.

Five advanced mutant lines derived from IP564 initial genotype were selected for this analysis based on their resistance to *Psp*. They were inoculated with races 1 and 6 of *Psp* and served as an mRNA source together with non-inoculated plants from each mutant line used as control. The susceptible initial genotype showed halo blight reaction and the mutant lines varied in their reaction to the pathogen races from resistance to moderate resistance, as expected.

A number of selected genes (LRR receptor serine/threonine protein kinase; serine/threonine protein kinase/LRR-containing protein ADP binding) potentially involved in *Psp* resistance were used (Table 18.1).

Relative quantification of gene expression was analysed using the comparative Ct method. The Ct value of one target gene was compared with another reference gene *Tub8* (e.g. housekeeping gene) using the formula $2^{-\Delta\Delta CT}$ in a single sample.

Quantitative PCR analysis of reference genes

Selection of reference genes is an essential consideration to increase the precision and quality of relative expression analysis by the qRT-PCR method. For validating the comparative Ct method, the efficiency (E) of the target amplification (gene of interest) and the efficiency of the reference amplification must be approximately equal. The efficiency was then calculated based on the curve slope: $E\ (\%) = [(10^{-1/\text{slope}}) - 1] \times 100$. The desired amplification efficiencies range from 90% to 110%.

It was found that both reference genes showed high qRT-PCR efficiency rates; for *Act11* = 97.1 %; and *Tub8* = 103.8 with correlation ($R \geq 0.999$) (Fig. 18.3). However, *Tub8* performed with better stability under both inoculated and controlled (not inoculated) conditions. Thus, *Tub8* was selected as the preferred reference gene for the expression study in the common bean/*Psp* pathosystem.

By studying gene expression with real-time polymerase chain reaction (qRT-PCR), we aimed to investigate changes (increases or decreases) in the expression of the set of genes by measuring the level of gene-specific transcripts. The investigation monitored the response of each gene to the inoculation with two races of *Psp*, after 24, 48 and 72 h post inoculation.

The relative gene expression levels in the M_6 lines showed differential regulation of all selected transcripts upon *Psp* pathogen inoculation Figs 18.4 and 18.5. The expression levels of the target genes were dependent on the genotype, pathogen race level of aggressiveness and time post inoculation.

Plant interaction with race 1 of Psp

The transcripts of the selected genes responding to different time points were detected in resistant parental genotype and susceptible mutants but at different levels (Fig. 18.4). Overall, we observed greater induction of gene transcripts in 48 hpi and 72 hpi of interaction with race 1 of the pathogen compared with the 24 hpi. Having said that, *FSL2*, *FSL2.3*, *PTO1* and *RBG1-B* showed enhanced expression in inoculated resistant mutants compared with inoculated susceptible parental genotype at every time point after inoculation; the expression was significantly upregulated in all three time points for one mutant only (M192).

In contrast, *FLS2.2* and *PTO* showed inconsistent and low expression in challenged mutants until 72 h after infection, whereas infected susceptible parental genotype showed enhanced expression after 24 h post infection.

The result of expression analysis also showed that the mutant genotypes responded to the pathogen infection at a later time point than the susceptible genotype, thus proving their increased level of resistance. *Psp*-race 1 upregulated the expression of all the *P. vulgaris* defence-related genes analysed, with *FSL2.3* reaching the highest relative value. In the parental susceptible genotype compared with resistant mutants, only *FSL2*, *FSL2.3*, *PTO, PTO1* and *RBG1-B* were significantly upregulated in all resistant mutants, raising comparative expression values. Finally, M192 showed consistent and significant upregulation

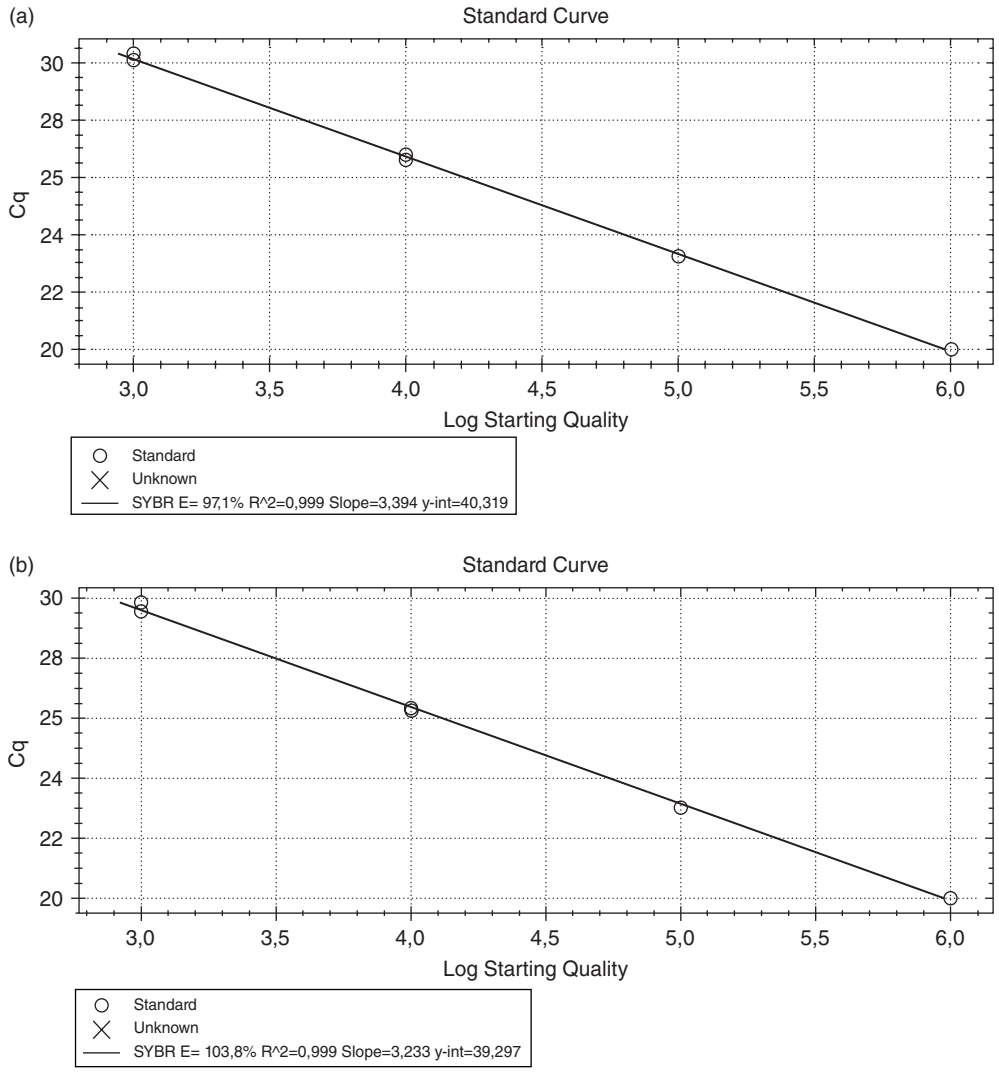

Fig. 18.3. Determination of qRT-PCR efficiencies of reference genes *Act11* **(a)** and *Tub8* **(b).** Cycle number of crossing point (Cq) versus cDNA (reverse transcribed total RNA) were plotted to calculate the slope. The corresponding real-time PCR efficiency was calculated according to equation: E (%) = [($10^{-1/slope}$) – 1] * 100.

of the expression ratio of all the analysed genes, with *FSL2, FSL2.3* and *PTO1* being the highest.

Plant interaction with race 6 of Psp

For the *Psp*–race 6 interaction, we observed greater inconsistency of gene expression among the mutant lines compared with race 1 (Fig. 18.5). The results included in Fig. 18.4 showed the following.

1. Like *Psp*–race 1, race 6 upregulated the expression of all the *P. vulgaris* defence-related genes analysed, with *FSL2.3* reaching the highest relative value again.

2. In parental susceptible genotype compared with resistant mutants, only *FSL2.3*, *PTO1* and *RBG1-B* were significantly upregulated in all time points, raising comparative expression values.

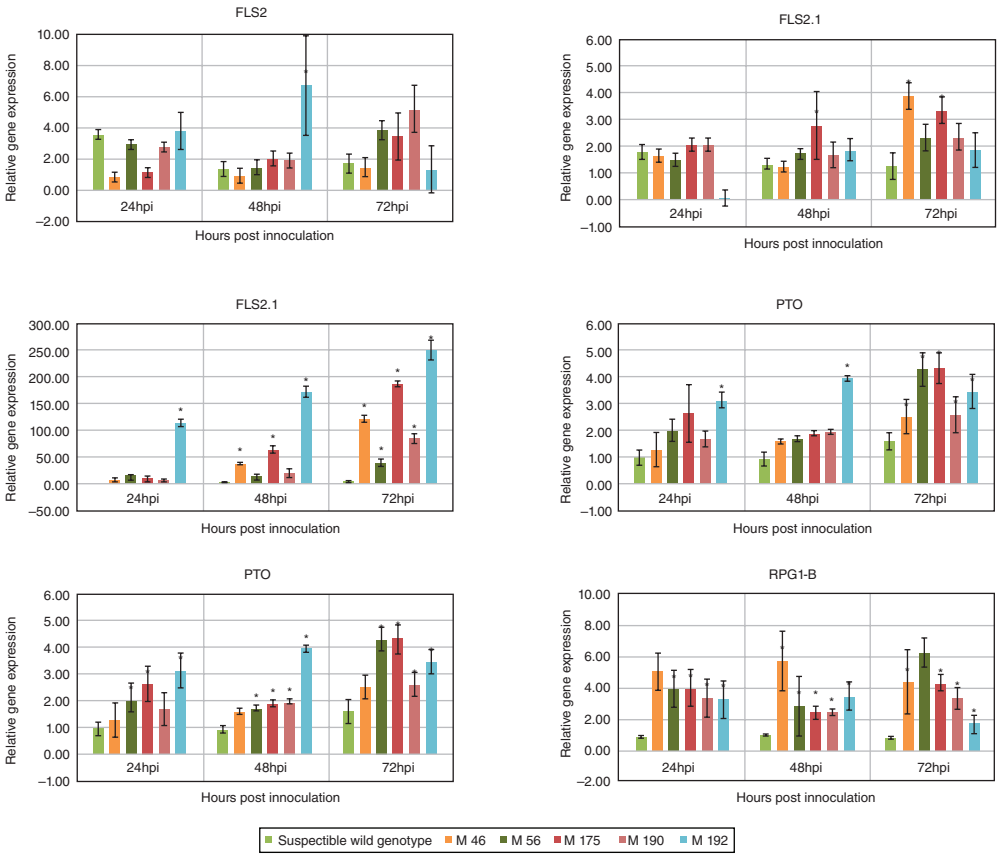

Fig. 18.4. Quantitative real-time PCR (qRT-PCR) analyses of six PR genes in response to *Psp* – Race 1 infection in susceptible (wild) and resistant mutant common bean genotypes. The data were analysed by the 2-ΔΔCt method. Probability values between infected wild type and mutant plants were estimated by the Student's *t*-test, and statistically significant differences ($p < 0.05$) are indicated with an asterisk.

3. Finally, M192 showed consistent and significantly upregulation of the expression ratio of all the analysed genes, except for *FLS2.1* and *RBG1-B.*

Although the avirulence functions of some effector proteins (e.g. *PTO*) have been described in detail (Kim *et al.*, 2002; Zhao *et al.*, 2003), the relatively constant regulation under both races of *Psp* indicates that these genes may be related to basic cell functions suppressed on infection.

The highest upregulation of *FLS2.3* may be related to the plant recognition mechanisms or to hypersensitive response (HR). It is now known that genes belonging to the family of PR proteins have been strongly linked to the resistance mechanisms of the plants against pathogens (Edreva, 2005).

4 Conclusion

Plant breeding achievements in Bulgaria confirm that experimental mutagenesis is a useful method capable of enhancing crop improvement. We have tested the chemical mutagen EMS in several concentrations on seeds from Bulgarian snap bean genotypes and developed advanced mutant lines. In these mutants, increased tolerance to the targeted halo blight and common bacterial blight diseases was observed under field conditions as well as in artificial inoculations under controlled (glasshouse) conditions. Individual plants with morphological and phenological mutations and increased resistance have been identified in the M_2 generation,

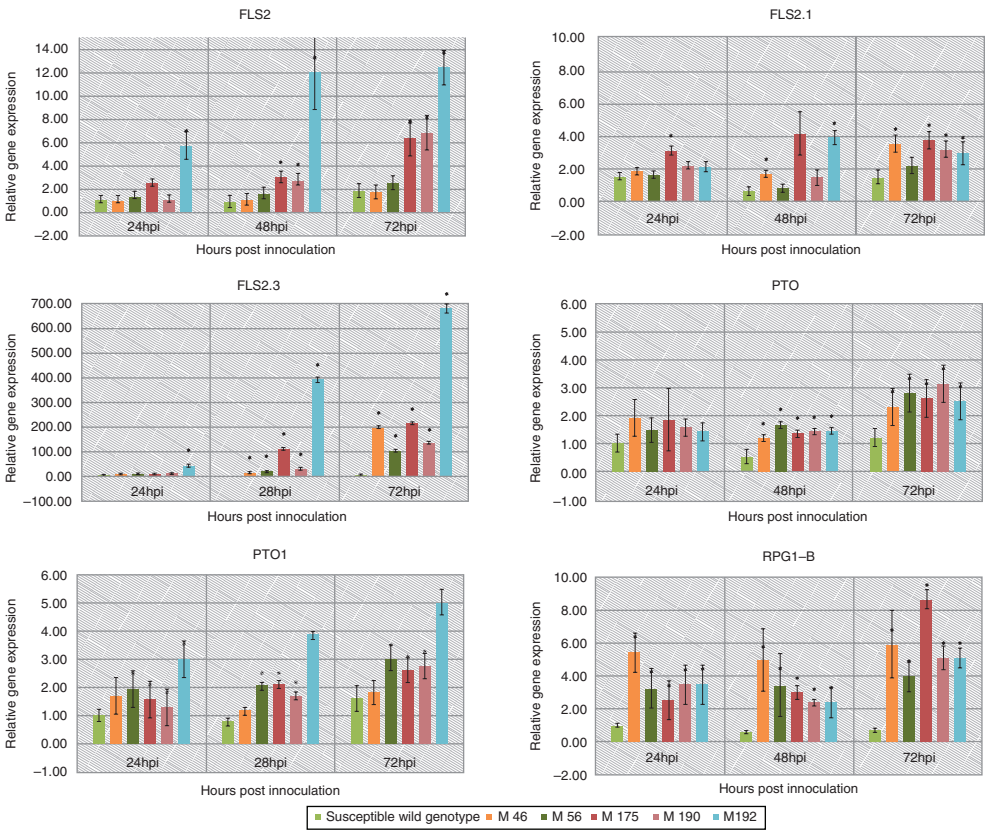

Fig. 18.5. Quantitative real-time PCR (qRT-PCR) analyses of six PR genes in response to *Psp* – Race 6 infection in susceptible (parental) and resistant (mutant) common bean genotypes. The data were analysed by the $2^{-\Delta\Delta Ct}$ method. Probability values between infected wile and mutant plants were estimated by the Student's *t*-test, and statistically significant differences ($p < 0.05$) are indicated with an asterisk.

further selected and tracked in the M_3, M_4 and M_5 generations. Agronomic and morphological traits in mutant lines different from the initial genotypes have been recorded for three successive mutant generations. Adding mutant lines with numerous beneficial agronomic traits to the pipeline of the bean breeding programme will boost competitiveness in international markets. However, there is still the need to overcome some undesirable side effects that have occurred from the mutagenic treatment by means of a conventional crossing and further selection programme.

In addition, this study has provided a valuable new insight into the molecular mechanisms of resistance to halo blight (*Psp*) in common bean by finding six genes to be differentially expressed in a time-dependent manner in bean leaves during the interaction with *Psp*. Resistance of the mutant plants to *Psp* was correlated with a high expression of PR genes.

The comparison of susceptible parental line IP564 and mutant lines was used to investigate the profile of gene expression in the interaction between two races of *Psp* and common bean at different stages of the infection process, and provided additional evidence for an active resistance response in the resistant mutant genotypes. The result of expression analysis shows that the mutant genotypes responded to the pathogen infection at a later time point than the susceptible genotype, thus proving their increased level of resistance. Furthermore, expression of most defence-related genes analysed was not significantly different in the mutants than in the parent in the earlier stages

of infection, but higher and significantly different in later stages. Data generated from this study will contribute to the understanding of the molecular mechanisms associated with plant defence against halo blight in common bean. Pyramiding of major resistance genes and QTLs associated with resistance may contribute to more durable resistance against this important pathogen. Moreover, our results also revealed a differential reaction against isolates of *Psp.*

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