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Pea transformation: History, current status and challenges

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Abstract: This review recapitulates the history, important milestones, the current status, and the perspectives of the pea (*Pisum sativum* L.) transformation as a tool for pea crop breeding. It summarises the developments of the pea transformation from the first methodological experiments to achieving the complete transformation and regeneration of genetically modified (GM) plants, transformation with the first genes of interest (GOI), to recent techniques of targeted genome editing. We show how recent biotechnological methods and genetic engineering may contribute to pea breeding in order to speed up the breeding process and for the creation of new pea cultivars. The focus is laid on genetic engineering which represents an excellent technology to enhance the pea gene pool with genes of interest which are not naturally present in the pea genome. Different methods of pea transformation are mentioned, as well as various GOI that have been used for pea transformation to date, all aimed at improving transgenic pea traits. Tolerance to herbicides or resistance to viruses, fungal pathogens, and insect pests belong, among others, to the pea traits that have already been modulated by methods of genetic engineering. The production of phytopharmaceuticals is also an important chapter in the use of genetically modified peas. We compare different methods of introducing transgenes to peas and also the usage of different selective and reporter genes. The transformation of other major legumes (soybeans, beans) is marginally mentioned. The effect of genetically modified (GM) peas on animal health (feeding tests, allergenicity) is summarised, the potential risks and benefits of pea modification are evaluated and also the prime expectations of GM peas and the real current state of this technology are compared. Unfortunately, this technology or, more precisely, the products created by this technology are under strict (albeit not scientifically-based) legislative control in the European Union.

Keywords: *Agrobacterium*; biotechnology in plant breeding; legumes; new breeding technology; pea; transformation/genetic modification

Fabaceae (syn. *Leguminosae*) is the third largest family of flowering plants, after *Orchidaceae* and *Asteraceae*, with over 650 genera and 20 000 species (Lewis et al. 2005). It is an extremely diverse family of worldwide distribution, encompassing everything from arctic alpine herbs, to annual xerophytes, and equatorial forest trees. Members of the family are characterised by a distinct fruit, termed a legume or pod, which gives the family its name (Mikić et al. 2011).

The tribe *Fabeae* (formerly *Vicieae*) contains some of humanity's most important grain legume crops, namely *Lathyrus* (grass pea/sweet pea/chickling vetches; about 160 species); *Lens* (lentils; 4 species); *Pisum* (peas; 3 species); *Vicia* (vetches/faba bean; about 140 species); and the monotypic genus *Vavilovia* (Smýkal et al. 2011). A study based on molecular data (Schaefer et al. 2012) positioned *Pisum* genetically between *Vicia* and *Lathyrus* and shows it to be closely allied to *Vavilovia*.

Among legumes, the term "pulses" refers only to dried seed crops, excluding those grown mostly for oil extraction (like soybeans), where dried peas, edible beans, lentils, chickpeas, cowpeas, mungbeans, the blackgram, and pigeon peas are the most common

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cultivated ones for human consumption due to their high nutritional value (Gatti et al. 2016). Legumes can interact symbiotically with specific soil-borne bacteria, the rhizobia, which allow the plant to fix atmospheric nitrogen and may help to protect them against some fungal pathogens (Chakraborty et al. 2003).

Legumes belong to ones of the most important crops worldwide, having major impacts on agriculture, the environment, animal/human nutrition and health (Graham & Vance 2003). Grain legumes significantly contribute to the total world food production. Legumes are the primary source of dietary proteins in many developing countries, where protein hunger and malnutrition are widespread (Mikić et al. 2011). One of the most important factors why the pea is important as a legume crop is because of its high protein value; which also has very low concentrations of detectable anti-nutritive factors, such as protease inhibitors, haemagglutinins, and alkaloids. However, an important limitation of the crop is that it is (like other grain legumes) a poor dietary source of sulfur-containing amino acids (Kotlarz et al. 2011; Schumacher et al. 2011).

The pea is considered to be one of the first domesticated plants in the world, together with the lentil (*L. culinaris* Medik.), chickpea (*Cicer arietinum* L.), bitter vetch (*Vicia ervilia* (L.) Willd.) and several cereal species (Zohary & Hopf 2000). The garden or field pea is cultivated worldwide in temperate climates, but *Pisum sativum* L. is naturally found in Europe, north-west Asia and extends south to temperate east Africa, while *P. fulvum* Sibth. and Sm. is restricted to the Middle East (Maxted & Ambrose 2001). A study of phylogeography, using a combination of plastid and nuclear markers, suggested that the wild pea spread from its centre of origin, the Middle East, eastwards to the Caucasus, Iran and Afghanistan, and westwards to the Mediterranean (Smýkal et al. 2011).

Peas remain one of the most important temperate pulse, fodder and vegetable crops. Garden peas (*P. sativum* var. *sativum*) are produced primarily for human consumption, field peas (*P. sativum* var. *arvense* (L.) Poiret) are traditionally produced for livestock and as a green manure. The pods are also eaten immature as a vegetable (e.g., mangetout, sugar snap peas or snow peas). In a number of developed countries, a significant proportion of the crop is now harvested in an immature state and frozen to make it a convenience food (Maxted & Ambrose 2001). The pea is well placed to meet the increased global demand for high protein human food and animal feed and to act as a leguminous break crop in diverse farming systems (Ambrose et al. 1997).

The top producer of green peas – by far – is China with 11.3 million tonnes in 2020, followed by India (5.7 million tonnes), the USA (0.28 million tonnes), France (0.27 million tonnes), Pakistan (0.22 million tonnes), Algeria (0.21 million tonnes), the United Kingdom (0.16 million tonnes), and Egypt (0.15 million tonnes). Peru and Spain completed the top 10 in 2020. The top producers of dry peas are Canada (4.6 million tonnes in 2020), the Russian Federation (2.7 million tonnes), and China (1.4 million tonnes), followed by the USA, India, France, Ukraine, Ethiopia, Germany, and Spain (FAO 2021).

The pea has also been traditionally used as a classical model plant in fundamental studies related to genetics and plant breeding, biochemistry and molecular biology (Fehr 1993; Gaikwad et al. 1999; Wen et al. 1999; Malysheva et al. 2001). The founding father of genetics, J.G. Mendel, studied many genera of plants in a monastery garden, but his famous research was on garden peas. Mendel deliberately chose the genus *Pisum*, since it fulfilled his three basic requirements for his experimental plants (simplified): constancy of characters over generations (true breeding), protection from foreign pollen during flowering and undisturbed fertility of hybrids and the offspring (Schwarzbach et al. 2014). He published the results of his seven-year work with an evaluation of more than 30 000 plants (Mendel 1866). The legacy of his work is taught to students all over the world, and the name of J.G. Mendel is inextricably linked to genetics.

Taxonomy and biology of *Pisum sativum* L.

Pisum is a very small genus and, at the present time, comprises only two species, *P. sativum* L. itself and *P. fulvum* Sibth. & Sm. Other species formerly assigned to it have been transferred to other genera (*P. formosanum* (Stev.) Alef. = *Vavilovia formosa* A. Fed.; monotypic genus) or reduced to synonymy, most frequently with *P. sativum* (Smartt 1990). The wild populations from which the domesticates probably arose were initially described as species in their own right, *P. elatius* Bieb. and *P. humile* Boiss. & Noe (syn. *P. syriacum* (Berger) Lehm. Nowadays, these species are regarded as a part of the biological species *P. sativum* in the broadest sense (Davis 1970; Ben-Ze'ev & Zohary 1973; Smartt 1990). In

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the wild subspecies *elatius*, morphological extremes are represented by the var. *elatius* and var. *pumilio* with the var. *brevipedunculatum* being intermediate (Smartt 1990; Table 1).

The pea is a highly self-pollinating annual herb, it is cleistogamic – pollination takes place before the flower opens. The pea is diploid ($2n = 2x = 14$), its genome size is ca 4.45 Gb (Doležel & Greilhuber 2010). Recently, more than 360 genes have been described and localised in *Pisum* (Świecicki 2019). All types of cultured peas are reciprocally easily crossable and the crosses within the "*elatius*", "*pumilio*" and "*sativum*" botanical varieties and between different accession of *P. fulvum* have been made without undue difficulty and have produced fertile progeny (Ben-Ze'ev & Zohary 1973). The wild pea species/forms represent valuable donors of resistances to biotic and abiotic factors (Coyne et al. 2020). The wild relatives of the pea are potentially significant contributors for genetic diversity in various adaptation traits, and have higher levels of disease resistance than the cultivated pea germplasm (Maxted 2000). Resistance to the pea weevil, *Bruchus pisorum* L. has been identified in the accessions of *P. fulvum* and is being transferred to *Pisum sativum* (Byrne et al. 2002, 2008). *Pisum fulvum* appears to be both more drought and heat tolerant with a more xeric distribution of native habitats in Israel than *P. elatius* and *P. humile* (Ladizinsky & Smartt 2000; Redden et al. 2005).

Seed germination in the pea is hypogeal. The pea plant is bushy or climbing, its stem length varies from 20 to 220 cm. The leaves are alternate, pinnate with 1–3 pairs of leaflets containing terminal branched tendril ovate or elliptic leaflets, 1.5–6 cm long (Duke 1981). The leaf type could be conventional, semi-leafless or leafless (Davies et al. 1985). Goldenberg (1965) found – as a result of spontaneous mutation – the *afila* plant type whose leaflets converted to tendrils. Jaranowski (1976) obtained a similar plant type via induced mutagenesis and started to use this type in breeding programmes. Despite the initial scepticism of pea breeders, caused by the expected decreasing seed yield as affected by the reduction in the plant assimilation area, the majority of recent field (dry-seed) pea cultivars are represented by the *afila* type. A similar situation can also be seen in garden (canning) peas. The leaf size, in most cases, increases up to the first node bearing the first flower. The stipules are large, leaflike and up to 10 cm long. The inflorescence of the pea is a raceme arising from the axil of the leaf. The corolla is white, pink, or purple; the pods can be swollen or compressed, short-stalked, straight or curved, 4–15 cm long, 1.5–2.5 cm wide, with 2–10 seeds, 2-valved, dehiscent on both sutures (Gritton 1980; Duke 1981). The node at which the first flower emerges is characteristic of a given variety; in temperate regions, the number of nodes at which the first flower emerges is reported to vary from four in the earliest to about 25 in late maturing types under field conditions (Gritton 1980). Flowers borne on the same peduncle produce pods that mature at different times, the youngest being at the tip. On a whole plant basis, flowering is sequential and upwards from node to node. The seed shape is globose or angled, smooth or wrinkled, whitish, grey, green, yellow or brownish; the thousand seed weight (TSW) varies from 100 to 360 g (Duke 1981). The size, shape and colour of the seeds are very variable traits; according to Blixt (1972), there are at least 45 known genes affecting the seed characteristics. Consumers prefer green-coloured pea cultivars; for animal feed, the seed colour is of marginal importance.

The total protein content in the pea seed is lower than in soybean meal and higher than in cereals. It is variable in particular cultivars, nevertheless it ranks at ca 25% on average (Bastianelli et al. 1995). Besides

Table 1. Taxonomy of *Pisum* (after Davis 1970 from Smartt 1990; modified)

Species	Subspecies	Variety
P. sativum L.	*sativum*	*sativum* *arvense* (L.) Pair
	elatius M. Bieb.	*elatius* (M. Bieb.) Alef *pumilio* Meikle *(P. humile* Boiss et Noe*)* *brevipedunculatum* Davis et Meikle
P. fulvum Sibth. et Sm.		
Vavilovia formosana (Stev.) Alef (*P. formosanum* (Stev.) Alef)		

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the cultivar effect, the soil and climatic conditions, as well as agrotechnological management, may substantially influence the seed protein content. The main part of the pea seed is constituted by starch. Peas, as an important starch source, may be divided based on the presence of the *R/r* alleles (r – *rugosus*); the starch grain characteristic is tightly connected with the seed shape (Hedley et al. 1986; Bhattacharrya et al. 1990; Wang & Hedley 1991): (1) Dry-seeded (protein) peas – *Pisum sativum* L. ssp. *sativum* var. *sativum* (genotype *RR* or *Rr*) characterised by oblong smooth seeds and oval, round-smooth, uncleaved and homogenous starch grains. (2) Garden (canning) peas – *Pisum sativum* L. ssp. *sativum* var. *medullare* (genotype *rr*) with wrinkled seeds exhibit cleaved starch grains with a broad extent of sizes (prevalence of small grains); the grains have irregular shape, frequently with radial grooves. Kooistra (1962) identified a mutation in the *rb* locus, which manifests similar to peas with wrinkled seeds, but with starch characteristics typical for round-seeded peas. Based on these findings, such genotypes are termed as intermediate ones; this second *rugosus* mutation is designated as *rb*. The presence of the *rb* mutation decreases the starch content to 35% of the fresh weight (FW); however, in contrast to *r*, the *rb* mutation increases the amylopectin content to 75% (Wang & Hedley 1993).

The pea as an economically and agronomically important crop

The pea belongs to the most commonly grown legumes worldwide (after the field bean and chickpea), it is grown in the whole temperate zone, predominantly as a spring crop for human food and animal feed. In the EU countries, peas represent the most important legume crop. It is grown mainly for seeds with a high protein content (21–24% crude protein), which is ca. two times higher when compared to cereals. However, the pea protein contains (similarly to other legumes) a low proportion of essential, sulfur-containing amino acids. Recently, consumers have shown increased interest in resistant starch and carotenoids in garden (canning) peas.

From the point of view of animal husbandry, there is a demand for decreasing antinutritional substances (mainly trypsin inhibitors and phytic acid), which limit the digestibility of proteins and some minerals, particularly phosphorus. A less known fact is that the pea is also utilised in the pharmaceutical industry and as a source of starch for special industrial utilisation (e.g., biodegradable plastics).

The most important environmental contribution of the pea is its fixation of atmospheric nitrogen by symbiotic bacteria and its excretion into the soil. In addition, there are other advantageous characteristics of peas, namely their phytosanitary effects, unique soil reclamation ability, which helps to improve the physical characteristics of the soil and their ability to bind macro- and microelements essential for plant nutrition. These facts are connected with a positive effect on the soil fertility and creating a balance of crop rotation complexes, where the pea plays the role of being the interrupter of the unilateral exploitation of nutrients and the maintainer of the soil microflora. Despite that the pea is attacked by various diseases and pests, placing it into a crop rotation scheme generally has a positive effect on the natural decrease in the expansion of harmful organisms in plant production management. As a result, there is a lower need for chemical agent applications, and this finally has a positive effect on the environment. The average yield increase of the subsequent crop (cereals) in the crop rotation system has been reported to be ca 1 tonne/ha, which approximately represents a 20% increase in production. Peas are relatively unresponsive to fertilisers, particularly nitrogen, additions are only necessary when nodulation is poor or fails completely. When the seeds are treated with the *Rhizobium*, care must be taken in the choice of fungicide seed treatments to prevent any potential toxicity (Muehlbauer et al. 1983).

Peas are adversely affected by numerous harmful organisms – viruses, fungi, and insects being the most important ones (Muehlbauer et al. 1983; Davies et al. 1985; van Emden et al. 1988, Kraft & Pleger 2001). In the temperate zone and, thus, in regions with the largest acreage of both field and garden peas, the most aggressive viruses are the *Pea enation mosaic virus* (PEMV), *Pea mosaic virus* (PMV), *Pea seed-borne mosaic virus* (PSbMV), *Pea streak virus* (PSV), and *Bean yellow mosaic virus*. The infected plants exhibit colour (lesions) and morphological changes (deformations) and, in general, they are subsequently more sensitive to fungal root disease complexes. Viruses are most frequently transmitted by insect vectors (aphids, thrips) or via infected seeds (PSbMV).

Root rot disease complex is caused by the soil oomycetes (*Pythium* ssp., *Aphanomyces euteiches*), whose importance has recently increased in tendency.

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Other causal agents of root rot are represented by fungi of the *Fusarium* genus (Sharma et al. 1998), namely *F. oxysporum* f.sp. *pisi* race 1 and 2 (McPhee et al. 1999) and *F. solani* f.sp. *pisi.* The main fungal pathogens of the above-ground plant parts are *Mycosphaerella pinodes*, *Ascochyta pisi, Phoma medicaginis.* Visual symptoms of the disease include lesions on the leaves, subsequently expanding to the pods and seeds and may participate in causing 75% seed yield loss. Downy mildew (*Erysiphe pisi*) has been recorded as spreading more increasingly in the last few decades (Tiwari et al. 1997).

The most harmful pea insect pests include *Acyrthosiphon pisum* (pea aphid), *Kakothrips robustus* (pea thrips), *Bruchus pisorum* (pea seed beetle), *Bruchus rufimanus*, *Callosobruchus chinensis* (Adzuki bean seed beetle), *Sitona lineatus* (bean weevil), *Cydia nigricana* (pea moth), *Delia platura* (bean seed fly) (Muehlbauer et al. 1983; van Emden et al. 1988; Kraft & Pleger 2001). The recent demand on pea breeders and growers on genetically-based resistance to harmful organisms is reflected by the increasing role of pea germplasm collections containing sources of resistance (McPhee et al. 1999; Redden et al. 2005), the utilisation of wild pea relatives as donors of traits absent in the pea genome (Maxted 2000; Byrne et al. 2008), and, finally, the use of gene engineering technologies to incorporate transgenes conferring resistance to biotic factors (viruses, fungi and insect pests; see the papers cited in this review).

Conventional pea breeding methods and their limits

Due to the high rate of self-pollination (99%; Gritton 1980), the adopted pea breeding strategies have been similar to other self-pollinating crop species and have generally involved hybridisation among cultivars or between cultivars, landraces, and primitive forms, followed by combinations of pedigree, bulk, backcross, or single-seed descent selection methods (Redden et al. 2005). Practically all field and canning pea cultivars were created by using two conventional approaches, i.e., (1) mostly by crossing of the parental genotypes, and (2) later also by mutagenesis (chemomutagenesis and radiomutagenesis) after 1950 (Blixt 1972; Jaranowski 1976) followed by selection in the framework of various breeding schemes. The desired phenotype was selected based on the expression of traits during vegetation (plant architecture, flower and seed characteristics, earliness, lodging, sensitivity/tolerance to biotic and abiotic factors) or after harvest (seed substances, both desired and undesired ones). The heritability and stability of the demanded traits had to be confirmed by repeated selfing and backcrossing when necessary (elimination of any undesired traits of one of the parents). Thus, the whole breeding process was quite lengthy and laborious – the standard time for releasing a new pea variety is ca. 10 to 15 years. Since 1970s, pea breeders tried to formulate and realise a pea ideotype, which would join together the maximum desired characteristics both from an agronomic and end-user point of view. Peas have a very long history in cultivation and, hence, the targeted selection of types or populations better suited to local farming systems and end-purpose requirements have occurred over hundreds of years. The transition from a wild type to a domesticated type has involved gradual changes in a number of major attributes mostly under simple genetic control. Over the last 50 years, breeders have made dramatic ideotype changes to better adapt the crop to broad-acre mechanised systems of farming (Redden et al. 2005). A major breakthrough came about when breeders combined a reduced crop height (e.g., the *le* gene) and the conversion of leaflets to tendrils (e.g., the *af* gene), described as the semidwarf, semi-leafless ideotype (Snoad 1985). This ideotype provides a number of benefits, such as reduced leafiness and excessive overshading, increased aeration and reduced disease spread in some environments, and improved ease of harvest of both the garden and field pea types as a consequence of the reduced lodging.

Since the 1960 s, the birth of plant biotechnology/tissue cultures *in vitro* was dated with later attempts at their application/implementation in plant breeding, including grain legumes. The main motive was to transfer part (namely the initial stages) of the breeding process to the laboratory with the objective of speeding-up/shortening the process. Many *in vitro* techniques were successively developed with various utilisation potential in the breeding of agricultural crops depending on the crop biology and especially on the mode of reproduction/propagation (seed- versus vegetatively-propagated crops, self-pollinated versus cross-pollinated crops, etc.). These techniques included micropropagation/clonal propagation, callus and suspension cultures, and their uses in the *in vitro* selection for tolerance to filtrates/toxins of phytopathogenic fungi, salinity or heavy metals, interspecific/intergeneric hybridisa-

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tion via protoplast fusion, quick homozygotization via doubled-haploid production in the microspore or anther cultures, and exploiting the "somaclonal variation" phenomenon. However, the crucial point and *condicio sine qua non* of all these techniques as potential tools in plant breeding was the ability of a particular species/crop to regenerate whole plants from isolated and cultured plant organs, tissues, cells or even protoplasts. It was found early that significant differences exist between plant species, plant families or even between monocots and dicots in the amenability to regenerate *in vitro*. Unfortunately, grain legume crops (including peas) belong to a poorly regenerating (= recalcitrant) group of plants. This fact slowed down the more rapid progress in the employment of biotechnologies in legume breeding when compared to other agricultural field crops or vegetables.

Genetic transformation/transgenesis became the breaking point in plant biotechnology (*Agrobacterium*-mediated transformation; particle bombardment), also successfully achieved in grain legumes, including peas (Puonti-Kaerlas et al. 1990; Schroeder et al. 1995; Jones et al. 1998). Rapid progress in this technology resulted in the release/registration of the first genetically modified (GM) crops (soybean, maize, cotton) and their subsequent massive extension and growth, namely in both North and South America (the USA, Canada, Brazil, Argentina) and Asia (India). Thus, in some countries, more than 90% of the total cultivation area of a particular crop (soybean, cotton) is represented by GM cultivars and farmers use their agronomic advantages and economic impact. A specific situation has arisen in Europe (the EU countries), where the public acceptance of GM technology/GM crops is very low, supported by the negative role of the mass media and the unwillingness of politicians to pass legislation endorsing its use. Thus, the cultivation of GM crops is under very strict legislative regulation (mostly unsupported by scientific data) and a very complicated administrative process of GM crop registration and permission for growing them which represents a substantial barrier/obstacle for breeders and growers. European breeders then lose – when compared to those in technologically developed countries outside the EU – the possibility of the unique transfer of genes between biological kingdoms or incorporation of synthetically created (artificial) genes; European growers then lose their competitiveness in growing crops with novel unique characteristics both from an agronomic and consumer point of view. Recent modern cultivars of dry-seed and canning peas have reached - from the point of view of both agrotechnology and product quality for food and feed utilisation - their limits and further improvement via conventional methods has become more and more difficult. The available variability/diversity of important traits within the pea gene pool has been practically exploited, and the utilisation success of wild *Pisum* species/forms is sometimes reduced by the lowered crossability and laborious and time-consuming back-crossing. Classical (non-targeted) mutagenesis has low efficiency and is, thus, less frequently used. The utilisation of promising techniques of genetic engineering (classical transgenesis; targeted gene editing) is obstructed by legislative regulation connected with the discriminative administrative and financial demands for breeders/farmers in the EU. A new hope is represented by new breeding techniques (NBTs) based on targeted gene editing – nevertheless, this technology was also, after long discussions, integrated under the standard genetically modified organism (GMO) legislative process (Jorasch 2020; Purnhagen & Wesseler 2021). Thus, the resignation in genetic engineering technology in plant breeding has resulted in the utilisation of alternative approaches, of which, marker-assisted selection (MAS) based on various molecular DNA markers seems to be the most effective one.

Pea transformation has already been reviewed in a number of journal papers or book chapters (Davies et al. 1993; de Kathen & Jacobsen 1993; Atkins & Smith 1997; Malysheva et al. 2001; Morton et al. 2002; Grant & Cooper 2003; Somers et al. 2003; Dita et al. 2006; Bhowmik & Basu 2008; Nadolska-Orczyk 2008; Atif et al. 2013; Gatti et al. 2016), nevertheless, this review tries to provide a thorough summary of this topic.

History and milestones of pea transformation research

The performance of successful pea transformation was preceded in the 1980 s by a number of methodological experiments. The pea regeneration capacity was shown to be tissue-specific (Kunakh et al. 1984; Ezhova et al. 1985; Lutova & Zabelina 1988; Mallick & Rashid 1989) and to depend on the age of the tissue (Hussey & Gunn 1984; Ezhova et al. 1985; Kysely & Jacobsen 1990). Regeneration processes have been shown to be strongly genotype-dependent. Pea genetic forms differ in the callus formation capacity,

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shoot formation (Rubluo et al. 1984; Ezhova et al. 1985; Lutova & Zabelina 1988), root formation in callus cultures, and rooting of the shoots (Lutova & Zabelina 1988).

Pea regenerants were obtained on media with phytohormones via organogenesis from pre-existing meristems (Kartha et al. 1974), organogenesis *de novo* from pea explant tissues and the primary calli (Rubluo et al. 1984; Lutova & Zabelina 1988) and somatic embryogenesis in the primary callus (Jacobsen & Kysely 1985; Kysely & Jacobsen 1990). Shoot formation was induced on long-term calli obtained from pea stem apices, epicotyls, explants of stems, leaves and roots (Malmberg 1979; Hussey & Gunn 1984; Kunakh et al. 1984; Ezhova et al. 1985). Numerous studies have been conducted in various aspects regarding the tissue culture, regeneration, and genetic transformation of peas (Griga & Novák 1990; de Kathen & Jacobsen 1993; Atkins & Smith 1997; Švábová et al. 2008).

Testing the interactions between *Agrobacterium tumefaciens*/*A. rhizogenes* and the pea started in the late 1980s and early 1990s (Hussey et al. 1989; Schaerer & Pilet 1991). Pea intraspecific variability of morphogenetic responses to transformation with *Agrobacterium* strains was demonstrated for the first time by Hobbs et al. (1989). The first successful attempt to realise the genetic transformation in peas was made in 1990 (Puonti-Kaerlas et al. 1990; de Kathen & Jacobsen 1990). Both of these reports are related to gene coding the antibiotic resistance of transformed plants to hygromycin and kanamycin. An analysis of the transmission of the transferred DNA in the progeny of these plants was published two years later (Puonti-Kaerlas et al. 1992). The obtained plants were tetraploid. The authors suggested two possible reasons for this, the long culture period and high cytokinin and auxin concentrations in the medium.

The above-mentioned pioneering works were later followed by reports optimising pea transformation protocols and finally modifying the pea genome with "useful" transgenes (herbicide tolerance, insect and virus resistance). This topic is summarised in more detail below in the part of the article concerning pea transformation with useful genes (genes of interest, GOI).

Transformation of other legumes

In addition to peas, many other legume crops have been used for transformation experiments. The species of legumes most commonly used for transformation include beans (*Phaseolus vulgaris* L.), soybeans (*Glycine max* L.), faba beans (*Vicia faba* L.), chickpeas (*Cicer arietinum* L.), pigeon peas (*Cajanus cajan* L.), alfalfa (*Medicago sativa* L.), cowpeas (*Vigna unguiculata*) or the blackgram (*Vigna mungo* L.).

Transgenic beans (*Phaseolus vulgaris* L.) engineered to express viral antisense RNA sowing, delayed and attenuated symptoms to the *Bean golden mosaic geminivirus* (BMGV) was reported by Aragão et al. (1998). In 2011, a transgenic bean event (named EMB-PV051-1; Embrapa 5.1) resistant to BGMV was approved for cultivation and consumption in Brazil (Aragão 2014).

The faba bean (*Vicia faba* L.) was transformed for the first time by inoculating seedlings with an *Agrobacterium rhizogenes* strain carrying the binary vector pGSGluc1 containing the selection markers *nptII* and *uidA* as the signalling genes to detect the success of the DNA transfer (Schiemann & Eisenreich 1989). Jelenic et al. (2000) used stem segments of three commonly grown varieties of faba beans. Different levels of susceptibility of the varieties to *Agrobacterium* strains were confirmed. The first procedure successfully achieving fertile transgenic faba bean plants was described by Böttinger et al. (2001). The protocol was based on the *de novo* shoot regeneration from dedifferentiated stem segment cells. However, the callus induction and regeneration of transgenic shoots lasted 16–24 months. Although a biolistic approach to the introduction of foreign DNA in faba beans has been tested (Metry et al. 2007), the most significant progress has been made by Hanafy et al. (2005), who developed a procedure for the direct *in vitro* regeneration of shoots from transformed meristems isolated from germinated faba bean seeds and confirmed the presence of the transgene in the third generation of the transformants.

A number of traits have been improved by transformation in pigeon peas also, e.g., insect resistance (Krishna et al. 2011; Kaur et al. 2016; Ghosh et al. 2017), increased salt tolerance (Singh et al. 2020) and seed nutritional quality (Thu et al. 2007). Insect resistance (Solleti et al. 2008; Grazziotin et al. 2020) and virus resistance (Cruz & Aragão 2013; Kumar et al. 2017) were induced by transformation methods in the cowpea also.

Among various transformation technologies, the *Agrobacterium*-mediated transformation method has been shown to be effective for the production of transgenic soybeans (Hansen & Wright 1999).

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Many reports have been published related to the optimalisation of conditions to achieve a high yield of soybean transformation; *Agrobacterium* inoculation conditions, regeneration media components and various *Agrobacterium* strains have also been tested to improve the transformation efficiency (Donaldson & Simmonds 2000; Olhoft & Somers 2001; Paz et al. 2004; Liu et al. 2008). The most important modified trait in the history of soybean transformation has been the herbicide resistance. One of the first practical applications of the genetic engineering of soybeans was the development of soybean tolerance to glyphosate, the active component in the herbicide Roundup (Padgette et al. 1995). Since 1996, a series of genetically engineered varieties of glyphosate-resistant soybeans were introduced into cultivation, and the food and feed processing in the market by several agricultural biotechnology companies. Beside herbicide resistance, a number of scientific papers have been published on the soybean transformation concerning the improvement of different properties, e.g., heat tolerance (Zhu et al. 2006), drought tolerance (de Ronde et al. 2000, 2001, 2004; Ning et al. 2017); stress tolerance (Preiszner et al. 2001) and tolerance to biotic stresses: viruses (Wang et al. 2001; Furutani et al. 2006), fungi (Li et al. 2004; Salehi et al. 2005) or nematodes (Steeves et al. 2006). Improvement in the nutritional quality of soybeans by methods of genetic engineering has been performed by Chiera et al. (2004), Flores et al. (2008), Arun et al. (2014) and Valentine et al. (2017). The progress in soybean transformation has been summarised in the number of reviews (e.g., Kocsy et al. 2007; Homrich et al. 2012; Lee et al. 2013b; Bhowmik et al. 2021).

Biotech soybeans covered 48% of the global biotech crop area with 91.9 million ha in 2019. At the same time, 74% of the soybeans grown worldwide were biotech cultivars. The top producers of biotech soybeans are Brazil (35.1 million ha of biotech soybeans in 2019, surpassing the US biotech soybean area for the first time) and the USA (30.43 million ha in 2019), followed by Argentina, Canada, Paraguay, South Africa, Bolivia and Uruguay (www.isaaa.org).

Methods of introduction of transgenes in peas

Despite the progress in pea transformation, the efficiency of transformation procedures in peas has not been satisfactory, therefore a number of works have focused on the optimisation of transformation methods (Nadolska-Orczyk 2008; Švábová & Griga 2008; Atif et al. 2013; Aftabi et al. 2018). A successful pea transformation was reported 30 years ago (Puonti-Kaerlas et al. 1990), nevertheless the efficiency of transformation protocols has been relatively low (in the range 0.1–6.5% as described by Davies et al. 1993; Schroeder et al. 1993; Bean et al. 1997; Jones et al. 1998; Grant et al. 1998; Polowick et al. 2000; exceptionally over 10% – Nadolska-Orczyk & Orczyk 2000; Grant & Cooper 2006).

Compared to cereals and oilseeds, less advancement has been seen in grain legumes using *in vitro* culture technique, mainly due to their recalcitrant nature. A poor regeneration rate and high genotype dependency further complicate the use of tissue culture in grain legumes and their genetic improvement.

The prerequisite for gene transfer into host cells are either procedures requiring *in vitro* cultures (agrobacterial transformation, particle bombardment and their innumerable modifications) or more recent protocols that do not require *in vitro* regeneration (electroporation, vacuum infiltration or *in vivo* injection methods). These methods are discussed in more detail in the next sections.

Agrobacterium-mediated pea transformation

To investigate the causative agent of the tumour-like outgrowths later named "crown gall disease," infected root tissues were isolated. The bacteria described as *Agrobacterium tumefaciens* were then presented (Conn 1942). The construction of a disarmed Ti-plasmid by deletion of the oncogenes, and the opine biosynthetic coding gene makes the plasmid suitable as a gene vector. *Agrobacterium*-mediated transformation is a complicated mechanism, which includes signal recognition from the plant host to *A. tumefaciens*, transfer DNA (T-DNA) processing and travelling in the plant host cell, T-DNA integrating to the plant host genome and its expression in the plant host cell. The mechanism of the T-DNA transfer is facilitated by a set of virulent genes located on the Ti-plasmid-borne genes (reviewed by Tzfira & Citovsky 2006; Hwang et al. 2017).

Grain legumes are, in general, one of the least amenable groups to transformation amongst dicotyledonous crops, although they are usually susceptible to *Agrobacterium* infection (Babaoglu et al. 2000). A number of protocols have been reported for *Agrobacterium*-mediated gene transfer in peas (Schroeder et al. 1993, 1994; Bean et al. 1997; Grant et al. 1998; Nadolska-Orczyk & Orczyk 2000; Polowick et al. 2000;

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Pniewski & Kapusta 2005; Švábová et al. 2008). The requirements for the successful application of the *Agrobacterium*-mediated transformation method are a well-working, short-term regeneration procedure, a responsive plant genotype, an appropriate type of explant and also a suitable agrobacterial strain.

Important factors of plant transformation in general are: transformation efficiency, occurrence of chimerism, genetic stability of the transformants (occurrence of mendelian versus non-mendelian segregation) and transformation productivity in terms of time (from transformation to obtaining fertile plants; as a long-lasting *in vitro* culture increases the possibility of somaclonal variation). The transformation events related to the different position/location of the transgenes in the targeted genome has also been considered to be one of the key aspects affecting transgene stability. This factor is not taken into account when using techniques of targeted genome editing.

As stated above, the success of the transformation depends on the choice of the explant and its potential for totipotency. In *in vitro* systems, protocols are mostly based on organogenesis in the callus and on the stimulation or proliferation of organised meristematic tissue contained in the transformed explants (stem nodal explants, cotyledonary nodes, immature embryo segments/slices, immature cotyledons), and only exceptionally on regeneration via somatic embryogenesis. Since the beginning of the trials with *Agrobacterium*-mediated transformation of peas, different types of explants have been used. In pioneering works focusing on this topic, shoot cultures and seedling epicotyls (Puonti-Kaerlas et al. 1990), epicotyl segments and nodes (de Kathen & Jacobsen 1990), and thin cell layers from nodes (Nauerby et al. 1991) have been used as explants.

Over the subsequent years, immature cotyledons belonged to the repeatedly reported explants used for pea transformation. Their successful utilisation was demonstrated, for instance, by Grant et al. (1995). This approach took approximately 7 months from the explant to a seed-bearing primary regenerant. The method was later commented on and amended by Nadolska-Orczyk (2008). Timmerman-Vaughan et al. (2001) and Grant and Cooper (2006) also described the usage of immature cotyledons as explants. Within the last-mentioned work, at least 25 plasmids were used for the transformation, where transgenic plants of over 30 cultivars or breeding lines have been produced.

The lateral cotyledonary meristems of germinating *Pisum sativum* cv. Puget seeds were used to develop a reproducible *Agrobacterium tumefaciens*-mediated transformation system by Bean et al. (1997). This procedure exhibited distinct advantages as the highly regenerable cotyledonary meristems rapidly produced transgenic shoots without an intermediate callus phase. The cotyledonary meristems were identically used as the initial explants by Davies et al. (1993), Jones et al. (1998), and Welham and Domoney (2000). The protocol for the transformation of cotyledonary lateral buds (Davies et al. 1993; Bean et al.1997) was commented on and amended by Nadolska-Orczyk (2008). Švábová et al. (2005) also described a transformation system for peas using either *in vitro* cotyledonary meristem (axillary buds) transformation and a concurrently simpler *in vivo* plant regeneration protocol ("non-tissue" culture of imbibed adjusted seeds). For the non-tissue culture approach, mature seeds were imbibed for 24 h, where the seed coat and nearly one whole cotyledon was removed. Such types of explants were used for co-cultivation with *Agrobacterium*. Švábová et al. (2008) used the above-mentioned *in vitro* culture avoiding approach in parallel with the standard tissue culture approach (multiple shoot regeneration from cotyledonary nodes and somatic embryogenesis from the shoot apical meristems).

Krejčí et al. (2007) reported *Agrobacterium*-mediated gene transfer using embryonic segments as the explants to be more efficient compared with the use of stem segments and axillary buds. When stem segments were used as the explants, no shoot or plantlet regenerated from the callus. On the contrary, a large number of regenerated shoots were obtained from the other two methods.

An attempt at improving the *Agrobacterium*-mediated pea transformation protocol using embryonic segments as appropriate explants was published by Aftabi et al. (2018). In this study, the highest transformation efficiency was achieved with an infection time of 90 min and co-cultivation period of 2 days. The shoots elongated well and the number of shoots/explants were observed to increase significantly after the addition of zeatin to the selection medium.

The explant apparently most commonly used for *Agrobacterium*-mediated transformation of the pea has been the embryonic axis (or embryonic axis segments). Its utilisation was mentioned by Schröeder et al. (1993; 1995) and Shade et al. (1994). Polowick et al. (2000) used thin slices from developing em-

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bryo axes to obtain transgenic pea lines. Seven pea breeding lines adapted to western Canadian growing conditions and three different transformation vectors with different selection genes were used for the transformation. In total, 323 transgenic plants were recovered from 39 independent transformation events. Analogous explants were later used by Rolletschek et al. (2005), Richter et al. (2006), de Sousa-Majer et al. (2007), Mikschofsky et al. (2009), Negawo et al. (2013) and Negawo (2015).

Pniewski and Kapusta (2005) examined the effect of explant type on the transformation efficiency using both axis and cotyledon slices as well as explants consisting of both the embryo axis and the basal part of a cotyledon. In their experiments, the embryo axis was found to be unsuitable for transformation, as it died soon, especially on the selection medium, whereas the cotyledon slices formed a callus, but had a very limited regeneration capacity. However, authors obtained satisfactory results using "mixed" explants, containing both the embryo axis and the basal part of a cotyledon. A problem of using such explants is the possible development of non-transgenic or chimeric shoots arising from the remaining cotyledonary nodes. To reduce this obstacle, they removed any shoots developing during the first 2–3 weeks of culture. The transgenic plants were regenerated via organogenesis in the callus. Although such a system of regeneration extended the plant recovery to several months, the plants obtained were most likely not chimeric, but developed *de novo* from one initial cell.

Fan et al. (2011) reported a different approach to the pea *Agrobacterial* transformation using the vacuum infiltration of *Agrobacterium* to germinated seeds. This way, the authors achieved the efficient production of the human acidic fibroblast growth factor in pea plants. It was summarised that this method was highly efficient compared to the leaf injection method. The production cycle of plants for harvesting the recombinant protein was shortened from 30 days for the leaf injection to 15 days by applying the vacuum infiltration.

Besides the explant type, another critical factor for the successful transformation is the pea genotype used. The comparison of usability of different pea varieties/cultivars has been mentioned in several publications. Puonti-Kaerlas et al. (1990) tested five cultivars, from which only two (Stivo and Puget) regenerated transgenic plants. In other studies, the efficiency of transformation of various cultivars or breeding lines differed, but all the tested genotypes provided transgenic plants (Grant et al. 1995, 1998; Nadolska-Orczyk & Orczyk 2000; Polowick et al. 2000). The transformation efficiency of Polish pea cultivars with various regeneration capacity by using hypervirulent *Agrobacterium tumefaciens* strains was tested by Pniewski and Kapusta (2005). Six different pea varieties (Vladan, Ctirad, Cezar, Havel, Kelvedon Wonder, and Puget) were used for different methods of pea transformation by Krejčí et al. (2007). Transgenic plants were recovered from each genotype, but surprisingly, the transformation efficiency of the particular pea cultivars did not clearly correspond to their regeneration capacity.

Over the years, several results have been published describing significant differences observed using different *Agrobacterium* strains for pea transformation. In one of the first works dealing with pea transformation, Hobbs et al. (1989) tested combinations of three wild-type strains, A281, C58, and Ach5, and different pea genotypes for their potential use in the transformation. A281 was the most virulent of these as determined by the size and number of the tumours. The differences between the genotypes in terms of their response to inoculation were considerable. Subsequently, non-tumorigenic, disarmed *Agrobacterium tumefaciens* strains were used. The succinamopine strain EHA101 produced the highest percentage of transformed callus lines independently of the type of selection (kanamycin or hygromycin). However, for the less virulent strain LBA4404, the percentage of transformed calli was significantly higher for the hygromycin selection (63%) than for the kanamycin selection (17%). Pniewski and Kapusta (2005) tested the usage of three hypervirulent strains of *A. tumefaciens*: AgL0, AgL1, and EHA105 for a pea transformation. Strain AgL0 was found to be efficient for the majority of the cultivars, followed by AgL1 and EHA105. Nadolska-Orczyk and Orczyk (2000) used the agrobacterial strains EHA105, C58C1, and LBA4404 to examine the factors influencing the efficiency of the pea transformation. An acetosyringone supplement had no apparent influence on the efficiency of the transformation with EHA105, however, it did affect the rate of transformation when the moderately virulent C58C1 was used.

The agrobacterial transformation is the most used method for delivering genetic information into the pea. Despite the significant progress achieved in *Agrobacterium*-mediated transformation of peas and many of the above-described methodological modifications, the available protocols still do not

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represent a fairly routine technology. A robust reproducible and efficient plant regeneration system belongs to one of the most important prerequisites for successful genetic transformation.

Particle bombardment

Particle bombardment is an alternative procedure for those legumes which fail to respond to *Agrobacterium*-mediated gene transfer (Babaoglu et al. 2000). This method has been used in many cases and published in connection with soybean transformation (Finer & McMullen 1991; Hadi et al. 1996; Droste et al. 2000; Simmonds & Donaldson 2000; Reddy et al. 2003; Li et al. 2004; Khalafalla et al. 2005; Soto et al. 2017; Paes de Melo et al. 2020).

Particle bombardment was described as a simple and rapid method for the assessment of vector constructs in pea tissues by Warkentin et al. (1992). In their work, the effect of the promoter-leader sequences on the transient reporter gene expression in the particle bombarded pea tissues was examined. Jordan et al. (1992) mentioned that the particle bombardment of a pea embryo tissue held promise for the pea transformation as the frequency of the *Agrobacterium* transformation was extremely low. In their work, the utilisation of agrobacterial transformation and particle bombardment was compared. The usage of microprojectile bombardment of immature pea cotyledons for transient β-glucuronidase (GUS) expression was depicted by Özcan (1995).

Molnar (2008) summarised the findings thus far and investigated the effect of nitrogen gas pressure, the size of microprojectiles and the optimal shooting distance for the microprojectile bombardment transformation of peas.

Direct gene transformation methods

Chowrira et al. (1995, 1996) reported the successful transformation of peas without using *Agrobacterium*. In their work, they electroporated 3–4 week-old peas of the cultivar Sparkle at the apical node. From the progeny seeds, the transformation effectivity was assessed. Twenty-six percent of 120 of the progenies showed GUS expression and transformation was confirmed by the Southern analysis. The electroporation step requires specific, critical conditions, and although the method does not require a tissue culture regeneration protocol, selection among the progeny is very time consuming.

Genome editing

Gene editing is based on the use of engineered nucleases and cellular DNA repair pathways to make precise, targeted changes in the genome of an organism. The development of gene-editing methodologies began nearly three decades ago with the key discovery that specific double-stranded breaks can be introduced in chromosomes using a meganuclease I-SceI (Rouet et al. 1994). The efforts to develop an efficient gene-editing tool brought programmable zinc finger nucleases (ZFNs; Bibikova et al. 2002) and transcription activator-like effector nucleases (TALENs; Li et al. 2011; Zhang et al. 2011). The real breakthrough in this technology came with the RNA-guided Cas9 nuclease from the type II prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR), an adaptive immune system for genome editing in eukaryotic cells (Cong et al. 2013; Mali et al. 2013).

The above-described methods represent effective tools for introducing site-specific double stranded DNA breaks and targeted cleavage of genomic DNA. The indubitable advance of these methods is that the progeny of the transformed plant can result in transgene free lines with segregated selection genes and mutations created in the gene of interest. These techniques make it possible to introduce plant genome modifications, which are indistinguishable from those introduced by conventional breeding and chemical or physical mutagenesis (Belhaj et al. 2013). It is important to note that particle bombardment and *Agrobacterium*-mediated systems have been further utilised in gene editing technologies to integrate vectors into plant cells.

Gene-editing methods have been successfully established for the soybean, cowpea, chickpea, and model legumes, such as *Medicago truncatula* and *Lotus japonicus*. However, the recalcitrance of other legumes to *in vitro* gene transfer and regeneration has posed a serious challenge in the application of gene editing (Bhowmik et al. 2021).

The most researched legume in the terms of gene editing methods has been the soybean. The targeted mutagenesis in soybean using the CRISPR-Cas9 system was published by Michno et al. (2015), Sun et al. (2015), Du et al. (2016), and Curtin et al. (2018). CRISPR-Cas9 was used for the mediated targeted disruption of FAD2-2 microsomal omega-6 desaturase in the soybean (Al Amin et al. 2019) or for the mutagenesis of *GmSPL9* genes which alter the plant architecture (Bao et al. 2019).

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Medicago truncatula was used as a model legume in a number of CRISPR/Cas9 studies (Michno et al. 2015; Curtin et al. 2018; Wolabu et al. 2020). Meng et al. (2016) developed an optimised *Agrobacterium*-delivered CRISPR/Cas9 system, which could successfully induce targeted genome modifications in the *M. truncatula*. Using this system, the authors obtained monoallelic and biallelic homozygous mutants in the T0 generation. This optimised *Agrobacterium*-mediated CRISPR/Cas9 system can efficiently induce sequence-specific genome mutagenesis at the T0 generation in *M. truncatula*, which significantly expands the toolbox for forage legume functional genomic studies (Meng et al. 2019).

CRISPR/Cas9 system is also being mentioned in connection with nitrogen fixation research in legumes. Wang et al. (2020) knocked out the *AZC_2928* gene in *Azorhizobium caulinodans* with the use of the genome editing tool, CRISPR/Cas9. The results showed that AZC_2928 plays an extremely important role in regulating the formation of chemotaxis and biofilm. Both chemotaxis and biofilm formation play an important role in nitrogen-fixing bacteria and their interaction with their host plants.

Gene editing represents a powerful tool in genetic engineering. The successful application of genome editing for legume (pea) improvement will depend on the availability of efficient protocols for the transformation and regeneration of whole plants, along with a conducive regulatory environment and evidence of public acceptance of the edited crops.

Selective and reporter genes used for pea transformation

The inclusion of an antibiotic or an herbicide in the culture medium is being used to select transformed cells and tissues from which transgenic plants are regenerated. The first obtained transgenic pea plants were hygromycin resistant (Puonti-Kaerlas et al. 1990). Transgenic pea regenerants expressing GUS were described by Nauerby et al. (1991). Transgenic pea seeds containing *bar* and *nptII* genes were achieved by Schroeder et al. (1993). Davies et al. (1993) observed that a number of shoots escaped kanamycin selection, but the increase of kanamycin concentration concurrently reduced the effectiveness of the procedure. Compared with phosphinotricin, kanamycin selection seemed to be long-lasting and often resulted in phenotypically abnormal plants (Bean et al. 1997). In contrast, all the phosphinotricin-resistant plants were phenotypically unchanged in an experiment reported by Nadolska-Orczyk (2008). Kanamycin selection was described as an ineffective by Schroeder et al. (1993) and Grant et al. (1995). Nevertheless, Grant et al. (1998) acquired a range of transformed pea cultivars, proving the selection efficiency of kanamycin. The successful acquisition of pea transformants using kanamycin selection has subsequently been showed by a number of other authors (Nadolska-Orczyk & Orczyk 2000; Švábová et al. 2005; Krejčí et al. 2007; Švábová et al. 2008).

There have been no contradictory results in the case of phosphinotricin as a selection agent, as demonstrated by several publications (Schroeder et al. 1993; Grant et al. 1995; Bean et al. 1997; Nadolska-Orczyk & Orczyk 2000; Nadolska-Orczyk 2008; Švábová et al. 2008; Aftabi et al. 2018).

Four different selection genes (*hpt*, *nptII*, *dhfr*, and *bar*) were used for the pea transformation by Nadolska-Orczyk and Orczyk (2000). However, no plants were selected on a hygromycin B and methotrexate-containing media, while 103 plants were selected on a phosphinotricin-containing media and 94 plants on a kanamycin-containing media, indicating the different efficiency of the selection genes.

In addition to the selection genes, reporter genes are important parts of constructs for the transformation. Expression of the β-glucuronidase (*uidA*) gene (Jefferson et al. 1987) remains a useful marker for rapid assessments of the success of gene delivery to plant cells and its successful use in pea transformation has been demonstrated many times (de Kathlen & Jacobsen 1990; Nauerby et al. 1991; Shade et al. 1994; Chowrira et al. 1995; Švábová et al. 2005; Krejčí et al. 2007). Also, the expression of the green fluorescent protein (*gfp*) gene from the jellyfish *Aequorea victoria* (Molinier et al. 2000) provides a very useful, non-destructive approach for monitoring the gene transfer and expression in plant tissues. *gfp* has been used as a selection marker for the transient and stable transformation of embryogenic suspension cultures of soybeans, following the gene introduction by particle bombardment (Ponappa et al. 1999). It was used as a selection marker for the production of recombinant proteins in the pea transient expression by agroinfection (Fan et al. 2011). Its successful usage for *Arobacterium*-mediated transformation of pigeon peas has been described by Karmakar et al. (2019). A summary of the selection and reporter genes used for the pea transformation in different studies is presented in Table 2.

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Table 2. Successfully achieved pea transformations: overview of the reporter and selection genes used

GOI/phenotype/intention	Reporter/selection gene	Explant	Reference
Stable transformation	*nptII*, *hpt*	shoot cultures and seedling epicotyl	Puonti-Kaerlas et al. (1990)
Stable transformation	*uidA*, *nptII*	epicotyl segments and nodes	de Kathen and Jacobsen (1990)
GUS assay	*uidA*	tin cell layers from nodes	Nauerby et al. (1991)
Stable transformation	*uidA*, *hpt*	protoplast	Puonti-Kaerlas et al. (1992)
Stable transformation	*nptII*, *bar*	embryonic axis	Schroeder et al. (1993)
Stable transformation	*uidA*, *bar*, *nptII*	cotyledonary meristems	Davies et al. (1993)
Resistance to *Bruchus pisorum*	*uidA*, *bar*	embryonic axis	Shade et al. (1994)
Stable inheritance and expression of transgenes	*bar*, *nptII*	immature cotyledons	Grant et al. (1995)
Resistance to *Bruchus pisorum*	*bar*	embryonic axis	Schroeder et al. (1995)
Stable transformation	*bar*, *nptII*	cotyledonary meristem	Bean et al. (1997)
Resistance to PEMV	not listed	axillary meristems	Chowrira et al. (1998)
Resistance to Alfalfa mosaic virus	*nptII*	immature cotyledons	Grant et al. (1998)
Resistance to PSbMV	*bar*	cotyledonary meristem	Jones et al. (1998)
Protease inhibitors	*bar*	embryonic axis	Charity et al. (1999)
Factors influencing transformation efficiency	*uidA*, *nptII*, *hpt*, *dhfr*, *bar*	immature cotyledons	Nadolska-Orczyk and Orczyk (2000)
Different genotypes and vectors	*uidA*, *nptII*, *pat*	embryogenic axis segments	Polowick et al. (2000)
Testing of trypsin/ chymotrypsin promoters	*bar*	cotyledonary meristems	Welham and Domoney (2000)
Coat protein from Alfalfa mosaic virus	*nptII*	immature cotyledons	Timmerman-Vaughan et al. (2001)
Transformation efficiency of Polish cultivars	*uidA*, *bar*, *nptII*	slice of an immature embryo, including embryonic axis and basal part of the cotyledon	Pniewski and Kapusta (2005)
Improved nitrogen status, higher seed protein	*bar*	embryonic axis	Rolletschek et al. (2005)
Two regeneration systems: i*n vitro* and i*n vivo*	*uidA*, *nptII*	cotyledonary nodes, seeds	Švábová et al. (2005, 2008)
Resistance to fungal pathogens	*bar*	sliced embryo axis	Richter et al. (2006)
Comparison of different types of explants	*uidA*, *bar*, *nptII*	stem segments, axillary buds, embryonic segments	Krejčí et al. (2007)
Resistance to pea weevil larvae	*bar*	embryonic axis	de Sousa-Majer et al. (2007)
Testing of cocultivation treatments	*uidA*, *bar*, *nptII*	cotyledonary nodes	Švábová and Griga (2008)
Vaccine against rabbit bleeding virus	*nptII*	embryogenic axis segments	Mikschofsky et al. (2009)
Vaccine against chicken coccidiosis	*bar*	epicotyl segments and apical meristem	Zimmerman et al. (2009)
Production of human acid fibroblast in pea plants	*gfp*	germinating pea seeds	Fan et al. (2011)
Resistance to insect pests, *cry1Ac* gene	*bar*	sliced embryo axis	Negawo et al. (2013); Negawo (2015)
Tolerance to salt stress	*bar*, *luc*	immature embryos	Ali et al. (2015, 2018)
Increased transformation efficiency	*bar*	embryonic segments	Aftabi et al. (2018)

GOI – genes of interest; GUS – β-glucuronidase; PEMV – *Pea enation mosaic virus*; PSbMV – *Pea seed-borne mosaic virus*

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Pea transformation with useful genes (GOI)

Agronomic and qualitative traits. The last decade of the 20th century was devoted to the development and optimalisation of pea transformation protocols, but also to pea modification by constructs with "useful" genes, namely conferring herbicide tolerance, insect and virus resistance (Shade et al. 1994; Grant et al. 1995; Chowrira et al. 1998; Jones et al. 1998; Charity et al. 1999). This research resulted in the successful proof of pea insect and virus resistance in field conditions (Morton et al. 2000; Timmerman-Vaughan et al. 2001).

One of the first useful genes to be inserted into the pea genome was the β-amylase inhibitor gene to induce resistance to bruchus (*Bruchus pisorum* L.) (Shade et al. 1994). Experiments have also been performed to induce resistance to the viral pathogens AMV (*Alfalfa mosaic virus*) (Grant et al. 1998; Timmerman-Vaughan et al. 2001), PSbMV (*Pea seed-borne mosaic virus*) (Jones et al. 1998) and PEMV (*Pea enation mosaic virus*) (Chowrira et al. 1998). Švábová et al. (2010) reported pea transformation experiments with a protease inhibitor from the wax moth *Galleria mellonela* L. (gene *gmSPI-2*).

Since the discovery of the phenomenon of RNA interference (Hamilton & Baulcombe 1999), genetic transformation has been used not only to increase variability by adding new genes, but to also silence genes by suppressing the gene transcription (TGS) or translation (PTGS; reviewed by Paszkowski & Whitham 2001). Nevertheless, a limited number of reports on the application of RNAi and targeted genome editing are available on legumes till date (Choudhury & Rajam 2021).

Gradually, transgenic pea lines with protease inhibitors (Charity et al. 1999) were produced. Morton et al. (2000) reported the results of field trials with peas transformed with α-amylase inhibitors αA1-1 and αA1-2 for resistance to the pea weevil. A one-year field trial using the cultivar Greenfeast and, on the other hand, a two-year trial with the cultivar Laura have been described. The transgenic Laura lines turned out to contain 50–70% more of the α-amylase inhibitor protein than Greenfeast.

Rolletschek et al. (2005) expressed an amino acid permease gene (*VfAAP1*) from *Vicia faba* L. in the seeds of *Pisum sativum* L. and *Vicia narbonensis* L. under the legumin B4 promoter. They observed that the *VfAAP1* gene expression increased the seed sink strength for nitrogen, improved the plant nitrogen status, which led to a higher seed protein.

Nifantova et al. (2005) produced transgenic pea plants resistant to the herbicide Pursuit by expressing a mutant acetolactate synthase gene. Prescott et al. (2005) showed that the transgenic expression of an alpha-amylase inhibitor (αAI) from the common bean (*Phaseolus vulgaris*) in the transgenic pea led to the synthesis of a structurally modified form of this inhibitor. This issue is discussed in more detail in this review in the chapter describing the effects of bruchid-resistant GM peas on animal health.

To induce resistance to fungal pathogens, the production of resveratrol in peas was achieved due to the gene expression for the polygalacturonase inhibiting protein from raspberries and stilbene synthase (Richter et al. 2006).

Negawo et al. (2013) used *Agrobacterium*-mediated transformation to improve insect resistance (tobacco budworm) in transgenic pea lines containing *cry1Ac* gene from *Bacillus thuringiensis*. The obtained transgenic pea lines expressing the *cry1Ac* gene were grown under a growth chamber and greenhouse conditions and used for molecular and functional characterisations (Negawo 2015). The results demonstrated the stable integration, inheritance and expression of the *cry1Ac* transgene.

Among grain legumes, peas are highly sensitive to salt stress. Ali et al. (2015) improved the salt stress tolerance response with transgenic pea plants overexpressing the Na+/H+ gene from *Arabidopsis thaliana*. In the follow up work, Ali et al. (2018) reported on the genetic stability and persistent features of salt stress tolerant transgenic peas over five generations. The transgenic plants also showed unexpected frost tolerance compared to wild-type plants.

The activity of a promoter from a pea enzyme inhibitor gene and its exploitation for seed quality improvement was investigated by Welham and Domoney (2000). The promoter from one of the two seed-expressed genes encoding trypsin/chymotrypsin inhibitors (TI) has been isolated and characterised in transgenic pea lines, following its re-introduction by the *Agrobacterium*-mediated transformation, as a TI promoter-beta-glucuronidase gene fusion. The promoter from this gene (TI1) directed expression of GUS enzyme at the late stages of embryogenesis, comparable to those determined for the activity of the homologous native TI genes. GUS expression was detected in the roots of plants subjected to drought stress conditions, indicating that the TI1

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gene, normally seed-specific in its expression, can be induced under these conditions. A second gene construct utilised the TI1 gene promoter for the direct expression of an antisense TI gene. The seed TI activities in some lines transformed with this construct were reduced significantly. The observed frequency of the non-transmission of the transgenes from the primary transformants was up to 80%.

Molecular farming. Plants are attractive expression systems for the economic production of recombinant proteins. Among different plant-based systems, the plant's seed is the leading platform and holds several advantages such as high protein yields and the stable storage of target proteins (Lau & Sun 2009). The field pea (*Pisum sativum* L.) appears well suited for the production of high-value molecules, such as recombinant antibodies, with well-established agricultural practices worldwide and seeds that are easily stored and distributed (Perrin et al. 2000).

Perrin et al. (2000) described the usage of transgenic pea seeds as bioreactors for the production of an antibody used in cancer diagnosis and therapy. In order to evaluate the suitability of peas for the production of biologically active antibodies, they transformed it with cDNA encoding the single-chain Fv fragment scFvT84.66 under the control of the seed-specific legumin A promoter. The antibody was targeted to the endoplasmic reticulum for better stability and high accumulation. Transgenic plants produced up to 9 μg per gram of fresh weight of the functional scFvT84.66 protein in their seeds. The transgene was stably inherited and expressed in the progeny, and the antibody remained active after storage in the dried transgenic seeds for two months at room temperature.

Saalbach et al. (2001) showed that recombinant antibodies can be accumulated in transgenic pea seeds in a homozygous transgenic line up to 2% of the total soluble seed protein. The expression was controlled by the seed-specific unknown seed protein (USP) promoter and the transgenic single-chain Fv antibody protein was retended in the endoplasmic reticulum. The stable inheritance, shown by investigation into the high-level accumulation in the R3 offspring was an important feature of this antibody production system.

The importance of the pea transformation (as well as transformation of other high-protein crops) has grown with the demand for the production of phytopharmaceuticals (herbal vaccines). In peas, a vaccine has been developed against the rabbit bleeding virus (Mikschofsky et al. 2009) or chicken coccidiosis (Zimmermann et al. 2009). Fan et al. (2011) described the production of the human acidic fibroblast growth factor in pea plants by agroinfection of the germinated seeds. The vacuum infiltration method was used, giving similar high yields to the leaf injection method, but it was more efficient. The successfully achieved pea transformations and modified traits in transgenic peas are summarised in Table 3.

Field trials with GM pea – environmental risk assessment and agronomic performance

Advanced methods of genetic engineering could help conventional breeding techniques by overcoming the sexual incompatibility of related or even distant plant species which may serve as donors of desired traits. The recent state-of-art techniques in classical plant transgenesis and new breeding techniques (NBTs) allow one to create a broad spectrum of GM crops with a plethora of GOI in laboratory/greenhouse conditions. Nevertheless, the journey from successful laboratory results to market is very long and includes several steps starting with the biological confirmation of the behaviour of created GM plant lines in complex field conditions (climatic and pedological conditions in a given region) and their comparison with parental lines and commercially grown cultivars of a particular crop (agronomic performance study). Of course, these field tests must satisfy the regulatory demands according to GM legislation which differs country by country. One of the general prerequisites for releasing a GM crop into the environment is an environmental risk assessment study in order to guarantee the unintended escape of transgene/s, mainly via pollen flow. This is relevant for commercial cultivars of particular crop in a given region (in order to avoid GM contamination of commercial seeds on the market) and for taxonomically closely related wild plant species (within a given genus or family). The risk assessment must mainly reflect a mode of reproduction of the crop (self-pollinated versus cross-pollinated crops) and the occurrence of hypothetically crossable relatives in a given region.

The pea as a self-pollinating/self-fertilising, cleistogamic crop that represents a species with a very low potential for outcrossing (less than 1%; Gritton 1980). The flowers open about 24 h after pollination (Cooper 1938), thus the probability of outcrossing is very low, nevertheless the intervention of wind or

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Table 3. Useful genes (genes of interest) successfully engineered and expressed in *Pisum sativum* L.

Trait/phenotype	Origin/donor of transgene	Gene product/gene symbol	Research status/objective obtained	Reference
Agronomic and qualitative traits				
Resistance to pea weevil (*Bruchus pisorum* L.)	common bean (*Phaseolus vulgaris* L.)	inhibitor of α-amylase expressed in pea seeds/α-AI-*Pv*	stable expression of α-AI-Pv; laboratory/greenhouse resistance to *B. pisorum* confirmed	Shade et al. (1994) Schroeder et al. (1995)
			field resistance to *B. pisorum* confirmed	Morton et al. (2000)
Resistance to *Pea enation mosaic virus* (PEMV)	PEMV	coat protein of PEMV/*cpPEMV*	resistance against PEMV confirmed in the greenhouse (T2—T4 GM pea plants)	Chowrira et al. (1998)
Resistance to *Pea seed-borne mosaic virus* (PSbMV) via PSTGS	PSbMV	viral replicase NIb/ *PSbMV NIb*	resistance against PSbMV confirmed in the greenhouse	Jones et al. (1998) Boogaart et al. (2004)
Resistance to *Alfalfa mosaic virus* (AMV)	*Alfalfa mosaic virus* (AMV)	coat protein of AMV/*cpAMV*	partial resistance to AMV under greenhouse and field conditions confirmed	Grant et al. (1998) Timmerman-Vaughan et al. (2001)
Resistance to cotton bollworm (*Helicoverpa armigera*)	*Nicotiana alata*	type II serine proteinase inhibitor Na-PI/*Na-PI*	bioassay i*n vitro*on isolated pea leaves (T1, T2 GM peas) – increased larval mortality, delayed growth and development	Charity et al. (1999)
Increased seed storage proteins synthesis	faba bean (*Vicia faba*)	amino acid permease /*VfAAP1*	*VfAAP1*expression increased seed sink, strength for nitrogen, improved plant nitrogen status and led to higher seed protein accumulation	Rolletschek et al. (2005)
Resistance to *Pea enation mosaic virus* (PEMV) and *Pea seed-borne mosaic virus* (PSbMV) via PTGS	PEMV, PSbMBV	whole coat protein PEMV/*cpPEMV*; coat protein fragments of PEMV and PSbMV in sense/antisense position/*cpPEMV* and *cpPSbMV*	resistance/tolerance against PEMV and PSbMV confirmed in T2—T3 GM pea plants in the greenhouse	Švábová et al. (2007a, 2009); Hanáček et al. (2010)
Insect resistance (*Sitona lineatus*, *Kakothrips pisivorus*, *Bruchus pisorum*)	wax moth (*Galleria mellonela*)	GM silk proteinase inhibitor/*gmSPI-2*	increased mortality of *S. lineatus* and *B. pisorum* larvae in the greenhouse feeding tests on GM peas	Hanáček et al. (2008) Griga et al. (2009) Švábová et al. (2007b, 2010)
The inhibition effect of antifungal transgenes on cell-wall degrading fungal enzymes (polygalacturonases)	raspberry (*Rubus idaeus*) – polygalacturonase; grape vine (*Vitis vinifera*) – stilbene synthase	polygalacturonase/*riPGIP*; stilbene synthase/ *Vst*; stacking of both genes by conventional crossing	inhibitory effect of GM peas (T7) on polygalacturonases from *Colletotrichum lupini* and *Stenocarpella maydis* in bioassay *in vitro*	Richter et al. (2006)

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Table 3 to be continued

Trait/phenotype	Origin/donor of transgene	Gene product/gene symbol	Research status/objective obtained	Reference
Agronomic and qualitative traits				
The effect of antifungal transgenes on pea root colonization with *Glomus intraradices*	raspberry (*Rubus idaeus*) – polygalacturonase; grape vine (*Vitis vinifera*) – stilbene synthase; *Sreptomyces olivaceoviridis*- chitinase	polygalacturonase/*riPGIP*; stilbene synthase/ *Vst*; chitinase/*Chit30*	antifungal transgenes had not any negative effect on pea root colonization with *Glomus intraradices*	Hassan et al. (2012)
Resistance to fungal diseases (*Trichoderma harzianum*)	*Sreptomyces olivaceoviridis* (chitinase), *Hordeum vulgare* (glucanase)	stacking of two antifungal genes: chitinase/*Chit30*; 1,3-β-glucanase/*gluc*	*in vitro* bioassay – inhibition of fungal spore germination	Amian et al. (2011)
Resistance to tobacco budworm (*Heliothis virescens*)	*Bacillus thuringiensis*	Cry1Ac protein (Bt-toxin)/*cry1Ac*	resistance to *Heliothis virescens* larvae confirmed in growth chamber insect bioassay in T2—T7 GM pea lines	Negawo et al. (2013, 2016)
Salt stress and frost tolerance	*Arabidopsis thaliana*	Na+/H+ antiporter/*AtNHX1*	salt stress and frost tolerance in GM peas confirmed in subsequent generations over a period of 6 years	Ali et al. (2015, 2018)
Molecular farming				
Cancer diagnosis and therapy	single-chain Fv fragment from monoclonal antibody	scFv from monoclonal antibody T84.66	active antibody production; transgene stably inherited and expressed in progeny	Perrin et al. (2000)
Recombinant protein production	single-chain Fv antibody gene used to immunomodulate abscisic acid	anti-ABA scFV gene fragment antibodies	high-level expression of single-chain Fv fragment antibody; stable inheritance of transgene	Saalbach et al. (2001)
Vaccine against *Rabbit haemorrhagic disease virus* (RHDV)	major structural protein of RHDV	VP60-based antigen	successful immunisation of rabbits with pea-derived vaccines	Mikschofsky et al. (2009)
Vaccine against chicken coccidiosis	immunised mice	anti-Eimeria scFvs antibody fragments	cost-effective antibody expression	Zimmerman et al. (2009)
Recombinant mitogen protein production	human	mammalian acidic fibroblast growth factor (aFGF)	successful recombinant protein production with the use of vacuum infiltration; shortened production cycle compared to leaf injection method	Fan et al. (2011)

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insect pollinators cannot be explicitly eliminated. According to the authors' knowledge, official field trials with GM peas were performed in Canada (Kahlon et al. 2018; Polowick et al. 2002;), Australia (de Sousa-Majer et al. 1999, 2000, 2001; Morton et al. 2000; de Sousa-Majer 2001), New Zealand (Timmerman-Vaughan et al. 2001) and the Czech Republic (Dostálová et al. 2004, 2005, 2009; Griga et al. 2008). In all of the above-mentioned countries, there are no wild relative species naturally crossable with culture peas, thus the performed tests were concentrated on the pollen flow between the GM peas and the commercially grown pea cultivars and the agronomic performance of the GM peas.

Polowick et al. (2002) tested the possible outcrossing of transgenic peas in field conditions of central Canada (Saskatchewan) (1997 and 1999). The transgenic lines recovered from the cv. Greenfeast via *Agrobacterium*-transformation have a dominant normal leaf form and contained the bi-functional fusion gene conferring β-glucuronidase (GUS) and neomycin phosphotransferase activities (Polowick et al. 2000). Three Canadian commercial cultivars (Montana, Carneval, Tipu) exhibiting a recessive semi-leafless trait and overlapping flowering times were chosen as the trap cultivars. The GM pea line PLP1 homozygous for the *gusA* gene (T5-generation) was sown (1997) as the central plot in four 15 m rows (100 seeds/row, with 15 cm spacing between the rows, on a north-south axis perpendicular to the prevailing westerly wind). Four blocks containing three trap varieties were seeded in random order (48 plots) on each side of the central transgenic plot. At the end of the growing season, all the pods were collected from each of the 48 trap plots and 100 randomly sampled seeds were grown in a growth cabinet. The leaf discs from the cultured plants were then assessed for the GUS expression and all the plants were scored for the presence of normal leaves. The experiment was repeated in 1999 with the T6 seeds. As a result, no GUS activity was detected in any plant from the offspring of the 1997 field trial. In the 1999 trial, the GUS activity was recorded in the offspring of two plots of cv. Montana and one plot of cv. Tipu. These three plots were located in close proximity to the GM pea plots. The GUS expressing plants also exhibited a dominant normal leaf type. In total, the mean frequency of outcrossing over the two-year trial was 0.07% (Montana 0.11%; Tipu 0.09%; Carneval 0.00%) based on ca. 9 000 tested offspring. The realised trials provided a demonstration of the transgene transference to commercial pea cultivars. However, the frequency of the transgene recovery in the trap plants was very low, in addition with any occurrence in only one year (1997) from the two tested years. The pollination could be possibly caused by wind or insect pollinators (pea aphids, bumblebees, ladybird beetle larvae, dipteran flies). The recorded outcrossing did not overcome a distance of 5 m, which indicates that a safety isolation distance between GM peas and commercially grown non-GM peas could be relatively short when compared to other crops (e.g., corn, *Brassicas*) (Polowick et al. 2002). In addition, the unintended pollen flow may be further restricted by buffering belts of non-GM peas surrounding GM-pea trials/fields, thus ensuring their safe co-existence.

Dostálová et al. (2004, 2005) conducted field trials (2001–2004) to assess the rate of natural outcrossing between commercial field pea cultivars in central European environmental conditions, namely in the Czech Republic. The objective of the research was to collect data for the official release of GM pea into the environment for agronomic performance trials (Rakouský et al. 2004). Two non-GM pea cvs. differing in flower colour, seed coat colour and the whole plant habit with overlapping flowering periods were used. The dry-seeded cv. Zekon (*P. sativum* ssp. *sativum* L.) with recessive traits (white flowering, colourless seed coat, green cotyledons, semi-leafless type) served as the trap variety. The fodder pea cv. Arvika (*P. sativum* ssp. *arvense* L.) with dominant traits (violet-flowering, coloured seed coat, yellow cotyledons, normal leaf type) was used as a pollen donor. Two 5 m^2 plots of trap cv. Zekon were surrounded by ten 5 m^2 plots of the pollen donor cv. Arvika and *vice versa*. The harvesting of the seeds of both cvs. was carried out separately. All the harvested seeds of cv. Zekon (2001, 2002) were sown each successive year (2002, 2003; plot ca. 430 m^2) and the occurrence of F_1 hybrids expressing the dominant genes was recorded (40 000 F_1 plants evaluated yearly). No plants expressing dominant genes were recorded within the F_1 populations in either 2002 or 2003. Detailed observations of the insects regularly visiting/damaging the pea flower buds/flowers (pests, pollinators) were carried out in the framework of the experiment (2001–2004). The most frequent taxa were the pea weevil (*Bruchus pisorum* L.), the pea aphid (*Acyrthosiphon pisum* L.), pea trips (*Kakothrips robustus* Uzel), the honey-bee (*Apis mellifera* L.), and bumble-bees (*Bombus* sp.).

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Based on the literature data, *Bruchus pisorum* L. (Clement et al. 1988; Clement 1992; Polowick et al. 2002; Dostálová et al. 2005) and bumble-bees (*Bombus* sp.) (Loennig 1983, 1984, 1985) are the most probable candidates for cross-pollination either in the unopened or just opened pea flowers.

As a conclusion, the reliability of experimentally obtained data on natural pea outcrossing may be affected by several factors (Dostálová et al. 2005), namely (1) the selection of suitable parent genotypes (overlapping flowering periods; better or worse affinity for cross-pollination due to a complex of physiological/biochemical characteristics affecting the pollen-stigma affinity of the included genotypes; competition in its own versus foreign pollen germination and growth; easy/difficult scorability of genetic markers demonstrating the hybrid state (Oliveira 1963; Loennig 1983; Giordano et al. 1991; Bogdanova & Berdnikov 2000), (2) the spatial distance between the pollen donor and recipient plants (Oliveira 1963; Polowick et al. 2002), (3) geographical/climatic conditions, particularly during the flowering period – temperate/subtropical/tropical regions (Govorov 1928; Harland 1948; Oliveira 1963; Giordano et al. 1991) and (4) the incidence of possible insect pollinators (Loennig 1983, 1984, 1985; Clement et al. 1988; Clement 1992; Polowick et al. 2002; Dostálová et al. 2005).

In Europe, the most advanced results on pea transformation with GOI were reported by the laboratory of Hans-Jörg Jacobsen from Hannover University, Germany (Richter et al. 2006; Hassan et al. 2010; Amian et al. 2011). The effort to test/confirm positive laboratory results in official GM field trials in Germany was unfortunately stopped due to the high risk of damage of these trials by ecological terrorists and the general low acceptance of GM technology/crops/products by German society. The originally promising negotiation to start these trials at the North Dakota State University, USA (Meldolesi 2010) were, however, also stopped due to objections of US-pea growers (H.-J. Jacobsen, personal communication). Thus, the final destination for the contained GM pea field trials (2013–2015) became Alberta, Canada (Kahlon et al. 2018). Two European pea cultivars (Baroness, Sponsor) were transformed with four antifungal genes encoding for disease resistance, namely, 1-3 β glucanase (G), endochitinase (C) (belonging to the PR proteins family), polygalacturonase (PGIPs), stilbene synthase (V). GM lines with genes inserted either individually or stacked through crossing (Richter et al. 2006; Hassan et al. 2010; Amian et al. 2011) showed disease tolerance under laboratory conditions – *in vitro* test with *Trichoderma harzianum* (Hassan et al. 2009; Amian et al. 2011). The GM pea lines were tested for their efficacy against Fusarium root rot (*Fusarium avenaceum*) in confined trials over three years in comparison with two parental German cvs. and three Canadian cvs. (cv. Agassiz: resistant to *Erysiphe pisi* and moderately susceptible to *Mycosphaerella pinodes*; AC Earlystar: resistant to *E.p.*, moderately resistant to *M.p.* and *Fusarium oxysporum*; cv. AAC Royce: resistant to *E.p.*, moderately susceptible *M.p.* and *F.ox.*). The GM lines with single gene insertions (G, C, V), double gene insertion (V : P), triple gene insertion (P : C : G) and four gene insertion (V : P : G : C) were used. The analysis of the root and soil samples for pathogen presence showed *Fusarium* ssp., namely *F. solani*, *F. avenaceum*, and *F. redolens* as being the most abundant, and *F. acuminatum*, *F. oxysporum* and *F. equiseti* as being less abundant. The presence of other common soil inhibiting fungi like *Rhizoctonia* ssp., *Rhizopus* ssp., *Trichoderma* ssp. and *Clonostachys rosea* were also recorded. The disease symptomology and severity for the above and below ground plant parts were recorded after destructive sampling at 8 weeks after planting. The fresh weight was recorded as an indicator of the potential yield. As a result, the authors were not able to identify the GM pea lines that outperformed the parental lines or the well adapted Canadian lines in the presence of disease over the course of three consequent field seasons. Also, no advantage of gene pyramiding over having individual genes was recorded, contrary to the initial hypothesis. The authors saw the possible reasons in the different behaviour of the GM plants *in vitro* (successful inhibition of fungal growth) as compared to the greenhouse and even field conditions (broad spectrum of fungal pathogens). Furthermore, the transgenes had variable relative gene expression in the roots as compared to the shoots in general. Finally, the major constraint in the co-expression of different transgenes is that the gene expression remains uncoordinated even with physically linked genes and the transcriptional silencing of the transgene may occur (Kahlon et al. 2018).

Timmerman-Vaughan et al. (2001) tested the agronomic performance of GM peas expressing the coat protein gene of the *Alfalfa mosaic virus* (AMV). In the greenhouse test, five lines (T1) with improved virus resistance were identified. A field test was

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then conducted (in accordance with New Zealand Government legislation and regulations) with the progeny of four independently derived transgenic lines over the summer in 1998–1999 near Lincoln, New Zealand. The trial was planted in three blocks, one for each treatment: Block 1, to evaluate the performance of the uninoculated plants; Block 2, plants mechanically inoculated with the AMV strain NZ1 (Lincoln); and Block 3, plants mechanically inoculated with the AMV strain NZ34. Each block contained 12 plots of 11 randomly arranged GM lines (one line was replicated) and two plots each of the non-GM control lines. The entire trial was surrounded by two buffer rows of cv. Primo, and two buffer rows were planted between the adjacent blocks. Standard agronomic practices were used: fertilisation and irrigation, pre- and post-emergence herbicides, fungicide application, and hand weeding as needed. The field trials confirmed the previous greenhouse tests, i.e., transgenic pea plants expressing a chimeric *cpAMV NZ1* gene had improved resistance to AMV. Molecular characterisation of resistant and susceptible plants suggests that resistance is coat protein (CP) mediated, since resistance was only observed in plants that accumulated the CP (detected on western blots). The authors concluded that a useful level of field resistance can be obtained in transgenic pea lines expressing the AMV CP. Nevertheless, this resistance should be proved in the conditions of naturally occurring, aphid-borne ASMV epidemics, including infection with a number of strains.

The most progressive results as related to the development of insect-resistant GM peas (bruchid-resistance) including extensive proof of the behaviour of the inserted transgenes both in glasshouse and field conditions were achieved by the team of T.J.V. Higgins from The Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia. The intensive concerted effort resulted in the development of stable GM pea lines expressing two a-amylase inhibitors (aAI-1 and aAI-2) from the common bean (*Phaseolus vulgaris* L.) (Shade et al. 1994; Schroeder et al. 1995), evidence of complete plant protection to bruchid beetles in the glasshouse-grown GM plants expressing a-AI-1 in pea seeds (Shade et al. 1994; Schroeder et al. 1995) and, finally, the confirmation of resistance of these GM lines against *Bruchus pisorum* L. in field conditions (Morton et al. 2000; de Sousa-Majer et al. 2001). The field experiments were performed with the pea cvs. Greenfeast and Laura bearing transgenes for a-AI-1 and a-AI-2 proteins in homozygous constitution ensuring the high seed-specific expression of both inhibitors. The field trials were conducted in 1996 and 1997 in New South Wales, Victoria, and Western Australia, in regions where peas are grown each year and bruchids overwinter in the surrounding areas and reinfest the trial plots. The severity of the natural infestation differs year by year and depends on many variables (temperature, humidity). Each plot in the standardly designed trial (randomised complete block with three replicates of each genotype) consisted of peas sown in two rows, 4 m long, 20 cm apart, at 40 seeds per row, and a 1.5 m border between each plot. The plants were hand-harvested at maturity, threshed, and cleaned. Two hundred to five hundred (200 to 500) randomly selected seeds from each plot were scored and the "percent adult emergence" was calculated as the percentage of the seeds containing larval entry holes ("infested seeds") that also had windows or exit holes. In some cases, the time taken for adult emergence was also monitored. In the 1996 trial with the cv. Greenfeast, the pea weevil larvae had entered 80% of the seeds harvested as evidenced by the larval entry holes. At 75 days post-harvest, an average of 98% of these larvae had developed into adults in the non-GM pea seeds, while only 7% of the larvae developed to adulthood in the seeds from plants transformed with the *aAI-1* gene. The remaining larvae died at the first or second instar. A similar dramatic reduction in the weevil emergence in αAI-1 GM lines was also observed in the 1997 field trials, over three different sites, with the cv. Laura. The yield data showed that the expression of aAI proteins in peas did not result in a yield penalty. Based on the 1997 trials, the gene that encodes the aAI-2 protein appeared to give less protection than the *aAI-1* gene. The observations indicated that aAI-2 had no effect on the final mortality rate of the developing larvae, but had a significant effect on their rate of development, delaying the emergence of the adults by about one month. Further analyses showed that the different efficacy of aAI-I and aAI-2 was not caused by their level of expression (content) in the pea seeds, but the differences in their particular chemical properties (optimum pH range for inhibiting activity, different affinity/inhibiting activity to multiple bruchid a-amylases). Based on these complex data, the authors (Morton et al. 2000) concluded that growing bruchid resistant GM peas may significantly reduce the use of chemical pesticides during vegetation and also the need for fumigation during seed storage.

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Thus, the cost to the pea growers and consumers may be reduced as well as the insecticide load on the environment. The possibility of development of field resistance in the wild pea weevil population as a result of growing aAI GM pea plants was studied in subsequent field experiments in 1999 and 2000 (de Sousa-Majer et al. 2001). Also, the effect of water and temperature stress on the level of production of the aAI in GM pea plants/seeds and subsequent bruchid larvae survival/mortality was shown. The use of pea seed mixtures comprised of pea weevil resistant (GM) seeds and non-GM pea susceptible seeds was demonstrated to be a viable on-farm control option for the pea weevil. The seed mixture strategy model reduces the pea weevil attack without selecting resistant populations, in addition, if it is combined with other on-farm practices like early harvesting of the pea crop (de Sousa-Majer et al. 2001).

Agritec Ltd. was the first and the only institution in Europe which performed official contained field trials with GM peas (Notification No. B/CZ/09/05; permitted period of release 2010–2019; https://webgate.ec.europa.eu/fip/GMO_Registers/GMO_Summary.php?NotificationNum=B/CZ/09/05&Cat=gmp). The experiments were focused on proving the effect of the inserted transgenes on the target organisms (viruses, fungal pathogens, and insect pests) and non-target organisms, and the agronomic performance of the GM lines. The field (dry-seed) peas – leafy and semileafless cvs. – were transformed via the *Agrobacterium*-mediated approach (Švábová et al. 2005; Švábová & Griga 2008) with various GOI, namely (1) the sequence of complete gene *cpPEMV* for the coat protein of PEMV (*Pea enation mosaic virus*) for activation of resistance induced by the pathogen infection; (2) the sequences of gene fragments *cpPSbMV* in the sense and antisense orientation for the coat protein of PSbMV (*Pea seed-borne mosaic virus*) for activation of resistance by the mechanism of post-transcriptional gene silencing (PTGS), (3) modified gene *gm-SPI2* for serine protease inhibitor from the labial glands of the wax moth (*Galleria mellonella* L.) to induce resistance to pea insect pests (*Bruchus pisorum* L., *Sitona lineatus* L.) and fungal diseases (*Fusarium* ssp.), (4) plant *Dof* (DNA-binding with one finger) gene, which is supposed to enhance the accumulation of seed proteins and, thus, could increase the yield of transgenic peas and (5) plant gene *L1L* (LEC1-LIKE) influencing the plant embryo development, especially during its early stage, which thus, could affect the earliness (sooner development) of transgenic peas. Some of the inserted traits were positively tested in the growth chamber or glasshouse (increased tolerance/resistance to PEMV and PSbMV after artificial infection with severe isolates of the particular viruses – Šafářová et al. 2008; Švábová et al. 2009; increased mortality of *Bruchus pisorum* L. larvae after feeding GM peas with *gm-SPI2* gene – Hanáček et al. 2008; Griga et al. 2009). Field trials were located at Vikýřovice, in the Olomouc region, northern Moravia, the total size of the site was about 1 000 m^2, with a 100–300 m^2 actual plot size for planting transgenic peas with conventional pea controls. The evaluation of the GM pea lines with various GOI was more difficult when compared to the growth chamber or glasshouse conditions, in which the experimental parameters could be better controlled (temperature, photoperiod and light intensity, humidity, artificial virus infection, artificial attack/invasion of insect eggs/larvae on the pea plants); there was a great variation in the occurrence of pests in the particular years, i.e., the incidence of aphids as virus vectors, incidence of overwintering *Bruchus* and *Sitona* adults, as influenced by the meteorological parameters (Kahlon et al. 2018). The complex evaluation of the recorded data has not been finished yet; nevertheless, the tested GM pea lines did not statistically overcome the non-GM controls as was observed in the glasshouse conditions (Kahlon et al. 2018). The compositional analysis of the seed substances (protein and starch content, trypsin inhibitors) did not show any statistical differences between the GM pea lines and the non-GM pea lines transformed with constructs for virus resistance (PEMV, PSbMV). These GM lines were also used for feeding tests with rats (Mares et al. 2014).

Under laboratory/greenhouse conditions, the effect of expressed transgenes may be easily detected (usually the reaction to a single challenge), while, in the field trials, the number of pathogens, differences in the soil moisture and weather variance more accurately represent the true agronomic effect (genotype x environment interaction) of the transgenes. Ideally, for addressing the efficacy of transgenic plants engineered for tolerance/resistance to both biotic and abiotic factors, multiyear and multi-location field trials are desired (Kahlon et al. 2018).

The effect of GM peas on animal health – feeding and allergenicity studies

Feeding studies in animals have been performed with two types of GM peas, namely lines with inserted

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transgene for common bean alfa-amylase inhibitor αAI-1Pv conferring bruchid resistance (Pusztai et al. 1999; Prescott et al. 2005; Collins et al. 2006; Li et al. 2006; Chen et al. 2009; Islam et al. 2009; Lee et al. 2013a) and lines with transgenes for coat proteins of pea attacking viruses (*cpPEMV*, *cpPSbMV*) conferring virus resistance (Mares et al. 2014). The tests were conducted on rats, mice, broiler chickens, and pigs.

The first study on the nutritional value of α-AI-*1Pv* containing transgenic peas (Pusztai et al. 1999) was conducted in parallel with the already ongoing GM pea field experiments in Australia (1996–2000; Morton et al. 2000; de Sousa-Majer et al. 2001). Male Hooded Lister rats weaned at 19 days of age were fed for 10 days with diets containing transgenic or parent peas at 300 and 650 g/kg, respectively, and with a 150 g protein/kg diet, supplemented with essential amino acids to target requirements. Another control was represented by the diet containing lactalbumin with or without 0.9 or 2.0 mg bean α-AI, i.e., the level equivalent to those in transgenic pea diets. The body weight, composition, nutritional performance, and relative weights of the selected organs, as well as the chemical composition of the selected organs and the enzyme levels in the small intestinal lumen and pancreas of rats fed for 10 days were recorded and compared. The results showed that the nutritional value of the diets containing transgenic or parent peas was remarkably similar, thus transgenic peas expressing the *α-AI* bean protein had no adverse effect on the starch digestibility and utilisation. The authors concluded, based on this short-term study, that GM peas may be used in the diet of mammals, including farm animals, particularly at the moderate levels of dietary inclusion recommended in commercial practice. Nevertheless, this initial study cannot be taken as proof that GM peas are fit for human consumption.

The rather optimistic vision of the first feeding study (Pusztai et al. 1999) was later corrected by a series of reports showing that the bean α-AI-1 containing transgenic pea may have some unintended negative effects when fed to experimental animals, namely mice (Prescott et al. 2005), pigs (Collins et al. 2006) or broiler chickens (Li et al. 2006). In the 15-day experiment (Collins et al. 2006), pigs were randomly allocated to one of the three dietary treatments: (1) an experimental basal wheat diet; basal wheat diet partly replaced with either (2) transgenic or (3) non-transgenic peas. The nutrient digestibility was determined at the terminal ileum (ileal and faecal digesta). The ileal dry matter digestibility was significantly reduced in the transgenic pea diet compared with non-transgenic peas (12.7 and 69.9%, respectively), which was largely due to the reduced starch digestibility. The crude protein digestibilities of GM-peas were similar to the non-GM peas, being 79.9 and 78.5%, respectively. The amino acid digestibility of both GM peas and non-GM peas were also similar. Nevertheless, the adverse effect of α-AI-1 on the amylase activity (and, thus, the starch digestibility) may be simply eliminated by heating the peas above 90 °C – this treatment resulted in similar amylase activity as compared to non-GM peas as well as to the pure α-amylase control.

Similar experiments with broiler chickens (Li et al. 2006) have shown that feeding 300 g/kg GM and non-GM peas for 40 days also resulted in ca. 50% reduced ileal starch digestibility. This reduction coincided with an 11% reduction in the growth rate of chickens fed the GM-pea diet. The metabolizable energy content of the GM pea was also significantly less than that of non-GM pea (5.08 and 12.12 MJ/kg dry matter (DM), respectively). The expression of α-AI-1 in peas did not affect the bird's health or utilisation of the dietary protein (the ileal digestion of protein and amino acids was unaffected). Thus, a significant ileal starch reduction in GM peas reduces the utility of this feedstuff in monogastric diets where efficient energy utilisation is required.

Prescott et al. (2005) studied the molecular architecture of the native common bean α-amylase inhibitor-1 (α-AI-1) (cvs. Pinto and Tendergreen) and the bean α-AI-1 expression in transgenic peas by Western immunoblot and MALDI-TOF-MS. The detailed comparison of the native and transgenically expressed bean α-AI-1 revealed differences in the banding profiles (α- and β-peptide chains), suggesting possible differences in the molecular structure, most probably the translational and post-translational modifications (glycosylation mode). The potential antigenicity of the transgenically expressed (and, thus, structurally altered) bean α-AI-1 in peas was tested in mice. The oral consumption of a diet from GM peas resulted in the detection of α-AI-1 specific IgG_1 at 2 weeks and its significant increase after 4 weeks, whose effect was not observed in mice after oral exposure of the diet from the non-GM peas, the GM lupin expressing sunflower seed albumin or the GM chickpea expressing bean α-AI-1. Finally, the consumption of the modified α-AI-1 and, not the native form, was predisposed to antigen-specific

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CD4+ th2-type inflammation. Thus, the transgenic expression of non-native proteins in plants may lead to the synthesis of structural variants possessing altered immunogenicity (Prescott et al. 2005).

The progress in the development of high-throughput molecular profiling technologies such as proteomics and metabolomics has led to the more precise characterisation of unintended changes in the composition of GM crops, including GM peas (Chen et al. 2009; Islam et al. 2009). A proteomic analysis (2-DE combined with MS) of GM pea lines expressing the bean α-AI-1 protein showed, in addition to the presence of α-AI-1, 33 other proteins which were differentially expressed/accumulated in GM pea lines compared with their non-GM parental line. Among these 33 proteins, three were associated with the expression of α-AI-1, the remaining 30 proteins seemed to be associated with transformation process *per se*. More than half of proteins with altered accumulation identified by the MALDI-TOF-TOF analysis were storage proteins (legumin, vicilin, convicilin, phaseolin, cupin, valosin-containing protein). Because seed storage proteins are known as common food allergens (Szymkiewicz & Jedrychowski 2006), the significantly increased reactions to food antigens detected in GM pea-fed mice (Prescott et al. 2005) might be connected with the elevated levels of these pea storage proteins. According to Chen et al. (2009), the variation in the storage protein expression/accumulation could be associated not only with expression of α-AI-1 in GM pea *per se*, but could be related to some other factors, namely (1) the position effects caused by the site of insertion, (2) insertion-site mutations resulting from *Agrobacterium*-mediated transformation or (3) genome-wide mutations not necessarily genetically linked to the transgene insertion site, but arising as a consequence of the tissue culture (= regeneration of transgenic lines) or *Agrobacterium* infection. Based on the above-mentioned findings on the unintended changes in the proteome of GM peas expressing the bean α-AI-1 protein (Chen et al. 2009; Islam et al. 2009) and the related increased immunogenicity of mice fed with a GM pea meal diet (Prescott et al. 2005), CSIRO discontinued the commercial development of bruchid-resistant GM peas.

Nevertheless, in 2012, a research team from the Medical University of Vienna subjected the previous unfavourable results/conclusions (Prescott et al. 2005) to a critical re-evaluation (Lee et al. 2013a). They studied the allergenicity of the α-AI-1 expressing GM peas, cowpeas, and chickpeas and compared them to the non-transgenic controls, Pinto and Tendergreen (the latter was the source of *α-AI-1* gene) in mice. The aim was to answer the question if the immunogenicity and allergenicity of α-AI-1 from these transgenic legumes was higher than the native α-AI-1 from the Pinto and Tendergreen beans. The evaluation included a comparison of antibody titres to α-AI-1 from each source. Furthermore, the antibody response to the twice weekly consumption of the pea, cowpea, chickpea, and bean meals for 4 weeks was tested. After the feeding period, the respiratory tract was challenged with α-AI-1 to evaluate the *in vivo* T lymphocyte responses. Finally, the adjuvant effect of α-AI-1 pea consumption on the initiation and exacerbation of non-cross-reactive ovalbumin (OVA)-induced an allergic lung disease. These extensive experiments revealed that a variation in antibody responses to α-AI-1 exists, but there was not an increased antibody response to the α-AI-1 from transgenic legumes compared to α-AI-1 from the beans. α-AI-1 from transgenic legumes and beans have differences in post-translational modifications (Prescott et al. 2005; Chen et al. 2009; Islam et al. 2009), which may subsequently modify the immunogenicity. However, the differences in the immunogenicity did not differentiate α-AI-1 from the transgenic legumes with those found in beans. α-AI-1 induced high IgG1 antibody titres and, thus, were immunogenic irrespective of the transgenic or non-transgenic source. The results are at odds with previous studies in which mice developed allergic response to α-AI-1 peas, but not to beans (Prescott et al. 2005, 2006). Lee et al. (2013a) offered some possible explanations: (1) different source of mice and their health status in the Australian and Austrian studies (absence of any pathological or commensal organisms or antibodies in the Austrian study; no data provided in the Australian study), (2) a difference between the two diets used in the source of the dietary protein (animal vs. plant). The question if the recorded immune and allergic reactions to α-AI-1 and pea lectin in mice may be biologically relevant to humans remains unanswered. The authors emphasised the importance of repeated experiments in independent laboratories and caution in interpretation of the results.

In fact, all the reported studies dealing with the feeding, allergenicity and chemical structure of transgressed protein in GM peas were performed with bruchid-resistant pea lines developed by CSIRO, Australia. The only exception is the report of Mares

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et al. (2014) describing the effect of virus-resistant GM peas (PSbMV, PEMV) developed by Agritec, the Czech Republic (Švábová et al. 2009. The seeds of GM pea lines were provided from the official contained field experiments of Agritec (https://webgate.ec.europa.eu/fip/GMO_Registers/GMO_Summary.php?NotificationNum=B/CZ/09/05&Cat=gmp). Twenty-four (24) male, specific pathogen-free Wistar rats (Biotech, Konarovice, Czech Republic) were used in the study. At the beginning of the experiments, the animals were 28 days old and the differences in body weight were in a range ± 5 g. The experiment started after 8 days of a quarantine period and lasted 35 days. The animals were divided into three groups (each with 8 animals) and fed three feed mixtures: KS1 without any pea supplement (pea substituted by pollards from soybeans), KS2 containing 30% of the GM pea line derived from the cv. Raman, and KS3 containing 30% of the non-GM parental pea cv. Raman. The following parameters were monitored and calculated individually in the groups of rats: net intake of feed, conversion of feed, weight increment, and health status (anatomical pathology, bacterial, parasitological, and virological indicators). The animals were treated and fed every day (food and water provided *ad libitum*) and once a week they were weighed. The average weights at the end of the experiment were 343 g in the first group fed KS1, 331 g (KS2, GM-pea) and 348 g (KS3, non-GM pea). No statistically significant differences were noticed among the compared groups (the standard deviations (SDs) between the groups were less than 3%). The feed conversion values were 3.67 (KS1) 3.81 (KS2) and 3.61 (KS3), again no significant statistical differences between the tested groups were recorded. All the experimental animals were in good health condition without any growth abnormalities and changes in behaviour. The authors concluded that relatively high level of GM peas (30%) in the feed mixture had no statistically significant adverse effect on the weight increase and health status of the laboratory rats.

In the course of the transgenesis procedure, changes in the structure of expressed transgenic protein/s *per se* as well as the alterations in the proteome, as a whole, may be detected, may be caused by various factors (as mentioned above), which was also reported in GM peas expressing the bean α-AI-1 (Prescott et al. 2005; Chen et al. 2009; Islam et al. 2009). The differences in the proteome between various pea cultivars may also occur naturally (similar to the isozyme variation) as a result of long-term pea breeders' efforts directed on the broad spectrum of traits (and not specifically intended towards the protein content/composition changes). The reported changes in the GM pea protein/proteome structure are not so dramatic in order that their consumption, in an appropriate amount (as a part of the diet), should have a necessary negative impact on farm animals, and even on humans (Bhowmik & Basu 2008, and citations in this review). The latest high-throughput methods of proteomics, metabolomics, and lipidomics enable the exact comparison of GM versus non-GM plant products, which fact may serve as a robust background for reliable feeding and allergenicity tests in order to speed the commercialisation of novel GM plant products. This, of course, needs more intensive GMO-related research, however, the recent complicated EU GMO-legislation represents high uncertainty for investments into innovative plant breeding, mainly in the private sector (Jorasch 2020; Purnhagen & Wesseler 2021).

CONCLUSION AND PERSPECTIVES

More than thirty years after the first pioneering pea genetic transformation and complete transgenic pea plant regeneration (de Kathen & Jacobsen 1990; Puonti-Kaerlas et al. 1990), we can state that the genetic transformation technology in peas is relatively well elaborated upon, *Agrobacterium*-mediated transformation protocols being the most reliable and the most frequently used ones. A number of traits have been engineered into the pea, namely virus, fungus and insect resistance, and salt tolerance. Pea seeds were also used as bioreactors for the production of recombinant antibodies or phytopharmaceuticals (herbal vaccines). Despite the fact that the fundamental and methodological research on pea transgenesis resulted in promising achievements with the high application potential in pea breeding, the commercialisation of these results was not achieved either due to the fact that laboratory/glasshouse data were not confirmed by field experiments (e.g., Kahlon et al. 2018) or the harvested product (pea seed proteins) exhibited unintended characteristics (changed chemical structure, lowered starch digestibility, allergenicity in laboratory animals; Prescott et al. 2005; Chen et al. 2009; Islam et al. 2009). However, the main obstacle for the implementation of GM technology into pea breeding was, and is, the rigid GMO legislation in the EU connected with the common low public acceptance

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of GM products by the European people/community. That is why the majority of ambitious projects finish in the laboratory or greenhouse, because the release of GM crops into the environment (contained field experiments for agronomic performance) represents a complicated administrative procedure with an uncertain result (German or France experience; H.-J. Jacobsen, S. Ochatt – personal communication). This is valid – even in a higher extent – for the release of GM crops into the market (expensive testing when compared to conventional cultivars of a given crop). Thus, the only field experiments with GM peas in Europe were conducted in the Czech Republic, as mentioned before (https://webgate.ec.europa.eu/fip/GMO_Registers/GMO_Summary.php?NotificationNum=B/CZ/09/05&Cat=gmp. As a consequence, up to date, no GM pea cultivar for cultivation/utilisation in the world has been registered (in contrast to the soybean, maize, cotton or rapeseed).

The bruchid-resistant GM pea created by an international interdisciplinary research team in Australia was the one closest to the commercialisation stage. The concerted effort resulted in GM pea lines with a stable inherited high-level field resistance to *Bruchus pisorum* L. and sophisticated agrotechnology guaranteeing that *Bruchus*-resistant populations would not be evolved. Thus, the bruchid-resistant GM pea represents – despite the project breaking off – an excellent example of implementation of the modern science-based technology into practical pea breeding/improvement. The latest developments in molecular biology, namely NBTs (new breeding techniques) could overcome the shortcomings of previous approaches, particularly the random insertion of transgene/s, unnecessity of selection genes, etc., and thus represent a new hope for more precise and sophisticated genetic engineering.

Few things in science are as contentious or politically charged as genetically modified crops. In order to bring better public perception as a consequence of progressing molecular techniques, new approaches need to be applied. Expansion of new progressive techniques of targeted genome engineering (TALENs, CRISPR-Cas9) as an alternative to classical plant breeding and transgenic methods to improve crop plants is currently in progress. The CRISPR-Cas9 system (as well as other methods of targeted genome engineering) could be utilised in modifying pea qualities including the pea composition as was comparably proven in soybean (Du et al. 2016; Curtin et al. 2018; Al Amin et al. 2019; Bao et al. 2019).

While conventional GMOs are similarly regulated in all countries, GE crops (products of targeted genome editing) and their products are not regulated as GMOs in a growing number of countries - including many South American countries, the United States, Australia, and Japan, despite the EU's approach. In response to this extension of methods of precise genome editing, there is a need of reform with regards to the regulations governing genetically modified crops in Europe. Nevertheless, these methods, up to now, seem to be less controversial than classical methods of genetic engineering and there may be a chance that the above technologies could be classified as non-GM. This would have a positive impact on the plant biotechnology and breeding sectors, especially in Europe. The enormous interest of European private breeding companies in using new breeding techniques for a wide range of crop species was recently reported as well as the negative impact of the current regulatory situation in the EU on the decisions of companies in regards to investments in NBT-related research and development (R&D) activities for the EU market and beyond (Jorasch 2020; Purnhagen & Wesseler 2021). A favourable regulatory framework and public acceptance are important factors in realising CRISPR's potential benefits to global food security (Bhowmik et al. 2021). As the proceeding climatic change as well as the rapid growth in the world population are a reality, any reliable technology which helps the agricultural crops improvement would be utilised without superfluous – not scientifically-based regulation. The recent state-of-art discoveries in biotechnology and molecular biology of major world legumes (including peas) provides hope that NBTs will contribute to the creation of the next generation of legume crops.

REFERENCES

Aftabi M., Negawo A.T., Hassan F. (2018): Improved protocol for *Agrobacterium*-mediated transformation of pea (*Pisum sativum*). Molecular Biology, 7: 1000202.

Al Amin N., Ahmad N., Wu N., Pu X., Ma T., Du Y., Bo X., Wang N., Sharif R., Wang P. (2019): CRISPR-Cas9 mediated targeted disruption of FAD2-2 microsomal omega-6 desaturase in soybean (*Glycine max* L.). BMC Biotechnology, 19: 9.

Ali Z.N., Naseem S., Inam-ul-Haq M., Jacobsen H.J. (2015): Soil bacteria conferred positive relationship and improved salt stress tolerance in transgenic pea (*Pisum sativum* L.) harboring Na+/H+ antiporter. Turkish Journal of Botany, 39: 962–972.

https://doi.org/10.17221/24/2022-CJGPB

Ali Z., Saeed W., Naseem S., Ahmad F., Akrem A., Yasmeen N., Jacobsen H.J. (2018): Phenotypic evaluation of transgenic peas (*Pisum sativum* L.) harboring AtNHX1 demonstrates stable gene expression and conserved morphology in subsequent generations. Turkish Journal of Botany, 42: 150–158.

Ambrose S.H., Butler B.M., Hanson D.B., Hunter-Anderson R.L., Krueger H.W. (1997): Stable isotopic analysis of human diet in the Marianas Archipelago, Western Pacific. American Journal of Physical Anthropology, 104: 343–361.

Amian A., Papenbrock J., Jacobsen H.-J., Hassan F. (2011): Enhancing transgenic pea (*Pisum sativum* L.) resistance against fungal diseases through stacking of two antifungal genes (chitinase and glucanase). GM Crops, 2: 104–109.

Aragão F.J.L. (2014): GM plants with RNAi-golden mosaic resistant bean. BMC Proceedings, 8: O24.

Aragão F., Ribeiro S., Barros L., Brasileiro A.C.M., Maxwell D.P., Rech E.L., Faria J.C. (1998): Transgenic beans (*Phaseolus vulgaris* L.) engineered to express viral antisense RNAs show delayed and attenuated symptoms to bean golden mosaic geminivirus. Molecular Breeding, 4: 491–499.

Arun M., Subramanyam K., Theboral J., Sivanandhan G., Rajesh M., Kapil Dev G., Jaganath B., Manickavasagam M., Girija S., Ganapathi A. (2014): Transfer and targeted overexpression of γ-tocopherol methyltransferase (γ-TMT) gene using seed-specific promoter improves tocopherol composition in Indian soybean cultivars. Applied Biochemistry and Biotechnology, 172: 1763–1776.

Atif R.M., Patat-Ochatt E., Svabova L., Ondrej V., Klenoticova H., Jacas L., Griga M., Ochatt S. (2013): Gene transfer in legumes. Progress in Botany, 74: 37–100.

Atkins C., Smith P.M.C. (1997): Genetic transformation and regeneration of legumes. In: Legocki A., Bothe H., Puhler A. (eds.): Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture. NATO ASI Series, Vol. 39, Berlin, Springer: 283–304.

Babaoglu M., Davey M., Power J. (2000): Genetic engineering of grain legumes: Key transformation events. AgBiotechNet ABN, 2.

Bao A., Chen H., Chen L., Chen S., Hao Q., Guo W., Qiu D., Shan Z., Yang Z., Yuan S., Zhang C., Zhang X., Liu B., Kong F., Li X., Zhou X., Tran L.P., Cao D. (2019): CRISPR/Cas9-mediated targeted mutagenesis of GmSPL9 genes alters plant architecture in soybean. BMC Plant Biology, 19: 131.

Bastianelli D., Carrouee B., Grosjean F., Peyonnet C., Revol N., Weiss P., Wiseman J. (1995): Peas: Utilization in Animal Feeding. Paris, UNIP-ITCP.

Bean S., Gooding P.S., Mullineaux P.M., Davies D.R. (1997): A simple system for pea transformation. Plant Cell Reports, 16: 513–519.

Belhaj K., Chaparro-Garcia A., Kamoun S., Nekrasov V. (2013): Plant genome editing made easy: Targeted mutagenesis in model and crop plants using the CRISPR/Cas system. Plant Methods, 9: 39.

Ben-Ze'ev N., Zohary D. (1973): Species relationships in the genus *Pisum*. Israel Journal of Botany, 22: 73–91.

Bhattacharrya M.K., Smith A.M., Ellis T.H.N., Hedley C.L., Martin C. (1990): The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch-branching enzyme. Cell, 60: 115–122.

Bhowmik P.K., Basu S. (2008): Current developments, progress, issues and concerns in producing transgenic peas (*Pisum sativum* L.). Transgenic Plant Journal, 2: 138–150.

Bhowmik P., Konkin D., Polowick P., Hodgins C.L., Subedi M., Xiang D., Yu B., Patterson N., Rajagopalan N., Babic V., Ro D.-K., Tar'an B., Bandara M., Smyth S.J., Cui Y., Kagale, S. (2021): CRISPR/Cas9 gene editing in legume crops: Opportunities and challenges. Legume Science, 3: e96.

Bibikova M., Golic M., Golic K.G., Carroll D. (2002): Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. Genetics, 161: 1169–1175.

Blixt S. (1972): Mutation genetics in *Pisum*. Agri Hortique Genetica, 30: 1.

Bogdanova V.S., Berdnikov V.A. (2000): A study of potential ability for cross-pollination in pea originating from different parts of the world. Pisum Genetics, 32: 16–17.

Boogaart T. van den, Maule A.J., Davies J.W., Lomonossoff G.P. (2004): Sources of target specificity associated with the recovery against Pea seed-borne mosaic virus infection mediated by RNA silencing in pea. Molecular Plant Pathology, 5: 37–43.

Böttinger P., Steinmetz A., Schieder O., Pickard T. (2001): *Agrobacterium*-mediated transformation of *Vicia faba*. Molecular Breeding, 8: 243–254.

Byrne O., Galwey N., Hardie H. (2002): Searching for molecular markers for resistance to pea weevil. In: McComb J.A. (ed.): Plant Breeding for the 11th Millennium: Proceedings of the 12th Australasian Plant Breeding Conference. Perth, Sept 15–20, 2002: 362.

Byrne O.M., Hardie D.C., Khan T.N., Speijers J., Yan G. (2008): Genetic analysis of pod and seed resistance to pea weevil in a *Pisum sativum* × *P. fulvum* interspecific cross. Australian Journal of Agricultural Research, 59: 854–862.

Chakraborty U., Sarkar B., Chakraborty B.N. (2003): Protection of soybean rot by *Bradyrhizobium japonicum* and *Trichoderma harzianum* associated with changes in enzyme activities and phytoalexin production. Journal of Mycology and Plant Pathology, 33: 21–25.

https://doi.org/10.17221/24/2022-CJGPB

Charity J.A., Anderson M.A., Bittisnich D.J., Whitecross M., Higgins T.J.V. (1999): Transgenic tobacco and peas expressing a proteinase inhibitor from *Nicotiana alata* have increased insect resistance. Molecular Breeding, 5: 357–365.

Chen H., Bodulovic G., Hall P.J., Moore A., Higgins T.J., Djordjevic M.A., Rolfe B.G. (2009): Unintended changes in protein expression revealed by proteomic analysis of seeds from transgenic pea expressing a bean alpha-amylase inhibitor gene. Proteomics, 9: 4406–4415.

Chiera J.M., Finer J.J., Grabau E.A. (2004): Ectopic expression of a soybean phytase in developing seeds of *Glycine max* to improve phosphorus availability. Plant Molecular Biology, 56: 895–904.

Choudhury A., Rajam M.V. (2021): Genetic transformation of legumes: An update. Plant Cell Reports, 40: 1813–1830.

Chowrira G.M., Akella V., Lurquin P.F. (1995): Electroporation-mediated gene transfer into intact nodal meristems in planta. Molecular Biotechnology, 3: 17–23.

Chowrira G.M., Akella V., Fuerst P.E., Lurquin P.F. (1996): Transgenic grain legumes obtained by in planta electroporation-mediated gene transfer. Molecular Biotechnology, 5: 85–96.

Chowrira G.M., Cavileer T.D., Gupta S.K., Lurquin P.F., Berger P.H. (1998): Coat protein resistance to pea enation mosaic virus in transgenic *Pisum sativum* L. Transgenic Research, 7: 265–271.

Clement S.L. (1992): On the function of pea flower feeding by *Bruchus pisorum*. Entomologia Experimentalis et Applicata, 63: 115–121.

Clement S.L., Lathrop L.A., Muehlbauer F.J. (1988): More on the pea's nectaries and insect visitors. The Pisum Newsletter, 20: 3–4.

Collins C., Eason P., Dunshea F., Higgins T.J., King R. (2006): Starch but not protein digestibility is altered in pigs fed transgenic peas containing alpha-amylase inhibitor. Journal of the Science of Food and Agriculture, 86: 1894–1899.

Cong L., Ran F.A., Cox D., Lin S., Barretto R., Habib N., Hsu P.D., Wu X., Jiang W., Marraffini L.A., Zhang F. (2013): Multiplex genome engineering using CRISPR/Cas systems. Science, 339: 819–823.

Conn H.J. (1942): Validity of the genus *Alcaligenes*. Journal of Bacteriology, 44: 353–360.

Cooper D.C. (1938): Embryology of *Pisum sativum*. Botanical Gazette, 100: 123–132.

Coyne C., Kumar S., Wettberg E., Marques E., Berger J., Redden R., Ellis N., Brus J., Zablatzká L., Smykal P. (2020): Potential and limits of exploitation of crop wild relatives for pea, lentil, and chickpea improvement. Legume Science, 2: e36.

Cruz A., Aragão F. (2013): RNAi-based enhanced resistance to Cowpea severe mosaic virus and Cowpea aphid-borne mosaic virus in transgenic cowpea. Plant Pathology, 63: 831–837.

Curtin S.J., Xiong Y., Michno J.M., Campbell B.W., Stec A.O., Čermak T., Starker C., Voytas D.F., Eamens A.L., Stupar R.M. (2018): CRISPR/Cas9 and TALENs generate heritable mutations for genes involved in small RNA processing of *Glycine max* and *Medicago truncatula*. Plant Biotechnology Journal, 16: 1125–1137.

Davies D.R., Berry G.J., Heath M.C., Dawkins T.C.K. (1985): Peas (*Pisum sativum* L.). In: Summerfield R.J., Roberts E.H. (eds.): Grain Legume Crops. Collins, London: 147–198.

Davies D.R., Hamilton J., Mullineaux P. (1993): Transformation of peas. Plant Cell Reports, 12: 180–183.

Davis P.H. (1970): *Pisum* L. In: Davis P.H. (ed.): Flora of Turkey and the East Aegean Islands. Vol. 3, Edinburgh, Edinburgh University Press: 370–373.

de Kathen A., Jacobsen H.-J. (1990): *Agrobacterium tumefaciens*-mediated transformation of *Pisum sativum* L. using binary and cointegrate vectors. Plant Cell Reports, 9: 276–279.

de Kathen A., Jacobsen H.-J. (1993): Transformation of pea (*Pisum sativum* L.). In: Bajaj Y.P.S. (ed.): Biotechnology in Agriculture and Forestry. Vol. 23, Plant Protoplasts and Genetic Engineering IV, Berlin, Springer-Verlag: 331–347.

de Ronde J.A., Spreeth M.H., Cress W.A. (2000): Effect of antisense L-1-pyrroline-5-carboxylate reductase transgenic soybean plants subjected to osmotic and drought stress. Plant Growth Regulation, 32: 13–26.

de Ronde J.A., Cress W.A., Van Staden J. (2001): Interaction of osmotic and temperature stress on transgenic soybean. South African Journal of Botany, 67: 655–660.

de Ronde J.A., Cress W.A., Kruger G.H.J., Strasser R.J., van Staden J. (2004): Photosynthetic response of transgenic soybean plants, containing an *Arabidopsis P5CR* gene during heat and drought stress. Journal of Plant Physiology, 161: 1211–1224.

de Sousa-Majer M.J. (2001): Evaluation of Efficacy of Transgenic Peas Against Pea Weevil. A Final Report for the Grains Research Committee of Western Australia. GRC Project No.99/00-03. Perth, Department of Agriculture: 1–8.

de Sousa-Majer M.J., Hardie D.C., Roush R.T. (1999): Evaluation of efficacy of transgenic peas against the pea weevil (*Bruchus pisorum*) and the development of growing strategies that minimise the possibility of weevils developing resistance. In: Proc. Intern. Symp. Dev. Agric. Life Science and Technology toward 21st Century. Kyong Nam, Miryang: 4.

de Sousa-Majer M.J., Turner N.C., Hardie D.C., Morton R., Higgins T.J.V. (2000): Response of transgenic peas con-

https://doi.org/10.17221/24/2022-CJGPB

taining a seed specific a-amylase inhibitor gene to heat and water stresses. In: Abstract Book II, 21th Int. Congr. of Entomology, Brazil: 1007.

de Sousa-Majer M.J., Roush R.T., Turner N.C., Hardie D.C., Morton R., Higgins T.J.V. (2001): Field evaluation of transgenic peas for the design of strategies to minimise the evolution of resistance in pea weevil (*Bruchus pisorum*). In: Abstract Book IAC-Rothamsted Resistance 2001 Conference, Rothamsted: 120.

de Sousa-Majer M.J., Hardie D.C., Turner N.C., Higgins T.J. (2007): Bean alpha amylase inhibitor in transgenic peas inhibit the development of pea weevil larvae. Journal of Economic Entomology, 100: 1416–1422.

Dita M., Rispail N., Prats E., Rubiales D., Singh K. (2006): Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes. Euphytica, 147: 1–24.

Doležel J., Greilhuber J. (2010): Nuclear genome size. Are we getting closer? Cytometry, 77: 635–642.

Donaldson P.A., Simmonds D.H. (2000): Susceptibility to *Agrobacterium tumefaciens* and cotyledonary node transformation in short-season soybean. Plant Cell Reports, 19: 478–484.

Dostálová R., Seidenglanz M., Griga M. (2004): Monitoring of environmental risks of genetically modified pea (*Pisum sativum* L.) in CR: Model solution. In: Ovesná J., Kučera L. (eds.): Problems of Biological Safety, GMOs and International Involvement of the Czech Republic. Prague, Crop Research Institute: 25–34. (in Czech)

Dostálová R., Seidenglanz M., Griga M. (2005): Simulation and assessment of possible environmental risks associated with release of genetically modified peas (*Pisum sativum* L.) into environment in Central Europe. Czech Journal of Genetics and Plant Breeding, 41: 51–63.

Dostálová R., Smýkal P., Seidenglanz M., Griga M. (2009): Field assessment of cross-pollination rate in pea (*Pisum sativum* L.) as a background for release of genetically modified pea into the environment. In: Sehnal F., Drobník J. (eds.): White Book of Genetically Modified Crops. EU Regulations and Research Experience from the Czech Republic. České Budějovice, Biology Centre AS CR: 59.

Droste A., Pasquali G., Bodanese-Zanettini M. (2000): Integrated bombardment and *Agrobacterium* transformation system: An alternative method for soybean transformation. Plant Molecular Biology Reporter, 18: 51–59.

Du H., Zeng X., Zhao M., Cui X., Wang Q., Yang H., Cheng H., Yu D. (2016): Efficient targeted mutagenesis in soybean by TALENs and CRISPR/Cas9. Journal of Biotechnology, 217: 90–97.

Duke J.A. (1981): Handbook of Legumes of World Economic Importance. New York, Plenum.

Ezhova T.A., Bagrova A.M., Gostimskij S.A. (1985): Shoot transformation in callus tissues derived from stem apices, epicotyls, stem internodes, and leaves of various pea genotypes. Fiziologija Rastenij, 32: 513–520. (in Russian)

Fan Y., Li W., Wang J., Yajun F., Wei L., Junjie W., Jingying L., Meiying Y., Duo X., Xiaojuan Z., Xingzhi W. (2011): Efficient production of human acidic fibroblast growth factor in pea (*Pisum sativum* L.) plants by agroinfection of germinated seeds. BMC Biotechnology, 11: 45.

FAO (2021): FAOSTAT. Available at https://www.fao.org/faostat/en/#home (accessed Nov 17, 2021).

Fehr W.R. (1993): Principles of Cultivar Development. Vol. I, Theory and Technique. New York, Macmillan Publishing Company.

Finer J.J., McMullen M.D. (1991): Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. In vitro Cellular and Developmental Biology – Plant, 27: 175–182.

Flores T., Karpova O., Su X., Zeng P., Bilyeu K., Sleper D.A., Nguyen H.T., Zhang Z.J. (2008): Silencing of *GmFAD3* gene by siRNA leads to low alpha-linolenic acids (18:3) of fad3-mutant phenotype in soybean [*Glycine max* (Merr.)]. Transgenic Research, 5: 839–850.

Furutani N., Hidaka S., Kosaka Y., Shizukawa Y., Kanematsu S. (2006): Coat protein-mediated resistance to soybean mosaic virus in transgenic soybean. Breeding Science, 56: 119–124.

Gaikwad A., Tewari K., Kumar D., Chen W., Mukherjee S.K. (1999): Isolation and characterisation of the cDNA encoding aglycosylated accesory protein of pea chloroplast DNA polymerase. Nucleic Acids Research, 27: 3120–3129.

Gatti I., Guindón F., Bermejo C., Espósito A., Cointry E. (2016): *In vitro* tissue culture in breeding programs of leguminous pulses: Use and current status. Plant Cell, Tissue and Organ Culture, 127: 543–559.

Ghosh G., Ganguly S., Purohit A., Chaudhuri R.K., Das S., Chakraborti D. (2017): Transgenic pigeonpea events expressing Cry1Ac and Cry2Aa exhibit resistance to *Helicoverpa armigera*. Plant Cell Reports, 36: 1037–1051.

Giordano L.B., Marques M.R.C., Melo P.E. (1991): Estimates of natural outcrossing in peas in Brasilia – DF. Horticultura Brasileira, 9: 82–83. (in Portuguese)

Goldenberg J.B. (1965): "Afila", a new mutation in pea (*Pisum sativum* L.). Boletin Genetico, 1: 27–31.

Govorov L.I. (1928): The peas of Afghanistan. Trudy po Prikladnoj Botanike, Genetike i Selekcii, 19: 517–522. (in Russian)

Graham P., Vance C. (2003): Legumes: Importance and constraints to greater use. Plant Physiology, 131: 872–877.

Grant J.E., Cooper P.A. (2003): Genetic transformation in pea. In: Jaiwal P.K., Singh R.P. (eds.): Applied Genetics

https://doi.org/10.17221/24/2022-CJGPB

of Leguminosae Biotechnology. Focus on Biotechnology. Vol. 10B, Dordrecht, Springer: 23–34.

Grant J., Cooper P. (2006): Peas (*Pisum sativum* L.). Methods in Molecular Biology, 343: 337–346.

Grant J.E., Cooper P.A., McAra A.E., Frew T.J. (1995): Transformation of peas (*Pisum sativum* L.) using immature cotyledons. Plant Cell Reports, 15: 254–258.

Grant J.E., Cooper P.A., Gilpin J.B., Hoglund S.J., Reader J.K., Pither-Joyce M.D., Timmerman-Vaughan G.M. (1998): Kanamycin is effective for selecting transformed peas. Plant Science, 139: 159–164.

Grazziotin M., Cabral G., Ibrahim A., Machado R., Aragão F. (2020): Expression of the *Arcelin 1* gene from *Phaseolus vulgaris* L. in cowpea seeds (*Vigna unguiculata* L.) confers bruchid resistance. Annals of Applied Biology, 176: 268–274.

Griga M., Novák F.J. (1990): Pea (*Pisum sativum* L.). In: Bajaj Y.P.S. (ed.): Legumes and Oilseed Crops I. Biotechnology in Agriculture and Forestry, Vol. 10, Berlin, Heidelberg, Springer: 65–99.

Griga M., Smýkal P., Dostálová R., Seidenglanz M. (2008): Field assesment of outcrossing rate in pea (*Pisum sativum* L.), a cleistogamic crop, as a background for release of genetically modified pea into environment. In: Symposium Handbook of the 10th Int. Symp. Biosafety of Genetically Modified Organisms (ISBGMO), Wellington, Nov 16–21, 2008: 110.

Griga M., Švábová L., Sehnal F., Hanáček P., Reinöhl V., Horáček J. (2009): Development of transgenic pea (*Pisum sativum* L.) lines for improved tolerance to insect pests and fungal pathogenes. In: Sehnal F., Drobník J. (eds.): White Book of Genetically Modified Crops. České Budějovice, Biology Centre AS CR: 38.

Gritton E.T. (1980): Field pea. In: Fehr W.R., Hadley H.H. (eds.): Hybridization of Crop Plants. Madison, American Society of Agronomy: 347–356.

Hadi M.Z., McMullen M.D., Finer J.J. (1996): Transformation of 12 different plasmids into soybean via particle bombardment. Plant Cell Reports, 15: 500–505.

Hamilton A.J., Baulcombe D.C. (1999): A species of small antisense RNA in posttranscriptional gene silencing in plants. Science, 286: 950–952.

Hanáček P., Reinöhl V., Švábová L., Horáček J., Griga M. (2008): Optimization of vectors with genes conferring resistance to insect pests and fungal pathogens, their functional proof on tobacco and utilization for pea transformation. In: Workshop Functional Genomics and Proteomics in Plant Breeding, Brno, MUAF: 28–34.

Hanáček P., Rohrer M., Reinöhl V., Procházka S., Šafářová D., Navrátil M., Horáček J., Švábová L., Smýkal P., Griga M. (2010): Use of GMO for lowering the pesticide contamination of raw materials and foodstuffs. Potravinarstvo – Scientific Journal for Food Industry, 4: 272–280. (in Czech)

Hanafy M., Pickardt T., Kiesecker H., Jacobsen J. (2005): *Agrobacterium*-mediated transformation of faba bean (*Vicia faba* L.) using embryo axes. Euphytica, 142: 227–236.

Hansen G., Wright M.S. (1999): Recent advances in the transformation of plants. Trends in Plant Science, 4: 226–231.

Harland S.C. (1948): Inheritance of immunity to mildew in Peruvian forms of *Pisum sativum*. Heredity, 2: 263–269.

Hassan F., Meens J., Jacobsen H.J., Kiesecker H. (2009): A family 19 chitinase (Chit30) from *Streptomyces olivaceoviridis* ATCC 11238 expressed in transgenic pea affects the development of *T. harzianum* in vitro. Journal of Biotechnology, 143: 302–308.

Hassan F., Muller A., Jacobsen H. (2010): Gene stacking of antifungal genes in transgenic pea to enhance the level of resistance. *In Vitro* Cell Developmental Biology – Animal, 46: 1.

Hassan F., Noorian M.S., Jacobsen H.J. (2012): Effect of antifungal genes expressed in transgenic pea (*Pisum sativum* L.) on root colonization with *Glomus intraradices*. GM Crops Food, 3:301–309.

Hedley C.L., Smith C.M., Ambrose M.J., Cook S., Wang T.L. (1986): An analysis of seed development in *Pisum sativum*. II. The effect of the *r* locus on the growth and development of the seed. Annals of Botany, 58: 371–379.

Hobbs S.L.A., Jackson J.A., Mahon J.D. (1989): Specificity of strain and genotype in the susceptibility of pea to *Agrobacterium tumefaciens*. Plant Cell Reports, 8: 274–277.

Homrich M.S., Wiebke-Strohm B., Weber R.L., Bodanese-Zanettini M.H. (2012): Soybean genetic transformation: A valuable tool for the functional study of genes and the production of agronomically improved plants. Genetics and Molecular Biology, 35: 998–1010.

Hussey G., Gunn H.V. (1984): Plant production in pea (*Pisum sativum* L. cvs. Puget and Upton) from long-term callus with superficial meristems. Plant Science Letters, 37: 143–148.

Hussey G., Johnson R.D., Warren S. (1989): Transformation of meristematic cells in the shoot apex of cultured pea shoots by *Agrobacterium tumefaciens* and *A. rhizogenes*. Protoplasma, 148: 101–105.

Hwang H.-H., Yu M., Lai E.-M. (2017): *Agrobacterium*-mediated plant transformation: Biology and applications. The Arabidopsis Book, 15: e0186.

Islam N., Campbell P.M., Higgins T.J.V., Hirano H., Akhurst R.J. (2009): Transgenic peas expressing an α-amylase inhibitor gene from beans show altered expression and modification of endogenous proteins. Electrophoresis, 30: 1863–1868.

https://doi.org/10.17221/24/2022-CJGPB

Jacobsen H.J., Kysely W. (1985): Induction of *in vitro* regeneration via somatic embryogenesis in pea (*Pisum sativum*) and bean (*Phaseolus vulgaris*). In: Proc. Int. Symp. Gene Manipulation in Plant Breeding, Berlin (West), Sept 8–13, 1985: 445–448.

Jaranowski J.K. (1976): Gamma-ray induced mutations in *Pisum arvense*. Genetica Polonica, 17: 478–495.

Jefferson R.A., Kavanagh T.A., Bevan M.W. (1987): GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO Journal, 6: 3901–3907.

Jelenic S., Mitrikeski P.T., Papes D., Jelaska S. (2000): *Agrobacterium*-mediated transformation of broad bean *Vicia faba* L. Food Technology and Biotechnology, 38: 167–172.

Jones A.L., Johansen I.E., Bean S.J., Bach I., Maule A.J. (1998): Specifity of resistance to pea seed-borne mosaic potyvirus in transgenic peas expressing the viral replicase (*Nib*) gene. Journal of general Virology, 79: 3129–3137.

Jorasch P. (2020): Potential, challenges, and threats for the application of new breeding techniques by the private plant breeding sector in the EU. Frontiers in Plant Science, 11: 58211.

Jordan M.C., Rempel H., Hobbs S.L.A. (1992): Genetic transformation of *Pisum sativum* L. via *Agrobacterium tumefaciens* or particle bombardment. In: Proc. 1st Conf. Grain Legumes, Angers: 115–116.

Kahlon J.G., Jacobsen H.-J., Chatterton S., Hassan F., Bowness R., Hall L.M. (2018): Lack of efficacy of transgenic pea (*Pisum sativum* L.) stably expressing antifungal genes against *Fusarium* spp. in three years of confined field trials. GM Crops and Food, 9: 90–108.

Karmakar S., Molla K.A., Gayen D., Karmakar A., Das K., Sarkar S.N., Datta K., Datta S.K. (2019): Development of a rapid and highly efficient *Agrobacterium*-mediated transformation system for pigeon pea [*Cajanus cajan* (L.) Millsp]. GM Crops and Food, 10: 115–138.

Kartha K.K., Gamborg O.L., Constabel F. (1974): Regeneration of pea (*Pisum sativum* L.) plants from shoot apical meristems. Zeitschrift für Pflanzenphysiologie, 72: 172–176.

Kaur A., Sharma M., Sharma C., Kaur H., Kaur N., Sharma S., Arora R., Singh I., Sandhu J. (2016): Pod borer resistant transgenic pigeon pea (*Cajanus cajan* L.) expressing *cry1Ac* transgene generated through simplified *Agrobacterium* transformation of pricked embryo axes. Plant Cell, Tissue and Organ Culture, 127: 717–727.

Khalafalla M., Rahman S.M., El-Shemy H., Nakamoto Y., Wakasa K., Ishimoto M. (2005): Optimization of particle bombardment conditions by monitoring of transient sGFP(S65T) expression in transformed soybean. Breeding Science, 55: 257–263.

Kocsy G., Simon-Sarkadi L., Galiba G., de Ronde J.A. (2007): Transformation of soybean and use of transgenic lines in basic and applied research. Transgenic Plant Journal, 1: 129–144.

Kooistra E. (1962): On the differences between smooth and three types of wrinkled peas. Euphytica, 11: 357–373.

Kotlarz A., Sujak A., Strobel W., Grzesiak W. (2011): Chemical composition and nutritive value of protein of the pea seeds – effect of harvesting year and variety. Journal of Fruit and Ornamental Plant Research, 75: 57–69.

Kraft M., Pleger F.L. (2001): Compendium of Pea Diseases and Pests. 2nd Ed., St. Paul, The American Phytopathological Society Press.

Krejčí P., Matušková P., Hanáček P., Reinöhl V., Procházka S. (2007): The transformation of pea (*Pisum sativum* L.): Applicable methods of *Agrobacterium tumefaciens*-mediated gene transfer. Acta Physiologiae Plantarum, 29: 157–163.

Krishna G., Reddy P.S., Ramteke P.W., Rambabu P., Tawar K.B., Bhattacharya P. (2011): *Agrobacterium*-mediated genetic transformation of pigeon pea [*Cajanus cajan* (L.) Millsp.] for resistance to legume pod borer *Helicoverpa armigera*. Journal of Crop Science and Biotechnology, 14: 197–204.

Kumar S., Tanti B., Patil B.L., Mukherjee S.K., Sahoo L. (2017): RNAi-derived transgenic resistance to Mungbean yellow mosaic India virus in cowpea. PLoS ONE, 12: e0186786.

Kunakh V.A., Voityuk L.I., Alkhimova E.G., Alpatova L.K. (1984): Production of callus tissues and induction of organogenesis in *Pisum sativum*. Soviet Plant Physiology, 31: 430–435.

Kysely W., Jacobsen H.J. (1990): Somatic embryogenesis from pea embryos and shoot apices. Plant Cell, Tissue and Organ Culture, 20: 7–14.

Ladizinsky G., Smartt J. (2000): Opportunities for improved adaptation via further domestication. In: Knight R. (ed.): Linking Research and Marketing Opportunities for Pulses in the 21st Century. Current Plant Science and Biotechnology in Agriculture. Vol. 34, Dordrecht, Springer: 257–263.

Lau O.S., Sun S.S.M. (2009): Plant seeds as bioreactors for recombinant protein production. Biotechnology, 27: 1015–1022.

Lee R.Y., Reiner D., Dekan G., Moore A.E., Higgins T.J., Epstein M.M. (2013a): Genetically modified α-amylase inhibitor peas are not specifically allergenic in mice. PLoS ONE, 8: e0052972.

Lee H., Park S.Y., Zhang Z. (2013b): An overview of genetic transformation of soybean. In: Board J.E. (ed.): A Comprehensive Survey of International Soybean Research. London, IntechOpen: 489–506.

https://doi.org/10.17221/24/2022-CJGPB

Lewis G.P., Schrire B., Mackinder B., Lock M. (2005): Legumes of the World. Richmond, Kew Royal Botanic Gardens.

Li H.Y., Zhu Y.M., Chen Q., Conner R.L., Ding X.D., Li J., Zhang B.B. (2004): Production of transgenic soybean plants with two anti-fungal protein genes via *Agrobacterium* and particle bombardment. Biologia Plantarum, 48: 367–374.

Li T., Huang S., Zhao X., Wright D.A., Carpenter S., Spalding M.H., Weeks D.P., Yang B. (2011): Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. Nucleic Acids Research, 39: 6315–6325.

Li X., Higgins T.J.V., Bryden W.L. (2006): Biological response of broiler chickens fed peas (*Pisum sativum* L.) expressing the bean (*Phaseolus vulgaris* L.) α-amylase inhibitor transgene. Journal of the Science of Food and Agriculture, 86: 1900–1907.

Liu S.-J., Wei Z.-M., Huang J.-Q. (2008): The effect of co-cultivation and selection parameters on *Agrobacterium*-mediated transformation of Chinese soybean varieties. Plant Cell Reports, 27: 489–498.

Loennig W.E. (1983): Cross-fertilization in peas. The Pisum Newsletter, 15: 40.

Loennig W.E. (1984): Cross-fertilization in peas under different ecological conditions. The Pisum Newsletter, 16: 38–40.

Loennig W.E. (1985): The peas' nectaries and insect visitors. The Pisum Newsletter, 17: 47–49.

Lutova L.A., Zabelina Y.K. (1988): Callus and shoot *in vitro* formation in different forms of peas (*Pisum sativum* L.). Genetica, 24: 1632–1640.

Mali P., Yang L., Esvelt K.M., Aach J., Guell M., DiCarlo J.E., Norville J.E., Church G.M. (2013): RNA-guided human genome engineering via Cas9. Science, 339: 823–826.

Mallick M., Rashid A. (1989): Induction of multiple shoots from cotyledonary node of grain legumes pea and lentil. Biologia Plantarum, 31: 230–232.

Malmberg R.L. (1979): Regeneration of whole plants from callus culture of diverse genetic lines of *Pisum sativum* L. Planta, 146: 243–244.

Malysheva N.V., Pavlova Z.B., Chernysh N.S., Kravchenko L.V., Kislin Y.N., Chmelev V., Lutova L.A. (2001): Genetic transformation of pea (*Pisum sativum*). In: Bajaj Y.P.S. (ed.): Transgenic Crops II: Biotechnology in Agriculture and Forestry. Vol. 47, Berlin, Heidelberg Springer: 284–304.

Mares P., Jurikova T., Sochor J., Zeman L., Baron M., Mlcek J., Balla S. (2014): The influence of feeding GMO peas on growth of animal models. Potravinarstvo – Scientific Journal for Food Industry, 8: 20–24.

Maxted N. (2000): Are our germplasm collections museum items? In: Knigt R. (ed.): Linking Research and Marketing Opportunities for Pulses in the 21st century. Dortrecht, Kluwer Academic Publishers.

Maxted N., Ambrose M. (2001): Peas (*Pisum* L.). In: Maxted N., Bennett S.J. (eds.): Plant Genetic Resources of Legumes in the Mediterranean. Current Plant Science and Biotechnology in Agriculture, Vol. 39, Dordrecht, Springer: 181–190.

McPhee K., Abebe T., Kraft J.M., Muehlbauer F.J. (1999): Resistance to Fusarium wilt race 2 in the *Pisum* core collection. Journal of American Society of Horticulture Science, 124: 28–31.

Mendel J.G. (1866): "Versuche über Pflanzenhybriden." Verhandlungen des naturforschenden Vereines in Brünn, Bd. IV für das Jahr, 1865 Abhandlungen: 3–47. (in English available at http://www.mendelweb.org/Mendel.plain.html)

Meldolesi A. (2010): Pea trials flee to US. Nature Biotechnology, 28: 8.

Meng Y., Hou Y., Wang H., Ji R., Liu B., Wen J., Niu L., Lin H. (2016): Targeted mutagenesis by CRISPR/Cas9 system in the model legume *Medicago truncatula*. Plant Cell Reports, 36: 371–374.

Meng Y., Hou Y., Wang H., Ji R., Liu B., Wen J., Meng Y., Wang Ch., Yin P., Zhu B., Zhang P., Niu L., Lin H. (2019): Targeted mutagenesis by an optimized *Agrobacterium*-delivered CRISPR/Cas9 system in the model legume *Medicago truncatula*. In: de Bruijn F. (ed.): The Model Legume *Medicago truncatula*. John Wiley & Sons, Inc.: 1015–1018.

Metry E.A., Ismail R.M., Hussien G.M., Nasr El Din T.M., El Itriby H.A. (2007): Regeneration and microprojectile-mediated transformation in *Vicia faba* L. Arab Journal of Biotechnology, 10: 23–36.

Michno J., Wang X., Liu J., Curtin S., Kono T., Stupar R. (2015): CRISPR/Cas mutagenesis of soybean and *Medicago truncatula* using a new web-tool and a modified Cas9 enzyme. GM Crops Food, 6: 243–252.

Mikić A., Rubiales D., Smýkal P., Stoddard F.L. (2011): The legume manifesto: (Net)workers on Fabaceae, unite! Field and and Vegetable Crops Research, 48: 253–258.

Mikschofsky H., Schirrmeier H., Keil G.M., Lange B., Polowick P.L., Keller W., Broer I. (2009): Pea-derived vaccines demonstrate high immunogenicity and protection in rabbits against rabbit haemorrhagic disease virus. Plant Biotechnology Journal, 6: 537–49.

Molinier J., Himber C., Hahne G. (2000): Use of green fluorescent protein for detection of transformed shoots and homozygous offspring. Plant Cell Reports, 19: 219–223.

Molnar Z. (2008): Genetic transformation of pea by microprojectile bombardment. In: Kirti P.B. (ed.): Handbook of

https://doi.org/10.17221/24/2022-CJGPB

New Technologies for Genetic Improvement of Legumes. Boca Raton, CRC Press.

Morton R.L., Gollasch S., Schroeder H.E., Bateman K.S., Higgins T.J. (2002): Genetic technology in peas for improved field performance and enhanced grain quality. In: Khachatourians G.C., Scorza R., Nip W.K., Hui Y.H. (eds.): Transgenic Plants and Crops. 1st Ed., New York, CRC Press.

Morton R.L., Schroeder H.E., Bateman K.S., Chrispeels M.J., Armstrong E., Higgins T.J. (2000): Bean alpha-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions. Proceedings of National Academy of Sciences USA, 97: 3820–3825.

Muehlbauer F.J., Short R.W., Kraft J.M. (1983): Description and Culture of Dry Peas. USDA-ARS Agricultural Reviews and Manuals, Western Series No. 37. Western Region, Oakland: 92.

Nadolska-Orczyk A. (2008): *Agrobacterium*-mediated transformation of pea (*Pisum sativum* L.). In: Kirti P.B. (ed.): Handbook of New Technologies for Genetic Improvement of Legumes. London, Boca Raton: 187–202.

Nadolska-Orczyk A., Orczyk W. (2000): Study of the factors influencing *Agrobacterium* mediated transformation of pea (*Pisum sativum* L.). Molecular Breeding, 6: 185–194.

Nauerby B., Madsen M., Christiansen J., Wyndaele R. (1991): A rapid and efficient regeneration system for pea (*Pisum sativum*), suitable for transformation. Plant Cell Reports, 9: 676–679.

Negawo A.T. (2015): Transgenic Insect Resistance in Grain Legumes. [Ph.D. Thesis.] Hannover, Hannover University: 159.

Negawo A.T., Aftabi M., Jacobsen H.-J., Altosaar I., Hassan F.S. (2013): Insect resistant transgenic pea expressing *cry1Ac* gene product from *Bacillus thuringiensis*. Biological Control, 67: 293–300.

Negawo A.T., Baranek L., Jacobsen H.-J., Hassan F. (2016): Molecular and functional characterization of *cry1Ac* transgenic pea lines. GM Crops and Food, 7: 159–174.

Nifantova S.N., Simonenko I.U.V., Komarnitskii I.K., Kuchuk N.V. (2005): Production of transgenic pea (*Pisum sativum* L.) plants resistant to herbicide pusuit. Tsitilogiia Genetika, 39: 16–21.

Ning W., Zhai H., Yu J., Liang S., Yang X., Xing X., Huo J., Pang T., Yang Y., Bai X. (2017): Overexpression of *Glycine soja WRKY20* enhances drought tolerance and improves plant yields under drought stress in transgenic soybean. Molecular Breeding, 37: 19.

Olhoft P.O., Somers D.S. (2001): L-cysteine increases *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary-node cells. Plant Cell Reports, 20: 706–711.

Oliveira H.A. de (1963): Occurence of allogamy in pea. Revista de Olericultura, 3: 83–90. (in Portuguese)

Özcan S. (1995): Transient expression of GUS gene delivered into immature cotyledons of pea by microprojectile bombardment. Turkish Journal of Botany, 19: 423–426.

Padgette S.R., Kolacz K.H., Delannay X., Re D.B., LaVallee B.J., Tinius C.N., Rhodes W.K., Otero Y.I., Barry G.F., Eichholtz D.A., Peschke V.M., Nida D.L., Taylor N.B., Kishore G.M. (1995): Development, identification, and characterization of a glyphosate-tolerant soybean line. Crop Science, 35: 1451–1461.

Paes de Melo B., Lourenço-Tessutti I.T., Morgante C.V., Santos N.C., Pinheiro L.B., de Jesus Lins C.B., Silva M., Macedo L., Fontes E., Grossi-de-Sa M.F. (2020): Soybean embryonic axis transformation: Combining biolistic and *Agrobacterium*-mediated protocols to overcome typical complications of *in vitro* plant regeneration. Frontiers in Plant Science, 11: 1228.

Paszkowski J., Whitham S.A. (2001): Gene silencing and DNA methylation processes. Current Opinion in Plant Biology, 4: 123–129.

Paz M., Shou H., Guo Z., Zhang Z., Banerjee A.K., Wang K. (2004): Assessment of conditions affecting *Agrobacterium*-mediated soybean transformation using the cotyledonary node explant. Euphytica, 136: 167–179.

Perrin Y., Vaquero C., Gerrard I., Sack M., Drossard J., Stöger E., Christou P., Fischer R. (2000): Transgenic pea seeds as bioreactors for the production of a single-chain Fv fragment (scFV) antibody used in cancer diagnosis and therapy. Molecular Breeding, 6: 345–352.

Pniewski T., Kapusta J. (2005): Efficiency of transformation of Polish cultivars of pea (*Pisum sativum* L.) with various regeneration capacity by using hypervirulent *Agrobacterium tumefaciens* strains. Journal of Applied Genetics, 46: 139–147.

Polowick P.L., Quandt J., Mahon J.D. (2000): The ability of pea transformation technology to transfer genes into peas adapted to western Canadian growing conditions. Plant Science, 153: 161–170.

Polowick P.L., Vandenberg A., Mahon J.D. (2002): Field assessment of outcrossing from transgenic pea (*Pisum sativum* L.) plants. Transgenic Research, 11: 515–519.

Ponappa T., Brzozowski A., Finer J. (1999): Transient expression and stable transformation of soybean using the jellyfish green fluorescent protein. Plant Cell Reports, 19: 6–12.

Preiszner J., Van Toai T.T., Huynh L., Bolla R.I., Yen H.H. (2001): Structure and activity of a soybean Adh promoter in transgenic hairy roots. Plant Cell Reports, 20: 763–769.

Prescott V.E., Campbell P.M., Moore A., Mattes J., Rothenberg M.E., Foster P.S., Higgins T.J.V., Hogan S.P. (2005):

https://doi.org/10.17221/24/2022-CJGPB

Transgenic expression of bean alpha amylase inhibitor in pea results in altered structure and immunogenicity. Journal of Agricultural and Food Chemistry, 16: 9023–9030.

Prescott V.E., Forbes E., Foster P.S., Matthaei K., Hogan S.P. (2006): Mechanistic analysis of experimental food allergen-induced cutaneous reactions. Journal of Leukocyte Biology, 80: 258–266.

Puonti-Kaerlas J., Eriksson T., Engstrom P. (1990): Production of transgenic pea (*Pisum sativum* L.) plants by *Agrobacterium tumefaciens*-mediated gene transfer. Theoretical and Applied Genetics, 80: 246–252.

Puonti-Kaerlas J., Eriksson T., Engstrom P. (1992): Inheritance of a bacterial hygromycin phosphotransferase gene in the progeny of primary transgenic pea plants. Theoretical and Applied Genetics, 84: 443–450.

Purnhagen K., Wesseler J. (2021): EU regulation of new plant breeding technologies and their possible economic implications for the EU and beyond. Applied Economic Perspectives and Policy, 43: 1621–1637.

Pusztai A., Bardocz G.G., Alonso R., Chrispeels M.J., Schroeder H.E., Tabe L.M., Higgins T.J. (1999): Expression of the insecticidal bean alpha-amylase inhibitor transgene has minimal detrimental effect on the nutritional value of peas fed to rats at 30% of the diet. The Journal of Nutrition, 129: 1597–603.

Rakouský S., Ondřej M., Sehnal F., Habuštová O. Hussein H.M., Ovesná J., Kučera L., Kocourek F., Říha K., Dostálová R., Seidenglanz M., Tejklová E., Griga M. (2004): Transgenic plant products and their introduction into the environment and crop protection systems, a risk assessment. In: Nap J.-P., Atanassov A., Stiekema W.J. (eds.): Genomics for Biosafety in Plant Biotechnology. Amsterdam, IOS Press: 173–184.

Redden B., Leonforte T., Ford R., Croser J., Slattery J. (2005): Pea (*Pisum sativum* L.). In: Singh R.J., Jahuar P.P. (eds.): Genetic Resources, Chromosome Engineering, and Crop Improvement. Florida, CRC Press: 49–83.

Reddy M.S., Dinkins R.D., Collins G.B. (2003): Gene silencing in transgenic soybean plants transformed via particle bombardment. Plant Cell Reports, 21: 676–683.

Richter A., Jacobsen H.J., de Kathen A., de Lorenzo G., Briviba K., Hain R., Kiesecker H. (2006): Transgenic peas (*Pisum sativum*) expressing polygalcturonase inhibiting protein from raspberry and stilbene synthase from grapevine (*Vitis vinifera*). Plant Cell Reports, 25: 1166–1173.

Rolletschek H., Hosein F., Miranda M., Heim U., Götz K.P., Schlereth A., Borisjuk L., Saalbach I., Wobus U., Weber H. (2005): Ectopic expression of an amino acid transporter (VfAAP1) in seeds of *Vicia narbonensis* and pea increases storage proteins. Plant Physiology, 137: 1236–1249.

Rouet P., Smih F., Jasin M. (1994): Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Molecular and Cellular Biology, 14: 8096–8106.

Rubluo A., Kartha K.K., Mroginski L.A., Dyck J. (1984): Plant regeneration from pea leaflets cultured *in vitro* and genetic stability of regenerants. Journal of Plant Physiology, 117: 119–130.

Saalbach I., Giersberg M., Conrad U.D.O. (2001): High-level expression of a single-chain Fv fragment (scFv) antibody in transgenic pea seeds. Journal of Plant Physiology, 158: 529–533.

Šafářová D., Navrátil M., Petrusová J., Pokorný R., Piáková Z. (2008): Genetic and biological diversity of the Pea Seed-borne Mosaic Virus isolates occuring in Czech Republic. Acta Virologica, 52: 53–57.

Salehi A., Mohammadi M., Okhovvat S.M., Omidi M. (2005): Chitinase gene transformation through *Agrobacterium* and its explanation in soybean in order to induce resistance to root rot caused by *Rhizoctonia solani*. Communications in Agricultural and Applied Biological Sciences, 70: 399–406.

Schaefer H., Hechenleitner P., Santos-Guerra A., Sequeira M., Pennington R., Kenicer G., Carine M. (2012): Systematics, biogeography, and character evolution of the legume tribe Fabeae with special focus on the middle-Atlantic island lineages. BMC Evolutionary Biology, 12: 250.

Schaerer S., Pilet P.-E. (1991): Roots, explants and protoplasts from pea transformed with strains of *Agrobacterium tumefaciens* and *rhizogenes*. Plant Science, 78: 247–258.

Schiemann J., Eisenreich G. (1989): Transformation of field bean *Vicia faba* L. cells expression of a chimeric gene in cultured hairy roots and root-derived callus. Biochemie und Physiologie der Pflanzen, 185: 135–140.

Schroeder H.E., Schotz A.H., Wardley-Richardson T., Spencer D., Higgins T. (1993): Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.). Plant Physiology, 101: 751–757.

Shroeder H.E., Gollasch S., Table L.M., Higgins T.J.V. (1994): Recent advances in gene transfer to peas. Pisum Genetics, 26: 1–5.

Schroeder H.E., Gollasch S., Moore A., Tabe L.M., Craig S., Hardie D.C., Chrispeels M.J., Spencer D., Higgins T.J.V. (1995): Bean [alpha]-amylase inhibitor confers resistance to the pea weevil (*Bruchus pisorum*) in transgenic peas (*Pisum sativum* L.). Plant Physiology, 107: 1233–1239.

Schumacher H., Paulsen H.M., Gau A.E., Link W., Jürgens H.U., Sass O., Dieterich R. (2011): Seed protein amino acid composition of important local grain legumes *Lu-*

https://doi.org/10.17221/24/2022-CJGPB

pinus angustifolius L., *L. luteus* L., *Pisum sativum* L. and *Vicia faba* L. Plant Breeding, 130: 156–164.

Schwarzbach E., Smýkal P., Dostál O., Jarkovská M., Valová S. (2014): Gregor J. Mendel – Genetics founding father. Czech Journal of Genetics and Plant Breeding, 50: 43–51.

Shade R.E., Schroeder H.E., Pueyo J.J., Tabe L.M., Murdock L.L., Higgins T.J.V., Chrispeels M.J. (1994): Transgenic pea seeds expressing the α-amylase inhibitor of the common bean are resistant to bruchid beetles. Bio/Technology, 12: 793–795.

Sharma R.P., Sharma R.K., Munshi A.D. (1998): Breeding for *Fusarium* wilt resistance in pea (*Pisum sativum* L.). Annual Plant Protection Science, 6: 1–10.

Singh R., Sharma S., Kharb P., Saifi S., Tuteja N. (2020): OsRuvB transgene induces salt tolerance in pigeon pea. Journal of Plant Interactions, 15: 17–26.

Simmonds D.H., Donaldson P.A. (2000): Genotype screening for proliferative embryogenesis and biolistic transformation of short-season soybean genotypes. Plant Cell Reports, 19: 485–490.

Smartt J. (1990): Contents. In: Grain Legumes: Evolution and Genetic Resources. Cambridge, Cambridge University Press: V–Vi.

Smýkal P., Kenicer G., Flavell A., Corander J., Kosterin O., Redden R., Ellis N. (2011): Phylogeny, phylogeography and genetic diversity of the *Pisum* genus. Plant Genetic Resources, 9: 4–18.

Snoad B. (1985): The need for improved pea-crop ideotypes. In: Hebblethwaite P.D., Heath M.C., Dawkins T.C.K. (eds.): The Pea Crop. London, Butterworhts: 31–44.

Solleti S.K., Bakshi S., Purkayastha J., Panda S.K., Sahoo L. (2008): Transgenic cowpea (*Vigna unguiculata*) seeds expressing a bean alpha-amylase inhibitor 1 confer resistance to storage pests, bruchid beetles. Plant Cell Reports, 27: 1841–1850.

Somers D., Samac D., Olhoft P. (2003): Recent advances in legume transformation. Plant Physiology, 131: 892–899.

Soto N., Delgado C., Hernández Y., Rosabal Y., Ferreira A., Pujol M., Aragão F.J.L., Enríquez G.A. (2017): Efficient particle bombardment-mediated transformation of Cuban soybean (INCASoy-36) using glyphosate as a selective agent. Plant Cell, Tissue and Organ Culture, 128: 187–196.

Steeves R.M., Todd T.C., Essig J.S., Trick H.N. (2006): Transgenic soybean expressing siRNAs specific to a major sperm protein gene suppress *Heterodera glycines* reproduction. Functional Plant Biology, 33: 991–999.

Sun X., Hu Z., Chen R., Jiang Q., Song G., Zhang H., Xi Y. (2015): Targeted mutagenesis in soybean using the CRISPR-Cas9 system. Scientific Reports, 5: 1–10.

Švábová L. (2008): Metodika agrobakteriální genetické transformace hrachu (*Pisum sativum* L.). Šumperk, Agritec Plant Research. (in Czech)

Švábová L., Lebeda A. (2005): *In vitro* selection for improved plant resistance to toxin-producing pathogens. Journal of Phytopathology, 153: 52–64.

Švábová L., Griga M. (2008): The effect of cocultivation treatments on transformation efficiency in pea (*Pisum sativum* L.). Plant Cell, Tissue and Organ Culture, 95: 293–304.

Švábová L., Smýkal P., Griga M., Ondřej V. (2005): *Agrobacterium*-mediated transformation of *Pisum sativum in vitro* and *in vivo*. Biologia Plantarum, 49: 361–370.

Švábová L., Griga M., Horáček J., Smýkal P., Hanáček P., Reinöhl V., Navrátil M., Šafářová D., Poláková M. (2007a): Development of transgenic pea lines with improved tolerance to Pea Enation Mosaic Virus and Pea Seed-borne Mosaic Virus. In: Book of Abstracts of 6th Europ. Conf. Grain Legumes „Integrated Legume Biology for Sustainable Agriculture", Lisbon: 53–54.

Švábová L., Hanáček P., Horáček J., Reinöhl V., Griga M. (2007b): Transformation of pea to induce resistance to insect pests and fungal pathogens – first partial results. In: Workshop "Functional Genomics and Proteomics in Plant Breeding", Brno, MZLU: 24–30.

Švábová L., Smýkal P., Griga M. (2008): *Agrobacterium*-mediated transformation of pea (*Pisum sativum* L.): Transformant production *in vitro* and by non-tissue culture approach. In: Kharkwal M.C. (ed.): Proc. 4th Int. Food Legumes Research Conference (IFLRC-IV). New Delhi, Oct 18–22, 2005.

Švábová L., Griga M., Navrátil M., Šafařová D., Hanáček P., Reinöhl V., Horáček J., Smýkal P. (2009): Transgenic pea (*Pisum sativum* L.) lines with improved tolerance to Pea Enation Mosaic Virus and Pea Seed-borne Mosaic Virus. In: Sehnal F., Drobník J. (eds.): White Book of Genetically Modified Crops. EU Regulations and Research Experience from the Czech Republic. České Budějovice, Biology Centre AS CR: 32.

Švábová L., Atif R.M., Horáček J., Sehnal F., Jacas L., Hanáček P., Ochatt S., Seidenglanz M., Griga M. (2010): Genetic transformation of pea for improved tolerance/resistance to fungal pathogens and insect pests. In: Proc. 5th Int. Food Legumes Conference (IFLRC V) and 7th Europ. Conf. Grain Legumes (AEP VII), Antalya.

Świecicki W. (2019): The Cataloque of *Pisum* Genes. Poznań, Agencja Rekl. Kraetiff.

Szymkiewicz A., Jedrychowski L. (2006): Determination of pea proteins allergenicity with the use Balb/c mouse. Central European Journal of Immunology, 31: 63–69.

Thu T.T., Dewaele E., Trung L.Q., Claeys M., Jacobs M., Angenon G. (2007): Increasing lysine levels in pigeon-

https://doi.org/10.17221/24/2022-CJGPB

pea (*Cajanus cajan* (L.) Millsp) seeds through genetic engineering. Plant Cell, Tissue and Organ Culture, 91: 135–143.

Timmerman-Vaughan G., Pither-Joyce M., Cooper P., Russell A., Goulden D., Butler R., Grant J. (2001): Partial resistance of transgenic peas to Alfalfa Mosaic Virus under greenhouse and field conditions. Crop Science, 41: 846–853.

Tiwari K.R., Penner G.A., Waekentin T.D., Rashid K.Y. (1997): Pathogenic variation in *Erysiphe pisi*, the causal organism of powdery mildew of pea. Canadian Journal of Plant Pathology, 19: 267–271.

Tzfira T., Citovsky V. (2006): *Agrobacterium*-mediated genetic transformation of plants: Biology and biotechnology. Current Opinion in Biotechnology, 17: 147–154.

Valentine M., Tar J., Mookkan M., Firman J., Zhang Z. (2017): Silencing of soybean raffinose synthase gene reduced raffinose family oligosaccharides and increased true metabolizable energy of poultry feed. Frontiers in Plant Science, 8: 692.

van Emden H.F., Ball S.L., Rao M.R. (1988): Pest, disease and weed problems in pea, lentil, faba bean and chickpea. In: Summerfield R.J. (eds.): World Crops: Cool Season Food Legumes. Current Plant Science and Biotechnology in Agriculture. Vol. 5, Dordrecht, Springer: 519–534.

Wang T.L., Hedley C.L. (1991): Seed development in peas: Knowing your three ‚r's' (or four, or five). Seed Science Research, 1: 3–14.

Wang T.L., Hedley C.L. (1993): Genetics and developmental analysis of the seed. In: Casey R., Davies D.R. (eds.): Peas, Genetics, Molecular Biology and Biotechnology. Wallingford, CAB International: 83–120.

Wang X., Eggenberger A.L., Nutter F.W. Jr., Hill J.H. (2001): Pathogen-derived transgenic resistance to soybean mosaic virus in soybean. Molecular Breeding, 8: 119–127.

Wang X., Lv S., Liu T., Wei J., Qu S., Lu Y., Zhang J., Oo S., Zhang B., Pan X., Liu H. (2020): CRISPR/Cas9 genome editing shows the important role of *AZC_2928* gene in nitrogen-fixing bacteria of plants. Functional and Integrative Genomics, 20: 657–668.

Warkentin T.D., Jordan M.C., Hobbs S.L.A. (1992): Effect of promoter-leader sequences on transient reporter gene expression in particle bombarded pea (*Pisum sativum* L.) tissues. Plant Science, 87: 171–177.

Welham T., Domoney C. (2000): Temporal and spatial activity of a promoter from a pea enzyme inhibitor gene and its exploitation for seed quality improvement. Plant Science, 159: 289–299.

Wen F., Zhu Y., Hawes M.C. (1999): Effect of pectin methylesterase gene expression on pea root development. Plant Cell, 11: 1129–1140.

Wolabu T.W., Park J.J., Chen M., Cong L., Ge Y., Jiang Q., Debnath S., Li G., Wen J., Wang Z. (2020): Improving the genome editing effi-ciency of CRISPR/Cas9 in *Arabidopsis* and *Medicago truncatula*. Planta, 252: 15.

Zhang F., Cong L., Lodato S., Kosuri S., Church G.M., Arlotta P. (2011): Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. Nature Biotechnology, 29: 149–153.

Zhu B.G., Ye C.J., Lu H.Y., Chen X.J., Chai G.H., Chen J.N., Wang C. (2006): Identification and characterization of a novel heat shock transcription factor gene, GmHsfA1 in soybeans (*Glycine max*). Journal of Plant Research, 119: 247–256.

Zimmermann J., Saalbach I., Jahn D., Giersberg M., Haehnel S., Wedel J., Macek J., Zoufal K., Glünder G., Falkenburg D., Kiprijanov S. (2009): Antibody expressing pea seeds as fodder for prevention of gastrointestinal parasitic infections in chickens. BMC Biotechnology, 9: 79.

Zohary D., Hopf M. (2000): Domestication of Plants in the Old World. 3rd Ed., Oxford, University Press.

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