Exploring variation in pea protein composition by natural selection and genetic transformation

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To my beloved family

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1. General introduction

Plant proteins as a sustainable alternative to meat

Meat is a major protein supplier for humans. However meat production generates environmental pressure (Alexandratos 1995). On the contrary, plant proteins are produced in a more sustainable way compared to meat proteins. Developing novel plant protein foods for replacing meat proteins, as such or as food ingredients, will reduce the environmental pressure generated by the meat industry (Weaver et al. 2000). Examining options for a more sustainable food production, the PROtein Food Environment Technology And Society (PROFETAS) project has been developed. PROFETAS aimed to answer if the replacement of meat with plant proteins in food production is technically feasible, societal desirable and more sustainable.

An option is to design and produce a new generation of food products, the Novel Protein Foods (NPFs), based on plant proteins. These NPFs could possibly replace meat consumption, and subsequently reduce meat production, either directly by mimicking meat or indirectly as a meat analogue in terms of protein content. Previous studies (Linneman and Dijkstra 2002; Dijkstra et al. 2003) have shown that pea proteins would be a good choice for production of Novel Protein Foods (NPFs). Peas have large genetic variation with respect to their seed reserves, starch and proteins, which makes it possible to select new materials with appropriate properties for particular applications. Additionally the pea crop meets most of the requirements for sustainable crop production and there are strong perspectives of rapid pea crop improvements.

Agronomical traits of pea

Peas (*Pisum sativum* L.) are known to be cultivated as early as 7000-6000 B.C. in neolithic villages of the Near East and were used similar to our days for fodder and human food (Zohary and Hopf 1973). Peas are classified depending on their use in: field peas for foliage, market peas for human consumption, vining peas for canning or freezing and dried peas, partly for human consumption, and mostly for animal feed (Cousin 1997).

Samec and Nasinec (1996) classified the peas using random amplification of polymorphic DNA (RAPD) techniques in the subspecies *P. arvense, P. humile*, *sativum* and *P. abyssinicum*. Between the different species there is extensive genetic variability, for example, in plant height, leaf type, number of branches and pods.

Different centres of origin have been postulated for pea. Most probably pea has originated in Abyssinia and Afghanistan and was transferred to the Mediterranean later (Cousin 1997 and references therein).

Pea belongs to the *Papilionaceae* family. The calyx of the pea flower is green and has five united sepals whereas its corona contains 5 petals. The pea flower has ten stamens and one style surrounded by the petals. It contains one ovary which has up to 13 ovules. The stigma is receptive to pollen several days prior to

anthesis until one or more days after the flowers wilt. The pollen is viable from the time the anthers dehisce until several days after. Depending on the variety and the weather conditions, a mature pea seed needs 25 to 35 days after pollination to be formed. The pea flower is cleistogamous and as a result self-pollination occurs in a high frequency. Cross pollination is rare but insects as *Paratrigona lineata, Xylocopa* and *Megachile* can visit pea flowers, and can be responsible for natural hybridisation.

The world production of peas is about 10.5 million tons of dry pea and 7 million tons fresh peas (FAO 2001). It is grown in cool climates with an optimum daily temperature of 17°C, a minimum of 10°C and a maximum of 23°C. The normal growing period is 65 to 100 days for fresh pea, with an additional 20 days for dry peas. The growing period is extended under cool conditions. Young plants can tolerate light frost, but flowers and green pods are injured. Under irrigation, pea yields 2 to 3 ton/ha shelled fresh pea (70% to 80% moisture) and 0.6 and 0.8 ton/ha dry pea (12% moisture). Vigorous vegetative growth results in interplant competition, and subsequently in reduced yield (Cousin et al. 1985). Reduction of plant height and leaf area reduces interplant competition and increases yields. Therefore, all modern varieties are bred to be short plants with small leafs (the so-called semi-leafless varieties with the afila mutation (*af/af*), which replaces leaflets with branching tendrils (Marx 1987). Yield of pea can be affected significantly by pathogens such as powdery mildew, *Fusarium*, *Ascochyta*, Pea Common Mosaic virus, Pea Enation Mosaic virus and the bacteria *Pseudomonas*. Marker-assisted breeding is used to identify and introduce genetic *loci* into commercial pea varieties, which provide resistance to these pathogens (Grajal-Martin and Muehlbauer 2002).

The major component of pea is starch, which accounts for up to 50% of the seed dry matter (DM) (Borowska et al. 1996; Wang et al. 1998). Protein and total dietary fiber account for about 24% and 20% DM, respectively. Lipids are present in lower amounts (2.5% DM) (Black et al. 1998). High variation in starch and protein contents are often observed, whereas the variations in the contents of other components are usually lower (Borowska et al. 1996).

Seed storage proteins

Seed proteins provide the major source of amino acids in human nutrition, since they are used either directly in the human diet or indirectly as animal feed in the meat industry. Seed proteins are classified in three major categories: biologically active, structural, and storage proteins, with the last being more abundant. During seed germination and seedling-growth they are hydrolysed, and the resulting amino acids are used for the formation of other proteins (Muntz 1998). Depending on the solvent in which they can be dissolved, seed storage proteins are classified in the water-soluble albumins, the salt-soluble globulins, the ethanol-soluble prolamins, and the acid- or alkali-soluble glutelins (Marcone 1999 and references therein). Fractions enriched in specific seed storage proteins can be obtained by extraction with various solvents.

Pea globulins account for about 65-85% of the proteins present in the seeds (Owusu-Ansah and McCurdy 1991). The globulins have been subdivided into two major groups based on their sedimentation

coefficient: the 11S fraction (legumin) and the 7S fraction (vicilin, convicilin) (**Figure 1**). The globulins accumulate in the seed as multimeric proteins; mature legumins are hexamers, whereas vicilins and convicilin assemble as trimers. The pea globulins are also known to undergo post-translational processing. Legumin is processed into two subunits, which are connected by a disulfide bridge. Vicilins can be proteolytically cleaved at various positions. Different methodologies like high performance liquid chromatography (HPLC), capillary electrophoresis, and polyacrylamide gel electrophoresis (PAGE) (Van der Berg and Gabillard 1994) can be used for the separation and identification of the constituent proteins in the extract. Seed storage proteins have a wide range of usage. Defatted meals of protein-rich seeds like soybean and sunflower are used for animal feed.

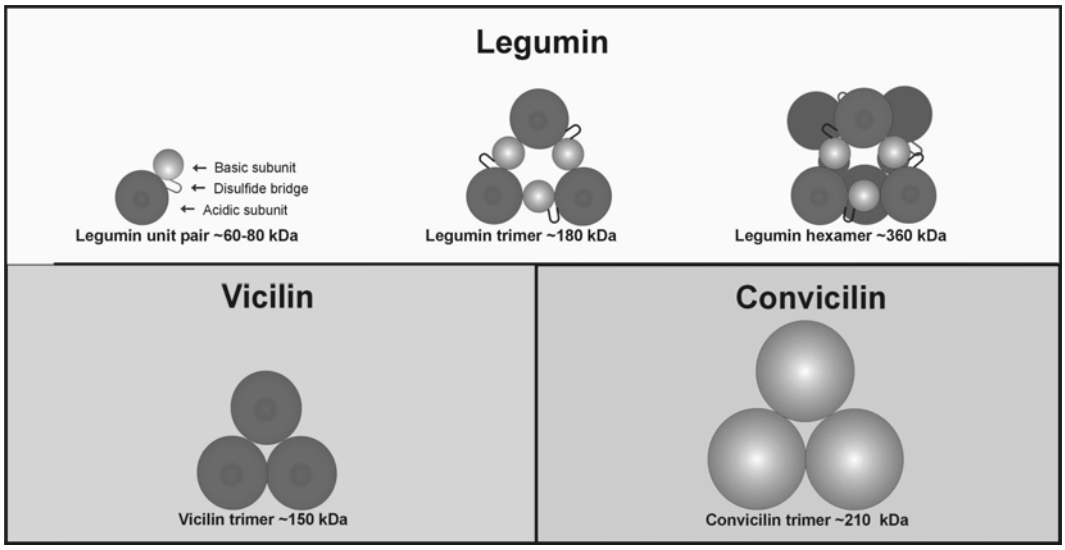

Figure 1. Schematic representation of pea globulins. Legumin is expressed as a protein of ~60-80 kDa, consisting of two subunits of about ~40 and ~20 kDa (the acidic and basic subunit, respectively), which remain linked by a disulfide bridge. Legumins form trimers, and subsequently hexamers with a molecular weight of ~180 and ~360 kDa, respectively. Vicilin is a protein of ~47-50 kDa, which subsequently forms trimers of a molecular weight of about ~150 kDa. Convicilin is a protein of about ~70 kDa, which can form trimers with a molecular weight of about ~210 kDa.

Isolates of seed storage proteins are used as food ingredients in products like sausages to increase their protein content and reduce their production cost (Lambert and Yarwood 1992).They are used instead of the more expensive proteins of animal origin as binding agents or emulsifiers in products like biscuits and snacks, or in dairy products. Studies have shown that the *in vivo* digestion of some seed proteins can result in the production of biologically active peptides, possessing activity similar to endogenous hormones; this offers interesting possibilities to use seed proteins as ingredients in so-called functional foods (Gorinstein et al. 2004 and references therein).

The most frequently used seed storage proteins for animal and human nutrition are the legume globulins, and in particular the soy bean globulins. There are various classes of different globulins, each with a number of isoforms. Studies in soy globulins have shown that they have different physico-chemical properties, and therefore potentially a different applicability in industrial processes (Lakemond and Vereijken 2003 and references therein). This, in combination with the fact that they are available in vast amounts and at low prices, has resulted in their use in a broad range of applications. However, not only soybean globulins but also pea (*Pisum sativum* L.) globulins have different physico-chemical properties (Wright and Bumstead 1984; Casey 2003). It has been shown that globulin-mixtures with different composition have different physico-chemical properties (O'Kane et al. 2004, 2005). They reported that the gelling behavior of protein mixtures, analogous to the ones commercially produced, is affected by the cultivar from which the proteins were isolated (O'Kane et al. 2005), and that one of the globulins, convicilin, has a negative effect on the gelling behaviour of pea proteins at near neutral pH (O'Kane et al. 2004). The differences in the properties of the various mixtures are of importance for industrial applications of pea globulins (Casey 1999 and references therein). Availability of pea seeds with a more defined, less heterogeneous protein composition (enriched in one particular globulins) will be of importance for the industrial use of pea. Seeds with different protein composition can be either obtained by exploiting the existing natural variation, or by using genetic modification, if natural variation is not enough. Pea lines lacking a particular globulin still need to be discovered in nature. However, it is questionable if such lines exist at all, because the various globulins are encoded by multigene families. The absence of one class of globulins in the seeds would require a simultaneous inhibition of the expression of several genes. In principle, such might be achieved by genetic modification or transformation of pea, at least when appropriate RNA inhibition constructs are used.

General aspects of plant regeneration and transformation

Genetic modification of plants requires a system to deliver genes to plant cells, plant cells capable to accept the genes, the gene's integration into the genome, development of the transformed cells into new functional plants, and last but not least a selection system which allows selective development of genetically modified cells.

Plant transformation

The gene delivery systems are divided in two groups: direct gene transfer and *Agrobacterium*-mediated gene transfer. In direct gene transfer, physical forces are used to deliver the gene into plant cells. Examples of direct gene transfer are particle bombardment and electroporation. In particle bombardment, the genes are coated on small metal particles, and these particles are shot into plant cells. In electroporation, plant cells and DNA are together in a solution, and an electric stimulus is used to transfer DNA into the plant cells.

In *Agrobacterium*-mediated transformation the natural capacity of the organism is used to transfer genes to plant cells. *Agrobacterium* is known to have this ability. In the past decades many laboratories around the world, as reviewed by Chilton (2001) have investigated the underlying mechanism for this. Nowadays, genetic modification has become a tool used in plant breeding for crop improvement. The first indication for *Agrobacterium's* ability to transform plant cells was obtained more than ninety years ago with the discovery that Agrobacterium was the causative agent of the crown gall disorder or disease (Smith and Townsend 1907). After more than half a century, Braun (1958) proved that indeed *Agrobacterium tumefaciens* causes the crown gall by inducing 'something' to the plants and introduced the term TIP (tumor inducing principle). In 1971, Hamilton and Fall reported that the components responsible for the transformation must be in an extra chromosomal element. That hypothesis was confirmed by the studies of Zaenen et al. (1974) and Chilton et al. (1977). Zaenen et al. (1974) discovered the presence of a large plasmid (TI plasmid) inside *Agrobacterium*, and subsequently Chilton at al. (1977) proved that genes were transferred from the TI plasmid to the plants. The DNA transferred to the plants is termed T-DNA, which is located in the plasmid between specific sequences of the TI plasmids, the so-called left and right border (Binns and Thomashow 1988 and references therein). The genes inducing transformation are termed virulence genes (*vir*), and they are located outside the two borders in a region called the virulence region. The *vir* genes are induced by phenolic compounds found in wounded tissues (Stachel et al. 1985 and references therein). Although many of the molecular characteristics of the transformation were unknown at that time, the first record on plants expressing foreign genes was achieved at the beginning of the 80's, using the TI plasmid as a vector for engineering transgenic tobacco (Herrera-Estrella 1983). From that time on, *Agrobacterium* was not only a plant pathogen, but also a tool for genetic engineering. However, the large size of the TI plasmid was inconvenient for cloning the gene of interest into the T-region, making the transformation procedure laborious. This became easier after the development of the binary system vector by Hoekema et al. (1983), which is currently the most commonly used gene delivery system. In this system, transformation is facilitated by two plasmids, which both co-reside in *Agrobacterium* and remain independent. One plasmid contains the genes of interest located on the T-DNA region, and the other plasmid contains a modified *vir* region (without tumor-genes). There are many wild type *Agrobacterium* stains capable of gene transfer, for example the strains A281, C58, A348 and 82.139. However, these stains are not broadly used nowadays, and they have been replaced by more virulent ones with modified disarmed (without tumor-genes) plasmids like strains LB4404, EHA105 AGL1 and AGL0. A critical step for successful transformation with *Agrobacterium tumefaciens* is the phase where plant cells and bacteria are mixed to allow transfer of the genes. In general, there are two different methods. In the first method, plant cells are mixed with *Agrobacterium* under *in vitro* conditions, and after a few days the bacteria are removed, and the plants cells are allowed to develop into plants using plant growth regulators. In the other method, whole plants are dipped in *Agrobacterium*, which are subsequently allowed to grow further under natural conditions. An example of the latter method is the floral dip transformation of *Arabidopsis thaliana* (Clough and Bent 1998). Unfortunately this latter method is

only applicable to a small number of plant species. In pea the methods for genetic modification all use *in vitro* techniques.

Regeneration of transgenic plants

Specialized *in vitro* techniques are used to allow the genetic modified cells to develop into plants (regeneration). Cells capable of regeneration can originate from different organs of the plants like meristems, leaves or stems. There is no general rule determining which organ is the best donor for cells capable of plant regeneration. Organs used in one crop can not be used in other crops. Thus the donor organs have to be determined experimentally for each species and sometimes each genotype.

Depending on the organ and the cells used for transformation, an appropriated regeneration system is required to allow genetically modified cells to develop into plants. Regeneration can be divided in adventitious and non-adventitious. If plants develop directly from existing meristems, the regeneration is non-adventitious. If plants develop from organs without meristems, as for example leaves, stems, or roots are used, the regeneration is adventitious. In this case, plants are regenerated from newly formed meristems. Adventitious regeneration is either through somatic embryogenesis (direct or indirect) or organogenesis (direct or indirect). In the direct systems of adventitious regeneration, explants cultured on a proper medium generate directly, without callus formation, embryos or shoots. In the indirect systems of adventitious regeneration, explants cultured on a proper medium generate first callus, which when subcultured on another medium generates embryos or shoots.

During transformation only a portion of the target cells are transformed by the gene transfer vector. Therefore, genetic modification requires a selection mechanism by which genetically modified cells are favoured to grow and divide over wild type cells. This is usually accomplished by coupling the gene(s) of interest to genes conveying resistance to antibiotics or herbicides. By applying antibiotics or herbicides to the medium, only transgenic cells are able to divide. In most crops, selection is applied during a callus phase (undifferentiated cells). After this selection phase, the callus is cultured for regeneration either via adventitious shoot formation as for example in potato (Heeres et al. 2002) and tobacco (Endo et al. 2002) or via somatic embryogenesis as for example in cassava (Raemakers et al. 2001) and rice (Vain et al. 1998). However, selection mechanisms are not always 100% efficient. As a result, regenerated plants are not composed of only transgenic cells but contain either, mixed transgenic and wild type cells or only wild type cells. The first type of plants is called ‘chimeras’ and the second ‘escapes’.

Recent developments in transformation and regeneration of important leguminous species

Production of transgenic plants has been reported in a broad range of leguminous species (reviewed recently by Somers et al. 2003). Legumes, including pea, are generally regarded as recalcitrant to transformation; however, some are easier transformed than others. Many of the available adventitious regeneration systems have been used for transformation of the different legumes (Somers et al. 2003; Ding et al. 2003), but the most successful regeneration methods are based on non-adventitious regeneration. This is because the adventitious methods have either a very low efficiency, are only applicable to a few genotypes of a species, or yield plants of very low quality due to somaclonal variation. In the next sections an overview is given of the different methods of genetic modification used in legumes species, including pea.

Tree species

Recently, methods for transformation via *Agrobacterium tumefaciens* have been reported for *Acacia mangium* (Xie and Hong 2002) and for *Robinia pseudoacacia* (Igasaki et al. 2000). In both species, regeneration of transgenic plants was achieved via organogenesis. For *Acacia mangium*, stem segments of rejuvenated shoots were used as explants (Xie and Hong 2002), whereas for *Robinia pseudoacacia* stem segments were used (Igasaki et al. 2000). In both cases, about 25% to 30% of the stem segments resulted in the production of transgenic plants.

Pasture and foliage species

Lotus japonicus: Transgenic *Lotus japonicus* plants were initially produced by Handberg and Stougaard (1992), and later by Lohar et al. (2001). Both methods include transformation of hypocotyl explants and regeneration via adventitious shoot organogenesis.

Barrel medic: *Medicago truncatula* was proposed as a model system for legume genomics (Cook 1999), because of the superior *in vitro* regeneration and transformation characteristics of the R108-1 genotype (Hoffmann et al. 1997; Trinh et al. 1998; Scholte et al. 2002). These authors produced barrel medic transgenics using leaves as explants, which were subsequently regenerated into plants through somatic embryogenesis. An efficient transformation procedure for barrel medic has also been developed by Kamate et al. (2000) by transforming parts of flowers, followed by regeneration through embryogenesis. Aiming to reduce the tissue culture work involved, Trieu and Harrison (1996) used different approaches from the previous groups, and they developed a method based on cotyledonary node explants, followed by regeneration of multiple shoots. Later, the same group (Trieu et al. 2000) described two *in planta* transformation procedures: one method based on infiltration of flowers, and the other on infiltration of seedlings.

Grains and pulses

Peanut: Using cotyledon explants from mature peanut seeds (*Arachis hypogaea* L.), Sharma and Anjaiah (2000) developed a regeneration procedure via adventitious shoot buds formation from cotyledons, which in combination with their transformation procedure resulted in one or more independently transformed shoots from up to 55% of the treated explants. Rohini and Rao (2000) co-cultivated wounded embryo axes of which one cotyledon was cut off. Embryos were allowed to develop without selection pressure of herbicides or antibiotics. Primary transformants were transferred to the greenhouse and GUS was used as reporter gene for the identification of the transgenic plants.

Soy bean: Transgenic soybeans have been produced via either Agrobacterium-mediated transformation (Hinchee et al. 1988; Parrott et al. 1989a; Di et al. 1996) or particle bombardment (McCabe et al. 1988; Christou et al. 1989; Falco et al. 1995) by using somatic embryogenesis from immature seeds or non-adventitious organogenesis from cotyledonary nodes of seedlings or germinating seeds. However, genetic modification of soybean is far from being a routine with most methods to give 0.2%-2% and rarely up to 6% efficiency. The main factors limiting soybean genetic modification are the low efficiency of T-DNA transfer, and the characteristics of the regeneration systems used to obtain plants. In recent studies, the low T-DNA transfer was improved by increasing the virulence of the *Agrobacterium* strain (Palanichelvam et al. 2000), and the addition of mixtures of thiol compounds (*L*-cysteine, dithiothreitol, and sodium thiosulfate) during the co-cultivation (Olhoft et al. 2003). However, less progress has been made in improving the regeneration systems (Somers et al. 2003). Somatic embryogenesis is genotype-specific and accompanied with high levels of somaclonal variation in the regenerated plants (Parrott et al. 1989b). Organogenesis is less genotype-dependent, and has become routine in several laboratories (Dan and Reighceri 1998). The regeneration is based on proliferation of meristems in the cotyledonary node. However, recovery of transgenic plants capable of transmitting the target genes to subsequently generations is very low (Christou et al. 1990). Meristems are highly complex, multicellular structures, and meristematic cells represent only a very low proportion of the total cells in the explant.

Lupin: By transforming shoot apices, Pigeaire et al. (1997) produced transgenic plants in *Lupinus angustifolius* L. The authors reported average transformation frequencies form 0.4% to 2.8%, depending on the genotype used. Li et al. (2000) generated transgenic yellow lupin (*Lupinus luteus* L.) plants, using meristems from the embryonic axis as explants. Resulting shoots that did not root *in vitro* were grafted onto rootstocks from seedlings of non-transgenic, narrow-leafed lupin (*Lupinus angustifolius* L.). The transformation efficiency of this procedure was 0.05%-0.75%.

Pea: Various protocols for genetic modification of pea have been described (Bean et al. 1997; Grant et al. 1998; Nadolska-Orczyk and Orczyk 2000; Polowick et al. 2000). In the procedure described by Polowick et al. (2000), segments of the embryogenic axis are used. Bean et al. (1997) and Nadolska-Orczyk and Orczyk (2000) used cotyledonary nodes, whereas Grant et al. (1995, 2003) used immature cotyledons as

starting material. In all procedures, multiple shoots are formed after infection with *Agrobacterium tumefaciens*, some of which are genetically modified. In these systems, about 2-5% of the initial seeds used for transformation formed transgenic plants. Limitations of the currently available transformation protocols are associated with the regeneration systems used, which either result in polyploidy and sterility, or in the production of high numbers of escapes and chimeric plants, or have a low repeatability (Puonti Kaerlas et al. 1990; De Kathen et al. 1990; Davies et al. 1993; Schroeder et al. 1993; Bean et al. 1997; Nadolska-Orczyk and Orczyk 2000; Polowick et al. 2000; Grant et al. 2003).

Faba bean: Recently, a reliable faba bean (*Vicia faba* L.) transformation method was reported by Böttinger et al. (2001). These authors produced transgenic faba bean using stem segments from aseptically germinated seedlings as explants. Via callus formation, transgenic shoot regeneration was achieved on a medium containing TDZ at a frequency of 10–30% of the initial explants used. **Table 1** shows a selection of recently published legume transformation protocols. It is shown that for grain legumes, mostly regeneration systems based on existing meristems are used, whereas for trees, pasture and forage species more indirect regeneration systems are used. The use of non adventitious regeneration approaches for grain legumes are probably due to the limited ability of these species to regenerate efficiently via adventitious regeneration (Polowick et al. 2000). However the non adventitious regeneration systems are not efficient, and have not yet resulted in routine transformation systems.

Table 1. Summary of transformation systems developed for legumes species

Species/genotype	Agrobacterium strain	Explant	Selection		Reference
			Gene	Agent	
Tree species					
Acacia mangium					
N	LBA4404	Rejuvenated shoots (non-a O)	*npt*II	G418	Xie and Hong (2002)
Black locust (*Robinia pseudoacacia*)					
N	GV3101	Stem and leaf segments (O)	*hpt*	Hyg	Igasaki et al. (2000)
Pasture and forage species					
Lotus japonicus					
Gifu	LBA4404, C58C1, GV2260	Hypocotyls (O)	*npt*II, *hpt*	Kan, Hyg	Handberg and Stougaard (1992)
Gifu B-129	AGL1	Hypocotyls (O)	*bar*	PPT	Lohar et al. (2001)
Barrel medic (*Medicago truncatula*)					
R108-1	A281, GV2260	Leaves (E)	*npt*II, *hph*	Kan, Hyg	Hoffmann et al. (1997)
R108-1(C3)	EHA105, GV3101	Leaves (E)	*npt*II, *hph*	Kan, Hyg	Trinh et al. (1998)
R108-1(C3), Jemalong J5	EHA105	Floral organs (E)	*npt*II	Kan	Kamaté et al. (2000)
R108-1(C3)	EHA105	Leaves (E)	*bar*, *npt*II	PPT, Kan	Scholte et al. (2002)
Jemalong	LBA4404	Cotyledons (non-a O)	*bar*	PPT	Trieu and Harrison (1996)
Jemalong	EHA105, ASE1, GV3101	Flowers, seedlings	*bar*	PPT	Trieu et al. (2000)
Grains, pulses, and other seed crops					
Peanut (*Arachis hypogaea*)					
JL-24	C58	Cotyledons (non-a O)	*npt*II	Kan	Sharma and Anjaiah (2000)
TMV-2	LBA4404	Embryo axes non-tissue culture (non-a O)	*gus*A	Visual	Rohini and Rao (2000)
Soybean (Glycine max L.)					
A3237	EHA101,EHA105	Cotyledonary node (non-a O)	*bar*	PPT	Zhang et al. (1999)
Bert	EHA101	Cotyledonary node (non-a O)	*hph*	Hyg	Olhoft et al. (2003)
Lupin (*Lupinus angustifolius*)					
Unicrop/Merrit	AgL0	Axillary shoot embryonic axis (non-a O)	*bar*	PPT	Pigeaire et al. (1997)
Yellow lupin (*Lupinus luteus*)					
Wodjil/Popiel/Teo/Juno	AgL0	Apical meristem (non-a O)	*bar*	PPT	Li et al. (2000)
Pea (*Pisum sativum*)					
94-A26/ Bolero/Hadlee/ Crown/ Courier/89T46.UK	AGL1	Immature cotyledons (non-a O)	*npt*II	Kan	Grant et al. (1998)
Laser, Heiga	EHA105, C58C1,LBA4404	Cotyledons (non-a O)	*npt*II, *bar*	Kan, PPT	Nadolska-Orczyk and Orczyk (2000)
Greenfeast/CDC Vienna/ S2-90-25E/ 93-4-18G/	EHA105	Embryonic axis (non-a O)	*bar*, *npt*II	PPT, Kan	Polowick et al. (2000)
Fava bean (*Vicia faba*)					
Mythos	EHA101,EHA105	Epicotyls (O,C)	*npt*II	Kan	Böttinger et al. (2001)

Abbreviations: N, Not identified; Tissue culture type: O, organogenesis; non-a O, non adventitious organogenesis; E, embryogenesis; and C, callus are indicated in parentheses.

Genetic engineering

Genetic engineering is used in plant breeding to improve crop characteristics or introduce new features, which would not be feasible (or would require a long period of time) by conventional breeding. Some typical examples, illustrating the potential uses of genetic modification, are given in the following paragraphs. However, it should be mentioned that there are (presumed as well as realistic) concerns about the safety of the transgenic crops in the environment, and of their products. The release of transgenic plants requires back-up of appropriate surveys in which the benefits and risks are conscientiously weighed.

Protein-rich seeds are an important source of amino acids in animal nutrition. However, some seeds like canola are deficient in the sulfur-containing amino acids, methionine and cysteine. This limits their nutritional value and makes non-ruminant animal diets based only on one plant source, inefficient to meet the animal's nutritional requirements. However, because non-ruminant animals can convert methionine to cysteine, seeds with increased methionine content can satisfy their total requirement for dietary sulfur-containing amino acids. With respect to this, Altenbach et al. (1992) expressed a chimeric gene encoding a methionine-rich protein from Brazil nut in the seeds. As a result the transgenic canola seeds accumulate the heterologous methionine-rich protein at levels which range from 1.7% to 4.0% of the total seed protein and contain up to 33% more methionine. As a result the nutritional value of canola meal was increased. Conveying pathogen resistance to plants is another application. Stem rot (caused by *Sclerotinia sclerotiorum*) is a serious fungal disease of soybean. The major factor of pathogenicity is the production of oxalic acid by *Sclerotinia*. Soybean plants transformed with the wheat germin gene (*gf*-2.8), encoding the protein oxalate oxidase (OxO), which oxidizes oxalic acid to carbon dioxide and hydrogen peroxide (H_2O_2), became capable of degrading oxalic acid, and thus became resistant to *Sclerotinia sclerotiorum* (Donaldson et al. 2001). Another application of genetic engineering is the use of plants as bio-reactors producing valuable products for the industry. This application is known as molecular farming (Twyman et al. 2003). A recent application of molecular farming is described by Börnkeet al. (2002) using potato. Palatinose is a structural isomer of sucrose with similar physico-chemical properties, but lower caloric value. This makes palatinose an ideal table sugar substitute. Börnke et al. (2002) introduced an *Erwinia rhapontici* gene, encoding sucrose isomerase catalysing the conversion of sucrose into palatinose, into potato plants under the control of a tuber-specific promoter. As a result high levels of palatinose were produced in storage tissues of the transgenic potato plants. In the future transgenic potatoes might be used for the commercial production of palatinose, reducing its production cost and making it more affordable to the consumers.

Another application of molecular farming is the production of antibodies (the so-called plantibodies) and other high-value products for pharmaceutical use (Stoger et al. 2002 and references therein). There are many advantages of using plants for the production of antibodies of which the large-scale production is the most important (Fischer et al. 2004). For plantibody production, various systems have been developed and many companies are already in final clinical trials before successfully launching such products commercially.

The clinically most advanced SIgA plantibody, called CaroRx™, recognizes and inhibits the binding of the major oral pathogen, *Streptoccocus mutans*, to teeth (Larrick et al. 2001). Another plantibody that is likely to result in a product for human medical applications is an antibody against the herpes simplex virus glycoprotein B. This antibody was expressed in soybean and shown to be effective in a model study using mice (Zeitlin et al. 1998).

Many plants have been tested for their capability of producing plantibodies, among others pea. Perrin et al. (2000) transformed peas with a cDNA encoding the single-chain Fv fragment scFvT84.66, which is derived from the monoclonal antibody T84.66 that recognizes the tumor-associated carcinoembryonic antigen, and is used for *in vivo* imaging of human cancers. Transgenic pea plants produced up to 9 μg per gram fresh weight of functional scFvT84.66 in their seeds. The antibody remained active after storage in dried transgenic seeds for 2 months at room temperature. These results indicated that pea and other legumes are suitable for molecular farming due to their well-established agricultural practices and their easily stored seeds.

In all the examples described above, genetic engineering was used to enhance a particular trait of plants, or to add a new function to plants. Technically, this is achieved by inserting sense copies of foreign genes (Van der Krol et al. 1990) into the plants. However, often genetic engineering is used to alter a function of a plant by inhibiting the production of an enzyme or protein. Technically, this is achieved by inserting antisense (Hamilton et al. 1990) DNA sequences, homologous to the target genes, into the plants. Sequences are designed to hybridize to specific regions of target mRNAs. Hybridization of the sequences to the mRNA interferes with the normal processing of that mRNA, resulting in reduced translation into the corresponding proteins.

Another technique used to alter a function of a plant by inhibiting the production of an enzyme or protein is the insertion of an interrupted inverted repeats (IIR) into the plant genome, as described in Mette et al. (2000). IIR are formed by a DNA fragment (approximately 500 bp), which has to be homologous to the target ORF for at least 80%. This fragment is present two times in the construct; in a sense and in an antisense orientation (inverted). Between the two parts, another part of DNA is present that is used as a spacer. It assists in making a hairpin shape in the dsRNAs formed by the transcripts of the IIR. The spacer can be any DNA fragment of at least 150 bp in size and preferably non homologous with the DNA sense or antisense fragments. This more efficient tool for gene silencing opens new perspectives for genetic modification. The precise mechanism of this method is not completely known. However the insertion of the DNA sequences into the plants, results in the formation of double-stranded RNA (dsRNA) transcripts homologous to the mRNA of the target gene. The formation of the dsRNA induces complicated mechanisms (Waterhouse et al. 2001 and references therein), which include the cleavage of the dsRNA into fragments of approximately 23 bp by enzymes of the Dicer-1 family. These fragments are subsequently processed into two ssRNA fragments, which can hybridize with mRNA of the target gene due to their homology. The formation of these

double stranded sequences initiates a mechanism which cleaves them, resulting in the silencing of the target gene.

Objectives and outline of this thesis

The work presented in this thesis was performed to obtain new lines of designer peas, which have a more suitable protein composition for the production of NPFs. Our first aim was to determine the existing natural variation in pea's globulin content and composition, in order to identify suitable lines for the production of NPFs. Ideally, one would like to have pea lines lacking one of the various classes of globulins. Since each class of globulins is encoded by a multi-gene family. It was anticipated that such ideal plant material would not exist, and that genetic engineering of pea would be necessary to provide more extreme variation in protein composition. Therefore, we also aimed at developing a more efficient protocol for genetic modification of pea (with respect to regeneration and transformation), and preparing efficient constructs for modulating pea protein composition, based on dsRNA directed ssRNA degradation.

Chapter 2 reports on the genetic variation in protein content and composition of pea aiming to identify lines with extreme variation in this respect. Additional focus was given to the level of post-translational processing of the various globulins in the different lines. A collection of seeds from 54 pea (*Pisum sativum* L.) lines, as well as from 5 pea wild relatives (taxa) (*P. sativum abyssinicum*, *P. arvense*, *P. elatius Marbre, P. elatius 1140175, P. elatius 1140176*), were subjected to gel electrophoretic analysis. The collection included wild-type (normal) and *afila* (semi-leafless) leaf shape varieties, and the seeds varied in external characteristics such as size, shape and seed coat colour.

A problem in genetic modification of pea is that pea is rather recalcitrant to transformation, and that the existing protocols for this are inefficient and laborious. Two new regeneration protocols were developed, which can be combined with a transformation procedure, resulting in a more routine method for generating transgenic pea lines than the currently available one (Schroeder et al. 1993; Grant et al. 1995; Bean et al. 1997; Nadolska-Orczyk and Orczyk 2000; Polowick et al. 2000). This is described in Chapters 3 and 4.

Chapter 5 reports the production of transgenic peas (*Pisum sativum* L.) via *Agrobacterium tumefaciens*-mediated transformation, using the regeneration system developed in Chapter 3. The developed transformation procedure uses luciferase as selectable marker. Transgenic plants containing an IIR construct made by fragments of a *leg*A gene have been produced. Protein analysis of the transformants indicated that the protein composition of the seeds was altered.

Chapter 6 gives a critical evaluation of the approaches that have been taken to fulfill the objectives of this thesis. The results, their implications and the opportunities for the creation of pea lines with improved properties for the food industry are discussed.

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2. Genetic variation in pea (*Pisum sativum* L.) seed globulin composition and their *in planta* processing

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Abstract

A quantitative characterization of seeds from 59 pea (*Pisum sativum* L.) lines and relative taxa with various external characteristics and wide geographical origin was performed to explore the genetic variation of pea concerning its starch and protein content, and globulin composition. Pea lines, which produce round, wrinkled, flat, and round with dimpled seeds, have starch as the major reserve, with an average content of 46%. Protein content varied from 16.3% to 36.6% of the seed dry matter, with an overall average of 26.6%. Densitometric quantification of the individual globulins (legumin, vicilin, convicilin and globulin-related proteins) based on SDS-PAGE gels showed no lines lacking any particular globulin. Between the lines tested, variation was shown in both their total globulins content and in their globulin composition. The total globulin content ranged from 49.2% to 81.8% of the total pea protein extract (TPPE). Legumin content varied between 5.9% and 24.5% of the TPPE. Vicilin was the most abundant protein of pea, and its content varied between 26.3% and 52.0% of the TPPE. Both processed and non-processed vicilins occurred. The processed vicilin was the predominant one with values between 17.8% and 40.8%, whereas the non-processed ones constituted between 3.1% and 13.5% of the TPPE. Convicilin was the least abundant globulin and its content ranged from 3.9% to 8.3%. Finally, the globulin-related proteins were present in amounts ranging from 2.8% to 17.3%. They were less abundant in comparison with legumin and vicilin, but they showed the largest relative variation of the four globulin classes. Correlations between the different external characteristics and globulin composition were determined. Comparison with soybean showed that pea lines show more variety in the abundance of globulin proteins, enabling a wider range of food application.

Keywords: pea (*Pisum sativum*); seed proteins; globulins; legumin; vicilin; convicilin; SDS-PAGE; modification

Introduction

Peas (*Pisum sativum* L.), have a high nutritional value and are used as animal feed or as food ingredient. They are a relatively high-quality source of proteins and starch. However, peas and pea proteins are not widely used in food application due to the competitiveness of soybean. Soybean is readily available in bulk quantities, has better agronomic traits, and is studied in greater detail with respect to protein quality parameters (Casey 2003). A number of drawbacks are associated with the use of soybean in foods, such as its beany or greeny flavor and the presence of antinutritional factors (Lakemond and Vereijken 2003 and references therein). This, together with the concern about the introduction of GMO soybean, has roused the interest in alternative vegetable protein sources, including pea.

The major component of pea is starch, which accounts for up to 50% of the seed dry matter (DM) (Borowska et al. 1996; Wang et al. 1998). Protein and total dietary fiber account for about 24% and 20% DM, respectively. Lipids are present in lower amounts (2.5% DM) (Black et al. 1998). High variation in starch and protein contents are often observed, whereas the variations in the contents of other components are usually lower (Borowska et al. 1996).

According to the Osborne fractionation (Osborne and Campbell 1898) pea proteins can be classified into two major classes: the salt-soluble globulins, and the water-soluble albumins. Both fractions account in total for approximately 80% of the total seed protein content. All globulins and some albumins are storage proteins, which are used as nitrogen sources for the new embryos after seed germination. The globulins have been subdivided into two major groups based on their sedimentation coefficient: the 11S fraction (legumin) and the 7S fraction (vicilin, convicilin). These two groups differ considerably in molecular weight and structure.

Legumin is expressed as a protein of 60-80 kDa, which is usually present in a hexameric form. Three families of legumin polypeptides can be distinguished based on sequence similarities: *Leg*A (consisting of *leg*A, A2, B, C, and E), *Leg*J (consisting of *leg*J, K, L, and M), and *Leg*S (the only member) (Matta et al. 1981; Casey and Domoney 1999). The representatives of the first two families have molecular weights of approximately 60-65 kDa, whereas *Leg*S has a molecular weight of about 80 kDa. Each legumin polypeptide (like all globulins) has a signal peptide for import into the endoplasmic reticulum (ER), which is removed co-translationally during ER entry. Subsequently, legumin polypeptides form trimers, which are transported into a pre-vacuolar compartment (Muntz 1998). Here, they are processed by a vacuolar processing enzyme into two polypeptides of about 40 and 20 kDa (referred to us as the acidic and basic polypeptide, respectively), which remain linked by a disulfide bridge (**Figure 1**). After processing, the trimers assemble into hexamers forming the mature protein.

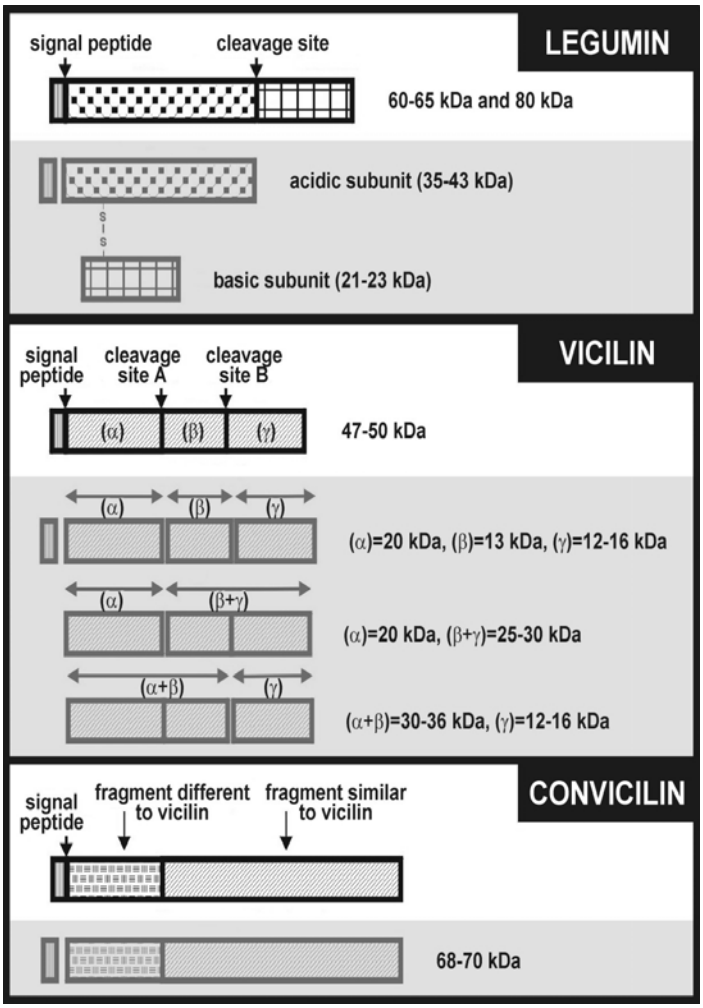

Figure 1. Schematic representation of globulin processing in pea. All globulins have a signal peptide for import into the endoplasmic reticulum (ER), which is removed co-translationally during ER entry. **Legumins** are processed by a vacuolar processing enzyme into two subunits of about 40 and 20 kDa (the acidic and basic subunit, respectively), which remain linked by a disulfide bridge. **Vicilins** have two potential cleavage sites (A and B) depending on the isoform. Cleavage at both sites yields fragments of about 20 kDa (α), 13 kDa (β) and 12-16 kDa (γ); cleavage at site A yields fragments of about 20 kDa (α) and of 25-30 kDa (β+γ); cleavage at site B gives 30-36 kDa (α+β) and 12-16 kDa fragments (γ). **Convicilin** are not known to undergo post-/co-translational modifications other than removal of the signal peptide.

Vicilin is a protein of 47-50 kDa, which can form trimers of a molecular weight of about 150 kDa (Gatehouse et al. 1981). Some, but not all vicilins, can undergo post-translational cleavage (Gatehouse et al. 1982). Two potential cleavage sites (A and B) are present in vicilin polypeptides, which can be processed separately (**Figure 1**). Cleavage at both sites yields fragments of about 20 kDa (α), 13 kDa (β) and 12-16 kDa (γ). Cleavage at site A alone yields fragments of about 20 kDa (α) and of 25-30 kDa (β+γ); cleavage at site B alone gives 30-36 kDa (α+β) and 12-16 kDa fragments (γ) (Gatehouse et al. 1982; Gatehouse et al. 1983). Processed polypeptides remain associated through non-covalent interactions (Casey and Domoney 1999; Gatehouse et al. 1982). Some of the vicilins are glycosylated and glycosylation occurs close to the C-terminus of the γ fragment (Badenoch-Jones 1981).

Convicilin is a protein of about 70 kDa, which can form trimers with a molecular weight of about 210 kDa. The occurrence of heteromeric trimers consisting of vicilin and convicilin polypeptides has been reported as well (Casey 2003; O'Kane 2004a). Convicilin is not known to undergo any post-/co-translational modifications other than removal of the signal peptide (**Figure 1**), and it is not glycosylated (Newbigin et al. 1990). Convicilin has extensive homology with vicilin from its amino acid residues 122-166 (depending on the isoform) to its C-terminus. It differs from vicilin by an N-terminal extension (Newbigin et al. 1990; Bown et al. 1988) which is highly charged in contrast to the rest of convicilin and to vicilin.

Apart from differences in structure and molecular weight, globulins can vary considerably in their physicochemical properties. Vicilin has a significantly higher foaming capacity than legumin, and a slightly lower emulsifying capacity (Casey 2003; Wright and Bumstead 1984). Various concentrations of legumin and vicilin, alone or in mixtures, can form good gels, depending on the conditions used. On the contrary, convicilin can hinder the gel formation of pea isolates (O'Kane et al. 2004b). Therefore, peas with a specific globulin composition, for instance enriched in legumin and vicilin, or lacking convicilin, would be desirable as a raw material for the food industry (Casey and Domoney 1999). In addition, the ability of an isolate to form good gels is not only matter of the ratio of globulins, but it also depends on the specific isoforms of the globulin present in the isolate (O'Kane et al. 2004c, 2005). The existing genetic variation for pea globulin composition might provide a useful resource for obtaining more appropriate seed material for food applications. It has been shown that there is considerable natural variation in pea protein content and composition (Casey et al. 1982; Gueguen and Barbot 1988; Baniel et al. 1999). These variations can be affected by genetic factors like the *r* locus, which influences starch biosynthesis and has pleiotropic effects in protein content and composition (Turner et al. 1990; Hughes et al. 2001). Very little information on *in planta* processing of proteins is available, although this may be an important criterion for application of pea proteins as ingredients in the food industry.

This study explores the genetic variation in starch and in protein content/composition of pea. Besides, the level of post-translational processing in the different lines was investigated. In order to include a wide range of natural genetic variation in the tested material, 59 genotypes were selected based on differences in

leaf and seed characteristics, and on a different geographic distribution. Results were correlated with external characteristics of seeds and plants to obtain phenotypical markers for initial screening in breeding programs.

Materials and methods

Seeds

A collection of seeds from 54 pea (*Pisum sativum* L.) lines, as well as from 5 pea wild relatives (*taxa*) (*Pisum sativum abyssinicum*, *Pisum arvense*, *Pisum elatius Marbre, Pisum elatius* 1140175, *Pisum elatius* 1140176), were obtained from Cebeco Zaden B.V (Lelystad, The Netherlands) and the Center for Genetic Resources (CGN) (Wageningen, The Netherlands), respectively. The lines originate from different parts of the world. They were grown by the provider at the same place and time, to eliminate compositional differences due to growth in different environments (except for the wild relatives). The collection includes wild-type (w.t.; normal leaves) and *afila* (*af*.; semi-leafless) leaf shape varieties. The seeds have diverse external characteristics such as weight, shape and seed coat color. Some representative examples are shown in **Figure 2**. The morphological characteristics of the different lines are summarized in **Table 1**.

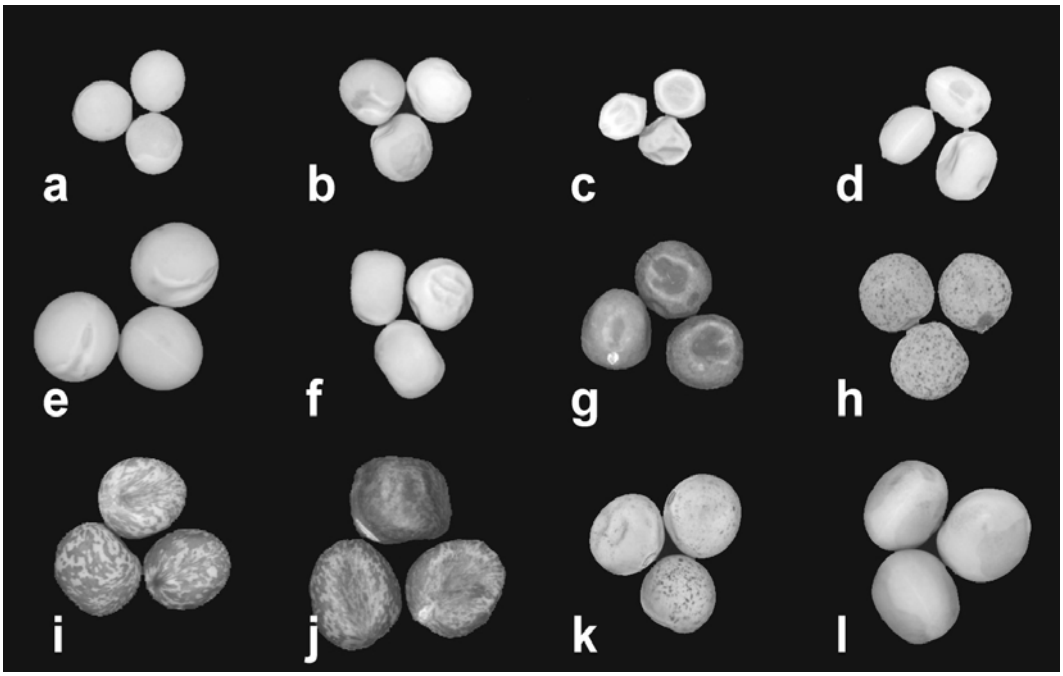

Figure 2. Different morphology (shape and color) of representative pea seeds used in this study. A, [Vreta] (round); B, [NGB 102920 Iran] (round-dimpled); C, [Cisca] (wrinkled); D, [6 S 41.4] (flat); E, [American Yellow] (yellow); F, [PV 13] (green); G, [Timo] (brown); H, [*Pisum elatius* 1145176] (brown-gray-spotted); I, [NGB 102149 Iran] (brown-green-spotted); J, [Courier] (brown-spotted); K, [NGB 101293 Jordan] (green-spotted); L, [NSA 93-0030-2] (green-yellow).

Table 1. Summary of the various genotypes used in this study with their morphological seed and leaf characteristics

Line	Name	Color [1]	Weight [2]	Shape [3]	Leaf [4]	Line	Name	Color	Weight	Shape	Leaf
1	93125-07 Canada	Gr	0.238	R-D	*af*	31	Classic	Yel	0.312	F	*af*
2	M 98 Canada	Yel	0.358	R-D	*af*	32	Baccara	Yel	0.361	R-D	*af*
3	Chileense grano de Oro	Yel	0.309	F	w.t.	33	Solara	Gr	0.282	R	*af*
4	KPMR 146 India	Yel	0.260	F	w.t.	34	NSA 93-0030-2	Gr-Yel	0.393	R	*Af*
5	MP 790 Canada	Yel	0.263	R	w.t.	35	CEB 1475.1	Yel	0.345	R	*af*
6	Russiche gele	Yel	0.427	R	w.t.	36	CEB 1466	Yel	0.231	R	*af*
7	Tanganyka	Yel	0.458	R	w.t.	37	FDP 9023/16	Yel	0.257	R	*af*
8	Russische voererwt groen	Gr	0.267	R	w.t.	38	484	Gr	0.335	R	*af*
9	Mexiaanse gele	Yel	0.260	R	w.t.	39	6 S 41.4	Gr	0.233	F	*af*
10	Amerikaanse gele	Yel	0.284	R	w.t.	40	A 5052/4	Gr	0.323	F	*af*
11	FAL 49110 Mongolie	Yel	0.216	R	w.t.	41	DS 4-9309	Gr-Yel	0.270	R	*af*
12	FAL 48919 Etiopie	Br-sp	0.262	F	w.t.	42	LPKE 8020	Gr	0.236	F	*af*
13	Fal 49142 China	Yel	0.224	R	w.t.	43	SCHW 67.89.35	Gr	0.360	R	*af*
14	NGB 102349 China	Yel	0.224	R	w.t.	44	SWS 97-112-13	Gr-Yel	0.363	R	*af*
15	NGB 102920 Iran	Gr	0.337	R-D	w.t.	45	UN 407	Gr-Yel	0.334	R	*af*
16	NGB 102149 Iran	Br-Gr-sp	0.179	F	w.t.	46	CEB 1475.2	Yel	0.233	R	*af*
17	NGB 101293 Jordaan	Gr-sp	0.163	F	w.t.	47	CEB 1488	Gr-Yel	0.280	R	*af*
18	NGB 100180 Hongarije	Br-Gr-sp	0.123	R	w.t.	48	CEB 1222	Gr	0.518	W	*af*
19	PV 13	Gr	0.202	F	w.t.	49	93126-610	Gr	0.486	W	*af*
20	Timo	Br	0.218	F	w.t.	50	CEB 1162	Gr	0.232	R	*af*
21	Vreta	Yel	0.223	R	w.t.	51	Finale	Gr	0.320	R	w.t.
22	Capella	Yel	0.237	R	*af*	52	Supra	Gr	0.473	W	*af*
23	Courier	Br-sp	0.272	F	*af*	53	Celica	Gr	0.346	F	*af*
24	Fallon	Yel	0.254	R	*af*	54	Espace	Gr	0.276	F	*af*
25	Tremont Scotch	Gr	0.180	F	w.t.	55	*Pisum sativum abyssinicum* (CGN 16636)*	Br	0.165	F	w.t.
26	Solido	Br	0.396	W	*af*	56	*Pisum elatius*, Marbre (CGN 03351)	Br-Gr-sp	0.213	F	w.t.
27	CEB 1312 (Racer)	Br-Gr-sp	0.224	F	*af*	57	*Pisum arvense* (CGN 10193)	Br-Gr-sp	0.062	F	w.t.
28	Mylosa (CEB 1811)	Gr	0.235	W	*af*	58	*Pisum elatius*, 1140175 (CGN 10205)	Br-Gray-sp	0.149	R	w.t.
29	Tango	Gr	0.164	R	w.t.	59	*Pisum elatius*, 1145176 (CGN 10206)	Br-Gray-sp	0.145	R	w.t.
30	Cisca	Gr	0.119	W	w.t.						

*CGN accession numbers

[1] Color abbreviations: Yel = yellow, Gr = green, Br = brown, sp = spotted. [2] Weight: average weight of 10 mature seeds (g). [3] Shape abbreviations: F = flat, W = wrinkled, R = round, R-D= round with dimples. [4]Leaf shape abbreviations: w.t. = wild type, *af* = semi-leafless (*afila* mutant).

Preparation of samples

For all the biochemical analyses performed in this study, samples were prepared as follows: seeds (about 1 g) were peeled and ground with a mortar and pestle at room temperature until a fine powder was obtained. This powder was dried at 40 °C in a vacuum desiccator in the presence of P_2O_5 until the weight remained constant (about 2 days).

Determination of the starch content

Ground pea seed (about 40 mg) was suspended in 2.5 mL 8 M HCl and 10 mL DMSO, and incubated for 1 h at 60 ºC. After incubation, 15 mL of H_2O, 4 mL of 5 M NaOH and 18.5 mL of 0.1 M sodium citrate buffer (pH 4.6) was added to obtain appropriate conditions for the enzymes used in the starch determination assay. After vigorous mixing, 1 mL of this suspension was centrifuged at 13,600 × *g* for 10 min, and 20 µL of the supernatant were used for enzymatic determination of starch. For that, a starch assay kit obtained from Boehringer (Mannheim, Germany) was used with a procedure slightly modified from the one described by the manufacturer. The modification consisted of a 5× reduction of the reaction volume of the enzymatic hydrolysis of the starch to adapt the procedure for determining the total amount of glucose with a micro-plate reader (Bio-Rad Labs 3550-UV, Hercules, CA). For each line, at least three independent samples were prepared and analyzed.

Protein content determination

Protein content was measured using the combustion (Dumas) method on a nitrogen and protein analyzer (model ThermoQuest NA 2100, CE Instruments, Milan, Italy). The sample (20-30 mg ground pea seed) was weighted into a sample cup and analyzed using D-methionine as an external standard. For each line, at least three independent samples were prepared and analyzed. The protein content was calculated using 6.25 as nitrogen to protein conversion factor (Hughes et al. 2001).

Determination of the composition of the extractable proteins

Total pea protein extract was prepared by stirring 100 mg of ground pea seed in 1.5 mL of 0.1 M Tris HCl buffer pH 8 for 1 h at 30 r.p.m. at room temperature. Subsequently, the samples were centrifuged at 1,500 × *g* for 7 min at 20 ºC to precipitate insoluble material. The protein composition of the supernatant, referred to as total pea protein extract (TPPE) was determined in triplicate by SDS-PAGE, without and with 2% (v/v) β-mercaptoethanol, on a Mini-Protean II electrophoresis system from Bio-Rad Labs (Hercules, CA), according to the instructions of the manufacturer. Five hundred µL of TPPE was diluted 1+1 (v/v) in sample buffer, consisting of 0.02 M Tris-HCl buffer (pH 8), 2 mM EDTA, 20 % glycerol, 2 % SDS, and 0.002 % Broomphenol Blue. The samples, sealed in 1.5 mL tubes, were heated in boiling water for 5 min and proteins were separated using 12% Tris-HCl polyacrylamide Ready Gels from Bio-Rad Labs (Hercules, CA). Gels were run at a constant voltage of 200 V, and were calibrated with low molecular mass markers ranging from 14-94 kDa from Amersham Biosc. (Uppsala, Sweden). 10 µL of sample was loaded into each well. The gels were run in a buffer solution of 0.025 M Tris, 0.19 M Glycine, and 0.1% SDS (pH 8.3). The gels were stained in a solution of 0.1 % Coomassie Brilliant Blue R250, 40% methanol, and 10% acetic acid, and destained in a solution of 30% methanol, and 10% acetic acid. The different bands were quantified by scanning densitometry, using a Molecular Dynamics Laser Scanner and the ImageQuant software package, both from Amersham Int. Plc (Buckinghamshire, U.K.). The proportions of legumin,

vicilin, convicilin and globulin-related proteins were calculated according to the area underneath the staining density peak. The bands were assigned to legumin, vicilin, convicilin according to their molecular weight reported in the literature, and based on relative motilities on SDS-PAGE gels of purified proteins from pea isolates (Matta et al. 1981; Casey and Domoney 1999; Gatehouse et al. 1981; Gatehouse et al. 1982; Gatehouse et al. 1983; O'Kane et al. 2004a; Newbigin et al. 1990; Casey et al. 1982) in a similar way as was done for soybean globulins (Yaklich 2001). The bands of a molecular weight of 60-65 kDa, which disappear after β-mercaptoethanol treatment, are referred to us as legumin. The bands of a molecular weight of 47-50 kDa were assigned as non-processed vicilins; processed vicilins were related to the bands of 36, 35, 33, 30, 25, 20, 16, 14-12 kDa. The bands with a molecular weight of 68-70 kDa were quantified as convicilins. The bands present on the SDS gel, which were not described in the literature as legumins vicilins, and convicilins, but which belong to the soluble fraction since they are co-precipitating with legumin and vicilin under acidic conditions (O'Kane et al. 2004a) were denoted as globulin-related proteins. They had a molecular weight between 52-66 and 45 kDa, which did not disappear under reducing conditions. The bands present in the gels not meeting the criteria for legumin, vicilin, convicilin, or globulin related proteins, were assigned as non-globulins.

Statistical analysis

To verify the statistical significance of the results, the values of mean and standard deviation (mean ± SD) were calculated with a 95% confidence interval (CI). When necessary, a T-test (Bonferroni; $p<0.05$) and a correlation coefficient test (Pearson 2-tailed; $p<0.01$) was performed with the statistical software package SPSS 10.0 for Windows (SPSS Inc, Chicago, Il).

Results

Starch and protein content

A qualitative and quantitative characterization of seeds from 59 pea lines was performed to explore the genetic variation of pea concerning its major seed reserves. Tested lines were grown under the same conditions and on randomized blocks (except the wild relatives of pea). Therefore, any variation found can be related to genetic differences in the plant material. **Figure 3** shows the natural variation in starch and protein contents of the 59 genotypes. The average starch content is 46.0% DM and varied from 27.6% to 56.3%. In all lines tested, the starch content is higher than the protein content, except in the wrinkled line 30 (Cisca). On average, the wrinkled lines showed significantly (T-test, Bonferroni) lower starch contents than the lines with other seed shapes, with an average of 38.5% compared to 46.7%, 48.0%, and 47.0% for flat, round with dimples, and round varieties, respectively. Nevertheless, certain wrinkled lines, such as 48 (CEB 1222) and 52 (Supra), had higher starch contents than lines with other seed shape types. No relationship was found between starch content and leaf shape or seed color. The *Pisum sativum* wild relatives have on average a starch content of about 43.0%, which is significantly lower than that of the *Pisum sativum* accessions (46.2%) (T-test, Bonferroni).

Results on protein content showed a variation from 16.3% to 36.6% DM with an overall average content of 26.6%. No significant differences were observed between varieties with different seed shapes (T-test, Bonferroni). Wrinkled varieties scored 27.7% compared to 26.9%, 26.4%, and 24.8% for flat, round, and round with dimples varieties, respectively. No significant differences were observed between lines with different leaf shapes, whereas differences were observed between lines with different seed colors (T-test, Bonferroni). The brown lines had on average significantly higher protein content (31.8%) than the green and yellow lines, which have average contents of 25.6% and 26.0%, respectively. The wild relatives had an average protein content of 33.9%, showing that the wild relatives have on average a significantly (T-test, Bonferroni) higher protein content than the *Pisum sativum* lines, which have an average of 25.9%. Nevertheless, there were also *Pisum sativum* lines scoring high when compared with the overall average protein content. For instance, line 17 (NGB 101293 Jordan) had a protein content of 31.9%, which falls in the range of that of the wild relatives (30.6%-36.6%).

Protein content correlated negatively with starch (significance –0.597; Pearson 2-tailed; $p<0.01$). **Figure 3** shows the sum of the protein and starch contents of the different pea lines. The average sum corresponds to a value of 72%. Only 13 out of 59 lines differed significantly from this average (standard error ± 0.05), which is much lower than the 36 and 39 lines out of 59 found for individual starch and protein contents, respectively. This indicates that pea's total reserve size remains more or less constant, but that differences in the starch to protein ratio are tolerated.

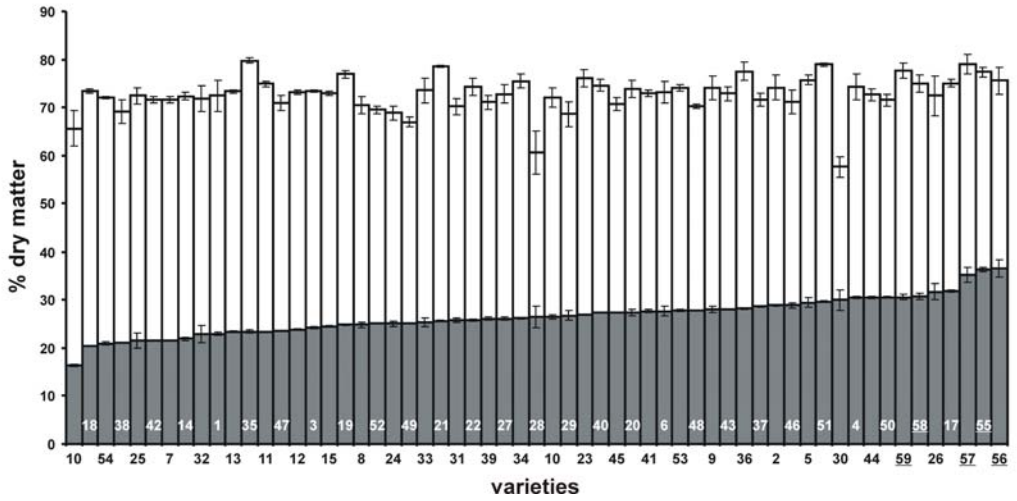

Figure 3. Protein (gray bars) and total reserve size (starch+protein; open bars) of the tested pea seed material. The various pea lines are ranked from low to high protein content. Values are the result of at least 3 replications (with standard deviation less than 1). The pea wild relatives are underlined.

Protein composition

Globulins were quantified based on SDS-PAGE analysis of the samples without β-mercaptoethanol, because in these samples, no overlap of legumin with processed vicilin subunits occurred. When β-mercaptoethanol was added, the bands of processed vicilins and those of the basic subunits of legumin overlapped. It should be noted that without β-mercaptoethanol some overlap of legumin and globulin-related protein bands was observed, which may overestimate the quantity of legumin.

However, the analysis of the protein samples treated with β-mercaptoethanol showed that this overestimation was negligible for all lines, and was not further accounted for. A representative SDS-PAGE gel is shown in **Figure 4**.

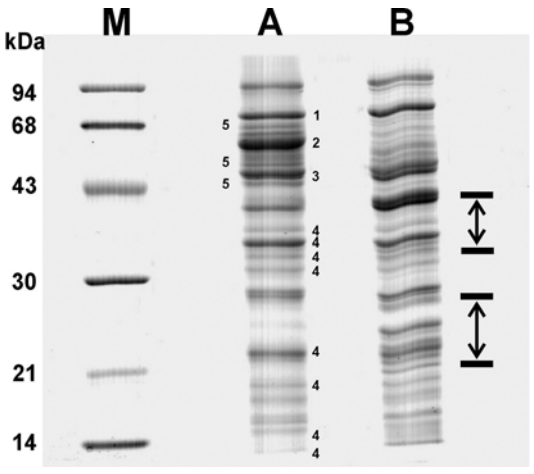

Figure 4. Representative 12% polyacrylamide gel electrophoretic separation of pea proteins contained in TPPE.
Lane M shows the marker; lane A shows TPPE without β–mercaptoethanol, and lane B shows TPPE with the addition of 2% β–mercaptoethanol. Bands indicated by 1, 2, 3, 4, and 5 correspond to convicilin, legumin, non-processed vicilin, processed vicilin and globulin-related proteins, respectively. The extra bands appearing in the region indicated by a and b upon β-mercaptoethanol treatment represent the acidic and basic subunits of legumin, respectively.

Table 2 shows the results of TPPE compositional analysis by densitometric measurement. Two main protein classes can be distinguished in TPPE, globulins and non-globulins (together 100% of the proteins). The non-globulins were not subdivided further, whereas the quantities of the globulins are presented both as total and for the individual classes. Globulins were the major category of seed proteins in all lines tested, except line 1.

Their contents varied between 49.2% and 81.8%, with an average content of 69.8%. No significant difference in globulin content was observed between the *Pisum sativum* species (69.7%) and the wild relatives (72.6%) (T-test, Bonferroni).

Densitometric quantification of the individual globulins showed that the legumin content varied between 5.9% and 24.5% of the proteins of TPPE. The wild relatives, contained on average 20.0% of legumin, which is significantly higher than the average of 14.0% found for the *Pisum sativum* lines (T-test, Bonferroni). Interestingly, legumin content correlated significantly (0.548) with that of total seed protein (Pearson 2-tailed); a similar relationship was found for processed vicilin (Pearson 2-tailed).

Table 2. Total and individual globulin composition of the tested material as percentage of the total protein content. Results are means of the determinations of three independent samples. Protein separation was performed by SDS gel electrophoresis using 12% polyacrylamide gels. The quantification of the individual bands was performed by scanning densitometry. Values are given % of total pea protein extract (TPPE).

Line	*n*Glob	Glob	Leg	Vicil	*np*Vicil	*p*Vicil	Conv	GlobRP	Line	*n*Glob	Glob	Leg	Vicil	*np*Vicil	*p*Vicil	Conv	GlobRP
1	50.8	49.2	8.3	32.4	9.2	23.2	5.7	2.8	31	35.6	64.4	11.5	32.6	10.7	21.9	5.5	14.8
2	33.5	66.5	15.1	35.1	10.4	24.7	5.7	10.6	32	29.3	70.7	14.3	33.8	11.4	22.4	5.4	17.2
3	34.7	65.3	10.5	37.4	8.5	28.9	6.1	11.2	33	28.8	71.2	21.3	29.7	5.0	24.7	7.6	12.7
4	21.5	78.5	18.4	45.5	11.3	34.2	7.2	7.3	34	33.5	66.5	15.4	30.9	11.3	19.7	5.6	14.7
5	28.2	71.8	14.1	38.6	6.6	31.9	7.4	11.8	35	30.6	69.4	16.8	35.6	8.5	27.1	7.6	9.4
6	30.1	69.9	13.4	39.4	9.0	30.4	6.2	10.9	36	35.2	64.8	16.9	33.5	7.6	25.9	5.2	9.2
7	24.3	75.7	11.2	47.9	10.4	37.4	4.2	12.4	37	34.7	65.3	11.4	33.2	10.0	23.2	7.5	13.1
8	32.8	67.2	13.6	38.9	10.3	28.6	5.9	8.9	38	29.2	70.8	14.2	34.8	13.3	21.4	5.8	16.0
9	25.7	74.3	18.5	41.4	9.2	32.3	5.4	9.0	39	34.3	65.7	10.5	37.8	7.9	29.9	5.8	11.6
10	29.8	70.2	17.8	37.1	7.2	29.9	4.5	10.8	40	34.3	65.7	10.5	36.7	7.7	28.9	6.4	12.2
11	30.7	69.3	14.2	39.5	6.8	32.6	3.9	11.7	41	35.8	64.2	7.3	37.1	7.3	29.8	5.9	13.9
12	27.5	72.5	13.6	37.9	10.8	27.1	7.4	13.6	42	26.0	74.0	13.3	44.1	12.8	31.3	6.1	10.6
13	27.0	73.0	13.4	41.3	9.6	31.6	5.2	13.2	43	30.4	69.6	14.4	34.6	11.8	22.8	5.8	14.7
14	28.6	71.4	13.9	38.3	8.9	29.4	5.2	13.9	44	40.5	59.5	10.2	30.4	7.0	23.4	6.3	12.5
15	31.0	69.0	12.3	34.2	6.3	27.9	5.1	17.3	45	33.0	67.0	16.0	33.6	11.7	22.0	6.5	10.8
16	23.5	76.5	5.9	48.2	12.8	35.4	8.3	14.0	46	29.1	70.9	16.1	37.0	9.6	27.4	7.5	10.4
17	25.6	74.4	16.5	38.1	7.2	31.0	7.8	12.0	47	28.8	71.2	18.5	31.8	11.7	20.1	6.6	14.2
18	19.2	80.8	10.9	52.3	12.0	40.3	6.6	10.9	48	19.3	80.7	13.9	50.8	10.8	40.0	5.4	10.7
19	26.6	73.4	16.2	41.6	8.8	32.8	5.2	10.4	49	34.5	65.5	12.0	33.8	10.6	23.2	5.7	14.0
20	22.3	77.7	16.6	42.8	11.7	31.1	8.3	10.0	50	31.7	68.3	18.1	26.3	8.5	17.8	7.6	16.3
21	38.1	61.9	14.5	29.1	7.1	22.0	6.7	11.6	51	29.5	70.5	13.5	38.8	7.8	31.0	6.3	11.8
22	26.9	73.1	15.4	38.6	7.5	31.1	7.8	11.3	52	32.6	67.4	20.7	30.6	7.3	23.3	4.3	11.8
23	23.2	76.8	14.3	41.9	12.6	29.3	7.0	13.6	53	37.3	62.7	14.8	29.6	8.5	21.0	6.7	11.6
24	27.7	72.3	12.4	39.7	7.3	32.4	7.4	12.8	54	37.2	62.8	13.0	29.1	6.3	22.8	7.0	13.7
25	31.1	68.9	9.3	41.9	7.6	34.3	5.0	12.6	55	23.0	77.0	21.4	38.5	13.5	25.0	6.3	10.8
26	28.2	71.8	21.0	33.7	9.4	24.3	6.1	11.0	56	18.2	81.8	19.4	41.1	8.7	32.3	6.8	14.6
27	29.6	70.4	16.4	37.0	7.6	29.5	5.5	11.5	57	26.8	73.2	24.5	31.2	7.3	23.9	6.9	10.6
28	33.2	66.8	9.4	38.4	7.7	30.8	7.5	11.4	58	36.3	63.7	17.4	26.4	3.1	23.3	5.3	14.7
29	32.4	67.6	14.3	36.3	6.8	29.5	5.6	11.4	59	32.4	67.6	17.1	31.5	5.1	26.4	6.3	12.7
30	22.7	77.3	11.0	45.8	5.1	40.8	4.2	16.2									

*n*Glob= non-globulins, Glob = globulins, Leg = legumins, Vicil = vicilins, *np*Vicil.= non-processed vicilins, *p*Vicil = processed vicilins, Conv = convicilins, GlobRP = globulin-related proteins.

A separate quantification of the LegA and LegJ families was not performed, since their similarities in molecular weight made it impossible to distinguish between members of these two families with SDS-PAGE. Vicilin was the most abundant protein of pea, and its content varied between 26.3% and 52.0% of the TPPE. There was no correlation between the amount of vicilin and legumin or that of vicilin and convicilin (Pearson 2-tailed). In all the materials tested, both processed and non-processed vicilins were found. The processed vicilin was the predominant of the two, with values between 17.8% and 40.8%, whereas the non-processed ones constituted between 3.1% and 13.5% of the TPPE. The amount of processed and non-processed vicilin did not correlate with each other (Pearson 2-tailed). Convicilin was the least abundant globulin having an average content of 6.1%. Its content ranged from 3.9% to 8.3%. Finally, the globulin-related proteins were present in amounts ranging from 2.8% to 17.3% of the TPPE; they showed the largest relative variation of the four globulin classes.

The TPPE compositional analysis showed that none of the lines lacked any particular globulin. All four classes of globulins were present in all lines investigated, including the wild relatives. However, significant differences were observed between the different lines, not only in their globulin content of TPPE, but also in their globulin composition. **Figure 5** shows a number of examples in which the content of one class of proteins is particularly high or low. It can be seen that the differences within the globulin fractions were not correlated with the content of the individual globulins; there were no apparent links between a high content of one globulin and a low content of another one. Line 1 (93125-07 Canada) had the highest proportion of vicilin and convicilin, and the lowest proportion of globulin-related proteins (**Figure 5** pie diagram (**d**)).

Table 3 shows that the ratio of vicilin/legumin varied from 1.3 to 8.2, and that on average it was significantly higher in *Pisum sativum* than in the wild relatives, with averages of 2.8 and 1.7, respectively (T-test, Bonferroni). No significant differences were found in this ratio between lines with different external characteristics (such as leaf shape, seed shape, and seed color). The vicilin/convicilin ratio varied from 3.5 to 11.4 (**Table 3**).

Tables 2 and **3**, and **Figure 5**, clearly demonstrate that the extent of vicilin processing can differ considerably between lines; the ratio of processed to non-processed vicilins varied from 1.6 to 8, showing that the processed fraction is always higher than the non-processed one.

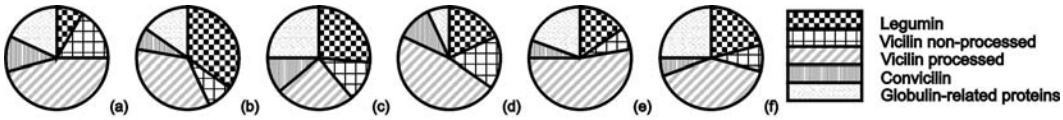

Figure 5. Examples of extremes in globulin composition in various pea lines. Diagrams (**a**) (line 16 [NGB 102149 Iran]) and (**b**) (line 57 [*Pisum arvense*, CGN 10193]) show the extremes in legumin composition, (**c**) (line 50 [CEB 1162]) and (**d**) (line 1 **[**93125-07 Canada]) the extremes in vicilin composition, (**e**) (line 30 [Cisca]) and (**d**) the extremes in convicilin composition, and the (**d**) and (**f**) (line 15 [NGB 102920 Iran]) the extremes in globulin-related proteins.

It appeared that this ratio was significantly lower in the *af* lines than in w.t., with averages of 5.6 and 6.8, respectively (T-test, Bonferroni). No differences between the wild relatives and *Pisum sativum* lines were observed. The ratio of vicilin to globulin-related proteins varied from 1.6 to 11.6 (**Table 3**), and significant differences in this ratio were neither found between lines with different external characteristics, nor between wild relatives and *Pisum sativum* lines (T-test, Bonferroni).

In **Figure 6,** this ratio has been categorized in 4 groups: <2, 2-4, 4-6, and >6. The majority of lines belongs to the group 2-4 (38 lines), whereas the least number of lines belongs to the group >6 (2 lines). The ratio between the two vicilin fractions does not vary significantly between lines with different seed shape (T-test, Bonferroni). In contrast, significant differences with respect to the *p*Vicil/*np*Vicil ratio were found between lines with different leaf shape and seed coat color (T-test, Bonferroni).

Table 3. Ratios between vicilin and the rest globulins, and between the two vicilin fractions of the tested lines. Results are based on the content of the individual globulins as % of the TPPE. For abbreviations see table 2.

Line	Vicil/ Leg	Vicil/ Conv	Vicil/ GlobRP	*p*Vicil/ *np*Vicil	Line	Vicil/ Leg	Vicil/ Conv	Vicil/ GlobRP	*p*Vicil/ *np*Vicil
1	3.9	5.7	11.6	2.5	31	2.8	5.9	2.2	2.0
2	2.3	6.2	3.3	2.4	32	2.4	6.3	2.0	2.0
3	3.6	6.1	3.3	3.4	33	1.4	3.9	2.3	4.9
4	2.5	6.3	6.2	3.0	34	2.0	5.5	2.1	1.7
5	2.7	5.2	3.3	4.8	35	2.1	4.7	3.8	3.2
6	2.9	6.4	3.6	3.4	36	2.0	6.4	3.6	3.4
7	4.3	11.4	3.9	3.6	37	2.9	4.4	2.5	2.3
8	2.9	6.6	4.4	2.8	38	2.5	6.0	2.2	1.6
9	2.2	7.7	4.6	3.5	39	3.6	6.5	3.3	3.8
10	2.1	8.2	3.4	4.2	40	3.5	5.7	3.0	3.8
11	2.8	10.1	3.4	4.8	41	5.1	6.3	2.7	4.1
12	2.8	5.1	2.8	2.5	42	3.3	7.2	4.2	2.4
13	3.1	7.9	3.1	3.3	43	2.4	6.0	2.4	1.9
14	2.8	7.4	2.8	3.3	44	3.0	4.8	2.4	3.3
15	2.8	6.7	2.0	4.4	45	2.1	5.2	3.1	1.9
16	8.2	5.8	3.4	2.8	46	2.3	4.9	3.6	2.9
17	2.3	4.9	3.2	4.3	47	1.7	4.8	2.2	1.7
18	4.8	7.9	4.8	3.4	48	3.7	9.4	4.7	3.7
19	2.6	8.0	4.0	3.7	49	2.8	5.9	2.4	2.2
20	2.6	5.2	4.3	2.7	50	1.5	3.5	1.6	2.1
21	2.0	4.3	2.5	3.1	51	2.9	6.2	3.3	4.0
22	2.5	4.9	3.4	4.1	52	1.5	7.1	2.6	3.2
23	2.9	6.0	3.1	2.3	53	2.0	4.4	2.6	2.5
24	3.2	5.4	3.1	4.4	54	2.2	4.2	2.1	3.6
25	4.5	8.4	3.3	4.5	55	1.8	6.1	3.6	1.9
26	1.6	5.5	3.1	2.6	56	2.1	6.0	2.8	3.7
27	2.3	6.7	3.2	3.9	57	1.3	4.5	2.9	3.3
28	4.1	5.1	3.4	4.0	58	1.5	5.0	1.8	7.5
29	2.5	6.5	3.2	4.3	59	1.8	5.0	2.5	5.2
30	4.2	10.9	2.8	8.0					

The average ratios of *af* and w.t. lines were 2.9 and 3.9, respectively, whereas the ratio of 6.3 found for brown-gray spotted seed-coat lines (*Pisum elatius* accessions) was significantly higher than that of 3.5, 3.4, 2.4, 2.4, and 2.6 for green, yellow, brown-spotted, brown, and green-yellow lines, respectively.

No differences with respect to the *p*Vicil/*np*Vicil ratio between wild relatives and *Pisum sativum* lines were observed.

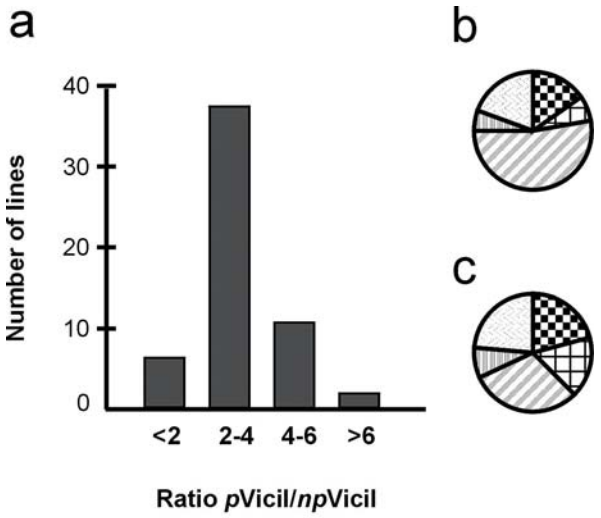

Figure 6. Extent of vicilin processing in the tested pea lines; (**a**) The ratio of processed to non-processed vicilin is categorized in 4 groups, and the number of lines belonging to each category are indicated. Pie diagrams (**b**) and (**c**) show the globulin composition of two extreme varieties with respect to processing, *p*Vicil/*np*Vicil <2 (line 38 [484]) and >6 (line 30 [Cisca]), respectively. For legend with the pie diagrams, see Fig. 5.

Discussion

In this study, the genetic variation of pea (P*isum sativum* L.) in its starch and protein content, and protein composition has been explored, aiming to identify lines with extreme variation. Such could be of importance for industrial applications of pea or its isolated proteins, since pea globulins have different physical properties (Casey and Domoney 1999). Additionally, it is known that starch and different mixtures of globulins give different extrusion products (Hughes et al. 2001). Our results with the 59 tested lines are discussed with respect to the quality of starch, pea meal, pea protein isolates, and purified globulins. The biodiversity identified has been analyzed for correlations with external seed and plant characteristics that might be used as an initial screen to assist in breeding programs.

External characteristics

The pea lines investigated, producing round, wrinkled, flat, and round with dimples pea seeds, have starch as the major pea seed reserve (average content of 46%). It is known that the starch content and quality are affected by the presence of the *r* locus, which results in the production of wrinkled instead of round peas, when near isogenic lines are compared (Wang et al. 1998; Stickland and Wilson 1983). The wrinkled varieties appeared to have on average lower starch content than the varieties with other seed shapes. Nevertheless, there are wrinkled varieties scoring higher on starch content than some non-wrinkled ones

(such as lines 48 and 52). This indicates that the genetic background of a line is crucial to overcome the effects of the *r/r* locus on starch content.

The protein content of the material tested varied considerably. Seed shape and leaf type did not seem to be related with protein content, but seed color was. The three brown pea lines tested had significantly higher protein contents than the other lines. Differences between green and yellow peas, which are the cultivated varieties for human consumption, were not observed, indicating that color can not be a selection criterion for pea protein content in breeding programs for human consumption. The wild relatives of pea have a protein content of 30-36%, which is above average, but below that of soybean (42-45%), the most important protein source for food applications (Toda et al. 2003).

We found a negative correlation between the starch and protein content of the lines tested, but the sum of these appeared to be remarkably constant. This demonstrates that the amounts of these two reserves are balanced. However, there are some exceptions to this (**Figure 3**), such as the lines 28 (Mylosa (CEB 1811)) and 30 (Cisca), which are both wrinkled peas; the other wrinkled lines do not deviate with respect to the sum of protein and starch content.

Protein composition

Pea lines lacking a specific globulin like for example convicilin would be desirable since convicilin's N-terminal extension reduces the gelation ability of pea globulin isolates (O'Kane et al. 2004c). Interestingly, no tested lines lacking any particular globulin have been found. The absence of such lines can be explained by the fact that globulins are encoded by multigene families (Domoney and Casey 1985).

The lines tested showed variation in both total globulin content and globulin composition. Vicilin is the most abundant globulin; it can be present in 8-fold higher amounts than legumin, and in up to 11-fold larger amounts than convicilin and globulin-related proteins. The ratio vicilin/legumin has been studied previously. Our results showed a higher vicilin/legumin ratio compared to that reported by others (Casey et al. 1982; Gueguen and Barbot 1988; Schroeder 1982). We did not find significant differences in the vicilin/legumin ratio between round and wrinkled peas. Others (Gueguen and Barbot 1988; Schroeder 1982), using rocket immunoelectrophoresis and ultracentrifugation, have reported that the ratio vicilin/legumin is slightly higher for wrinkled peas than for round peas. Probably, the differences in results between these studies and our study are due to different determination methods and plant material used.

All lines tested contained processed vicilin. This showed that the vicilin-processing enzyme, which has not been identified yet, is present and active in all lines. This gene could be a potential genetic marker for pea genetic studies. In all lines, the amount of processed vicilin was higher than that of non-processed vicilins. However, the amounts of processed vicilin and non-processed vicilin did not correlate with each other. This was expected, because not all vicilins are processed. Thus, the extent of processing is related to the availability of the isoform that can be cleaved, and not to the total amount of vicilin. It has been shown that fragmentation of a protein can alter its physicochemical properties (Periago et al. 1998). This indicates

that the varieties high in processed vicilin might have different properties compared to those low in processed vicilin. It would be worthwhile to explore the effect of the extent of *in planta* processing of vicilin in relation to the properties of protein mixtures further.

Globulins have different physico-chemical properties and this is reflected in their protein isolates. Gelation studies with globulin isolates derived from the varieties Classic, Solara, Finale, Supra, Espace (**Table 1**, lines 31, 33, 51, 52, and 54), which are varying in globulin composition, also showed a different gelation behavior (O'Kane et al. 2005). Similarly, it has been shown that genetic soybean variants in glycinin have different denaturation behavior (Lakemond et al. 2002). Our results showed significant biodiversity in globulin composition, which may have potential for applications in the food industry. In soybean, the globulins of which are widely used for various applications, the genetic variation for protein composition is much less than that observed for pea. For instance, the ratio 7S/11S globulins in soybean ranged from 0.47 to 0.79 (Fehr et al. 2003; Kim et al. 1995) whereas we have shown that the same ratio (excluding convicilin) varied between 1.2 to 8 in pea. Moreover, the content of glycinin, which is the soybean analogue to pea legumin, is always higher than that of β-conglycinin (the soybean analogue of pea vicilin+convicilin). On the contrary, in pea with some exceptions (Casey et al. 1982; Shroeder 1982) vicilin is always higher than legumin. This is another major difference in the globulin composition of pea and soybean, which could make the use of pea proteins, complementary to those from soybean, attractive to the food industry.

It is known that a low vicilin/convicilin ratio in pea or a low ratio of its soybean equivalents (the β subunit of β-conglycinin corresponds to vicilin; the α and α′ subunits of β-conglycinin correspond to convicilin) hinders gelation in both pea (O'Kane et al. 2004b) and soy isolates (Maruyama et al. 1999). Based on the data provided by Yaklich (1983) and Fehr et al. (2003) it can be calculated that the β/(α+α′) ratio of the subunits of β-conglycinin in soybean varies between 0.7 and 1.5 (Yaklich 2001; Fehr et al. 2003), whereas in pea the ratio vicilin/convicilin varies between 3.5 and 11.4. This shows that pea isolates have a more favorable protein composition for gelling applications compared to those from soybean. Moreover, the genetic variation for this trait appears to be larger in pea than in soybean, which might offer opportunities to reduce the convicilin content further.

Acknowledgments

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3. Regeneration of pea (*Pisum sativum* L.) by a cyclic organogenic system

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Abstract

In a five-step procedure, plants were regenerated from meristematic tissue initiated from nodal tissue in four pea cultivars ('Espace', 'Classic', 'Solara', and 'Puget'). In step 1, stem tissue with one node (1-cm size) was subcultured on medium containing thidiazuron. As a result multiple shoots were produced, appearing normal or swollen at their bases. The multiple shoots were subcultured in the same medium, resulting in the formation of a green hyperhydric tissue in the swollen bases of the multiple shoots, which is fully covered with small buds [bud-containing tissue (BCT)]. In step 2, BCT fragments were isolated and subcultured in the same medium and, as a result, they were able to reproduce themselves in a cyclic fashion. In step 3, subculture of BCT on medium supplemented with a combination of gibberelic acid, 6-benzyladenine and a-naphthalene acetic acid (NAA), resulted in the formation of shoots, which were rooted in step 4 on medium supplemented with 0.5 mg/l NAA, indole-3-acetic acid (IAA) or indole-3-butyric acid. In step 5, in vitro plants were transferred to the greenhouse for acclimatisation and further development. The four varieties tested were all able to produce meristematic tissue, suggesting that its production is genotype independent.

Keywords: pea (*Pisum sativum*); thidiazuron; TDZ; multiple shoots; bud containing tissues (BCT)

Introduction

Pea (*Pisum sativum* L.) is an important crop in Northern Europe. It is grown for its seeds, which are considered as a high quality and relatively cheap source of protein, used for cattle feed and in the human diet. Resistance to viruses, lowering of anti-nutritional factors, and improving protein composition and quality, are important goals in pea breeding. Improvement of these goals can probably only be accomplished with genetic modification, since peas' natural variation is limited (Christou 1997 and references therein). Pea, like most legume species is recalcitrant to genetic modification, although protocols for genetic modification of pea have been described (Schroeder et al. 1993; Grant et al. 1995; Bean et al. 1997; Nadolska-Orczyk and Orczyk 2000; Polowick et al. 2000). In the procedures described by Schroeder et al. (1993) and Polowick et al. (2000), segments of embryogenic axis are used. Bean et al. (1997) and Nadolska-Orczyk and Orczyk (2000) use cotyledonary nodes as starting material. After infection with *Agrobacterium tumefaciens*, multiple shoots are formed, some of which contain genetically modified shoots. However, these systems have drawbacks such as a low efficiency (about 2-5% of the initial seeds), high frequency of escapes and the occurrence of chimeric genetically modified plants (Bean et al. 1997; Grant et al. 2003). These disadvantages might be related to the fact that the majority of the shoots are regenerated from existing meristems, and might be reduced significantly if plants were regenerated from newly formed meristems (adventitious regeneration). Adventitious regeneration can be obtained either by somatic embryogenesis or by shoot organogenesis, and both types of regeneration can be either direct or indirect via a callus phase. The latter is preferred over direct regeneration for genetic modification as the callus phase is ideal for selection of genetically modified cells and, subsequently, plants. However, callus-based regeneration systems have the disadvantage that they have a much higher chance of yielding plants with somaclonal variation than direct regeneration. Somatic embryogenesis has been described in pea (Loiseau et al. 1998; Griga 1998, 2002 and references therein). In our own laboratory it was found that the described protocols are genotype-dependent, somatic embryos are regenerated directly without callus phase, and the efficiency is low (unpublished results). The multicellular origin of somatic embryos in pea (Loiseau et al. 1998; Griga et al. 2002) might be another constraint in employing this embryogenic system for genetic modification, as it might result in chimeric genetically modified somatic embryos, as was observed in cassava (Raemakers et al. 2001) and walnut (Escobar et al. 2000). Shoot organogenesis has been reported in pea using hypocotyls explants (Ochatt et al. 2000) and immature cotyledon explants (Grant et al. 1995). The protocol described for hypocotyls has a low regeneration frequency and is genotype-dependent (Ochatt et al. 2000) and has not been combined with genetic modification. The protocol described for cotyledons has been combined successfully with genetic modification. However, as in the other systems, multiple shoots are formed and as a result the efficiency is low, there is a large variation between experiments, and often a high frequency of escapes (Grant et al. 1998). In this report, we present a novel regeneration system with both adventitious and non-adventitious properties. An organogenic/meristematic tissue is formed from nodal tissue, which can be maintained in a cyclic fashion,

giving it callus-like properties. Furthermore, we describe the regeneration of fertile plants, which were grown successfully in the greenhouse.

Materials and methods

General

A step-by-step approach was used to test a range of pea (*Pisum sativum* L.) commercial varieties 'Espace', 'Classic', 'Solara', and 'Puget') with the aim of developing a novel regeneration protocol. Each experiment consisted of three replicates with 18-20 explants per replicate.

Media and growth conditions

Unless stated otherwise, basic medium contained Murashige and Skoog (1962) salts and vitamins, 30 g/l sucrose and 7.5 g/l micro agar (MS3). The pH was adjusted to 5.7 prior to autoclaving (121 °C for 15 min). All growth regulators were added filter-sterilised to the medium after autoclaving. Media were dispensed into 9-cm Petri dishes (25 ml) or into 9 cm jars (310 ml). All cultures were incubated in a growth chamber at a temperature of 24 °C, a photoperiod of 16 h and an irradiance of 40 μmol m^{-2} s^{-1}.

Preparation of plant material

Seeds from commercially important pea varieties ('Espace', green pea semi-leafless; 'Classic', yellow pea semi-leafless; 'Solara', green pea semi-leafless, kindly provided by Cebeco Zaden (Lelystad, The Netherlands), and 'Puget', green pea, semi-leafless, kindly provided by S. Bean, John Innes Centre, Norwich, UK), were surface- sterilised in 70% ethanol for 1 min, subsequently immersed in 1.6% sodium hypochloride for 20 min, and rinsed three times with sterile distilled water. The seeds were then cultured for 36 h in jars containing 50 ml water (10 seeds per jar) after which they were cultured on MS3 for further growth.

Step 1: Induction of bud-containing tissue on one-node stem explants

Shoots of *in vitro* plants were cut into one-node pieces of about 1 cm. Cuttings were placed on MS3 medium or MS3 medium supplemented with 1.1 mg/l or 2.2 mg/l thidiazuron (TDZ) or 0.55 mg/l or 1.1 mg/l 6-benzyladenine (BAP). Every 4 weeks, the newly formed shoots were subjected to the same procedure of cutting one-node stem pieces and subculture on the fresh medium. A vitrified callus-like bud containing tissue (BCT), covered with buds, was formed near the bases of the shoots.

Step 2: Isolation and multiplication of BCT

Isolated fragments (2–5 mm) of BCT were placed on MS3 medium or MS3 medium supplemented with 0.5 mg/l, 1.1 mg/l, 2.2 mg/l, 4.4 mg/l, or 8.8 mg/l TDZ or 0.25 mg/l, 0.55 mg/l, 1.1 mg/l, 2.2 mg/l, or 4.4 mg/l BAP. Every 4 weeks the developed BCT was subdivided and subcultured on the same medium.

Step 3: Regeneration of shoots from BCT

BCT multiplied for 6 months on MS3 supplemented with 2.2 mg/l TDZ was used as explant material for regeneration of plants. BCT cultured for 4 weeks on MS3 supplemented with 2.2 mg/l TDZ, was cultured on medium supplemented with B_5 salts and vitamins (Gamborg et al. 1968), 30 g/l sucrose, 7.5 g/l micro agar ($B_5$3) and all possible combinations of gibberellic acid (GA_3), a-naphthalene acetic acid (NAA) and BAP, at a concentration of 1 mg/l for 5 weeks. After 3 weeks the explants were transferred to the same medium.

Step 4: *In vitro* rooting of shoots derived from BCT

Shoots developed from 6-month-old BCT cultured on $B_5$3 medium supplemented with 1 mg/l GA_3 were subcultured in jars on $B_5$3 medium supplemented 0.5 mg/l IAA, or 0.5 mg/l IBA or 0.5 mg/l NAA (Ochatt et al. 2000) for rooting. After 5 weeks the apical part (5 cm) of the shoots was subcultured on the same medium for an additional period of 5 weeks. After this period, rooted and non-rooted plants were transferred to the greenhouse for acclimatisation.

Step 5: Greenhouse rooting and acclimatisation of plants derived from BCT

In vitro plants, with or without roots, produced as described above, were planted in pots containing standard potting soil and maintained under constant temperature of 18 °C and relative moisture of 100% for further development. Plants without roots were covered with plastic to reduce water evaporation prior to root formation.

Low temperature scanning electron microscopy

BCT samples cultured for 4 weeks on MS3 supplemented with 2.2 mg/l TDZ were placed on a specimen holder and immediately plunged into liquid N2 (-196 ºC). The frozen samples were transferred to a cryo-transfer unit (CT 1500-HF Oxford Instruments, Oxford, UK). The unit consists of a cryo-preparation chamber under high vacuum (10^{-6} Pa) dedicated to a low temperature scanning electron microscope (LT-SEM; JEOL, model 6300 F, Tokyo, Japan) and a cryo-stage inside the microscope. The specimens were placed inside the cryo-chamber at -85 °C, and freeze-dried for 2 min at -85 °C and 10^{-7} Torr. The samples were then sputter-coated with 10 nm platinum. LT-SEM was used to examine the coated callus-like tissue at 5-10 kV, keeping the temperature of the specimens at -180 °C.

Ploidy level analysis

To identify the ploidy level of the shoots produced, flow cytometry was performed by Plant Cytometry Services (Schijndel, The Netherlands).

Results

Induction of BCT on stem node explants

BCT was first observed in experiments where stem pieces were cultured on MS3 medium supplemented with 2.2 mg/l TDZ to maximise multiple shoot formation. BCT is characterised as a vitrified, dark green, callus-like tissue, round in shape, covered with buds, and was formed in the swollen parts of the multiple shoots. One-node explants of four commercial pea varieties were subcultured for three consecutive cycles on media with various amounts of cytokinins to optimise BCT initiation. The results obtained with cultivars Puget and Solara are shown in **Table 1**. Explants cultured on MS3 medium grew normally and formed rooted plants with one shoot per explant in the first cycle. Similarly, in the two subsequent subculture cycles, only plants with a single shoot were formed. However, only 20% of these plants formed roots compared to 100% in the first cycle. The same response was observed for the other two varieties. Addition of BAP to MS3 medium resulted in the formation of a single shoot without roots per cultured node stem explant in the first cycle. Similar results were obtained in the two subsequent subculture cycles. Multiple shoot formation was never observed in any of the four cultured varieties. In variety Solara, the addition of 1.1 mg/l BAP induced *in vitro* flower formation in 25% of the cultured node explants. However, *in vitro* seed formation was not observed. Addition of TDZ to MS3 medium resulted in the formation of multiple shoots (3–4 shoots per node explant) in the first cycle, of which 30% had swollen bases. In the second cycle all subcultured node stem explants formed multiple shoots, all swollen at their bases. Some explants formed BCT on the swollen bases of the multiple shoots. In Puget, BCT was produced in 4% of node explants on 1.1 mg/l TDZ and in 15% of node explants cultured on 2.2 mg/l TDZ. This increased in the third cycle to 10% and 85% of subcultured node stem explants cultured on 1.1 mg/l and 2.2 mg/l TDZ, respectively. In Espace and Classic, the frequencies were comparable to those of Solara and Puget (**Table 1**) in all cycles.

Table 1. Effects of 3 cycles of subculture of one node stem explants on medium supplemented with of BAP and TDZ in the pea (*Pisum sativum* L.) varieties Puget and Solara. Values are the mean of 3 replicates with 50-60 explants in total, SSR Single Shoots with Roots, SS Single Shoots without roots, MS Multiple Shoots without roots, BCT Bud Containing Callus on the bases of the multiple shoots; Pug *cv* Puget; Sol *cv* Solara.

Growth regulators	Response in first cycle (%)								Response in second cycle (%)								Response in third cycle (%)							
mg/l	SSR		SS		MS		BCT		SSR		SS		MS		BCT		SSR		SS		MS		BCT	
TDZ	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol
0	100	100	0	0	0	0	0	0	20	20	80	80	0	0	0	0	20	20	80	80	0	0	0	0
1.1	0	0	0	0	100	100	0	0	0	0	0	0	96	95	4	5	0	0	0	0	90	85	10	15
2.2	0	0	0	0	100	100	0	0	0	0	0	0	85	86	15	14	0	0	0	0	15	5	85	95
BAP																								
0.55	0	0	100	100	0	0	0	0	0	0	100	100	0	0	0	0	0	0	100	100	0	0	0	0
1.1	0	0	100	100	0	0	0	0	0	0	100	100	0	0	0	0	0	0	100	100	0	0	0	0

Isolation and multiplication of BCT

Preliminary results showed that BCT was not able to develop into plants while attached to the bases of the multiple shoots. On MS3 medium supplemented with 2.2 mg/l TDZ, the amount of BCT increased with time and the multiple shoots grew slowly. On MS3 medium supplemented with mixtures of BA, NAA and GA_3, the BCT died and the multiple shoots grew further. In the next set of experiments, BCT was isolated from the bases of the multiple shoots and pieces of 2–5 mm were cultured on MS3 medium supplemented with cytokinins (BAP or TDZ). The general response of BCT was equal for all four genotypes. **Table 2** shows the effects of cytokinins on BCT multiplication for the varieties Puget and Classic after 4 weeks of culture. On MS3 medium, around 40% of the explants of Puget and Classic survived and the rest of the explants turned brown within 2 weeks and died (**Table 2**). When MS3 medium was supplemented with 0.25–4.4 mg/l BAP, 38–50% of the explants from Classic survived, and for Puget this varied from 42% to 90%. Upon addition of 0.5–8.8 mg/l TDZ to MS3 medium, all explants survived and browning was not observed for Puget. For Classic all explants survived on MS3 medium supplemented with 4.4 mg/l or 8.8 mg/l TDZ, while at lower concentrations 47–72% of the explants survived. Surviving BCT pieces cultured on MS3 medium became light green, lost their hyperhydric nature, and formed one or two short and thick shoots. On MS3 medium supplemented with BAP, most explants formed only one or two short and thick shoots and, at a low frequency, short and thick shoots together with BCT (**Table 2**). Addition of BAP did not result in formation of pure BCT. When TDZ was supplemented to the MS3 medium, either thick short shoots were formed or new BCT was initiated, or a mixture of both. Lower concentrations of TDZ stimulated shoot formation whereas higher concentrations of TDZ stimulated BCT formation. On MS3 medium supplemented with 2.2 mg/l or more TDZ, 50–63% of the BCT pieces were reproduced in pure state (**Table 2**) in the variety Puget. In this way, BCT was maintained for more than 2 years by subculturing pieces of 2–5 mm every 4 weeks on MS3 supplemented with 2.2 mg/l TDZ. When larger pieces of 10–20 mm were used, BCT had a stronger tendency to form shoots instead of pure BCT and hence smaller pieces were preferred for routine purification and maintenance of BCT. LT-SEM was performed to illustrate the nature of BCT. **Figure 1b,c** shows that bud- like structures, which appeared on the surface of BCT, are bud or shoot initials. After 4 weeks of culture on MS3 medium supplemented with 2.2 mg/l TDZ, BCT was fully covered with meristems at a density of 1 per mm2. Some buds contained leaf primordia and shoot meristems, whereas others were at earlier stages (Fig. 1c).

Table 2. Effects of different concentrations of TDZ and BAP on the proliferation of bud containing tissue (BCT) in pea (*Pisum sativum* L.) varieties Puget and Classic. Values are the mean of 3 replicates with 50-60 explants in total.

Growth regulators	Explants surviving the treatment (%)		Explants producing only shoots [a] (%)		Explants producing a mixture of shoots [a]		Explants producing only BCT (%)	
TDZ	Puget	Classic	Puget	Classic	Puget	Classic	Puget	Classic
0	40	43	100	100	0	0	0	0
0.5	100	47	50	63	50	37	0	0
1.1	100	55	50	48	50	52	0	0
2.2	100	72	8	9	42	91	50	0
4.4	100	100	8	8	42	47	50	45
8.8	100	100	2	4	35	48	63	48
BAP								
0.25	42	40	100	100	0	0	0	0
0.55	90	39	94	100	6	0	0	0
1.1	90	38	94	100	6	0	0	0
2.2	80	47	94	97	6	3	0	0
4.4	75	50	90	96	10	4	0	0

[a] Shoots were short and thick (as shown in Fig. 2b).

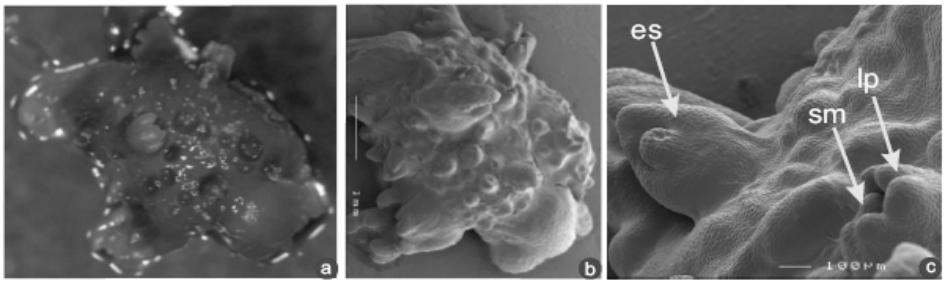

Figure 1a–c. Development of meristematic bud-containing tissue (BCT) after culture of one-node stem segments of pea on medium supplemented with 2.2 mg/l thidiazuron (TDZ). **a** Normal image of BCT. **b** Scanning electron micrograph of the same explant showing that globular structures on the callus surface are developing meristems. **c** Not all buds were in the same developmental stage: some formed leaf primordia (lp) and shoot meristems (sm), whereas others were in earlier stages (es)

Plant regeneration from BCT

BCT cultured for 4 weeks on MS3 medium supplemented with 2.2 mg/l TDZ was subcultured on $B_5$3 medium supplemented with various growth regulators for regeneration of shoots. In all four varieties tested, the response of BCT to the different media was similar. **Table 3** shows the results for the varieties Puget and Espace after 5 weeks of culture. Depending on the growth regulator used, between 40% and 90% of the explants survived, and the rest became brown and died. A higher survival rate was observed when BAP and NAA, with or without GA_3, were used, and a lower rate was seen in the absence of growth regulators or when GA_3 with or without NAA or BAP was used. Surviving explants formed shoots in all media tested after 5 weeks of culture. Two different types of shoot formation were observed depending on the presence or

absence of GA_3 in the medium. Addition of GA_3 to $B_5$3 medium, alone or in combination with NAA or BAP, resulted in the formation of a high number of shoots (10–22), which were hyperhydric. The shoots completely covered the surface of BCT, giving it a bushy appearance (Fig. 2a). When these shoots were isolated from BCT and subcultured in the same medium, their hyperhydric appearance shifted to a normal (non-hyperhydric) one and new shoots were formed from the remaining BCT cells at the bases of the shoots. On the contrary, on $B_5$3 medium without GA_3, only 2–10 non-hyperhydric shoots developed per explant (Fig. 2b). These shoots developed normally when they were isolated and subcultured on the same medium and they did not form any new shoots from their bases. Shoots produced on media containing GA_3 were thinner, and generally longer, compared to shoots obtained on medium without GA_3.

Table 3. Effects of different growth regulators on shoot production of 4 weeks old BCT in pea (*Pisum sativum* L.) varieties Puget and Espace (Results after 5 weeks of cultivation). Values are the mean of three replicates with 50-60 explants in total. *NAA* a-naphthalene acetic acid, *GA_3* gibberellic acid

Growth regulators mg/l			Explants survived the treatments (%)		Explants forming long thin shoots (%)		Explants forming short thick shoots (%)		# of shoots per responding explant	
GA_3	BAP	NAA	Puget	Espace	Puget	Espace	Puget	Espace	Puget	Espace
0	0	0	45	40	0	0	100	100	2[a]	3[a]
1	0	0	50	40	100	100	0	0	14[b]	12[b]
0	1	0	55	50	0	0	100	100	5[a]	5[a]
0	0	1	60	50	0	0	100	100	8[a]	6[a]
0	1	1	90	80	0	0	100	100	10[a]	8[a]
1	1	0	40	40	100	100	0	0	22[b]	20[b]
1	0	1	50	45	100	100	0	0	20[b]	19[b]
1	1	1	70	65	100	100	0	0	10[b]	10[b]

[a] Shoots were short and thick (as shown in Fig. 2b); [b] shoots were thin and long (as shown in Fig. 2a).

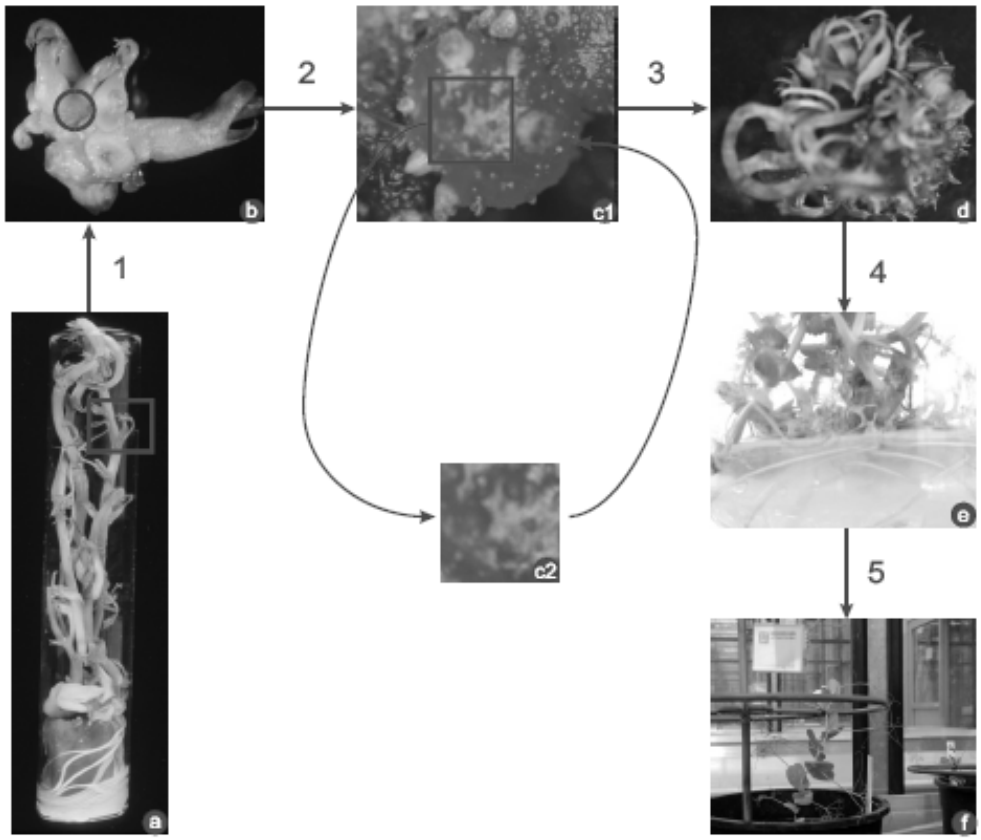

Figure 3a–f. A five-step procedure for the initiation of BCT and subsequent regeneration of plants in pea (*Pisum sativum* L.); steps 1–5 are indicated. **a** Pea plant obtained from seed with one-node explant (box), **b** production of swollen multiple shoots with meristematic BCT at their base (circle), **c1** isolation and subculture of the BCT shown in b resulting in pure BCT, **c2** subculturing BCT from c1 cut in fragments resulted in its reproduction in a cyclic manner, **d** formation of shoots from BCT, **e** rooting of shoots derived from BCT, **f** BCT-derived plant grown in the greenhouse (step 1: 8 weeks, step 2: 4 weeks, step 3: 5 weeks, step 4: 5 weeks (replication of this step optional since non-rooted in vitro plants can be transferred directly to the greenhouse), step 5: 6–8 weeks)

In vitro rooting of shoots derived from BCT

Preliminary experiments showed that shoots (2–3 cm) derived from BCT cultured on medium supplemented with cytokinins alone or together with NAA or GA_3 did not form roots on $B_5$3 medium supplemented with auxins. Also, shoots isolated from 2-year-old BCT were not capable of forming roots in the various media tested (not shown). In the results for the variety Puget presented in **Table 4**, shoots (2–3 cm in size) derived from 6-month-old BCT cultured for 5 weeks on medium supplemented with 1 mg/l GA_3 were used. Root formation was not observed in any treatment after 5 weeks of culture. The apical parts of shoots (5 cm) were subcultured in the same medium. In the second subculture, 50% of the shoots cultured in $B_5$3 medium supplemented with 0.5 mg/l IBA formed roots. When shoots were cultured in $B_5$3 medium supplemented with 0.5 mg/l NAA or 0.5 mg/l IAA, 24% and 9% of the plants rooted, respectively. Independent of the three auxins used, callus formation preceded root formation, but the roots formed were attached to the stem and functional. When IAA was used, more vigorously growing plants developed than when IBA or NAA were used. Smaller plants were produced in the presence of NAA, which also had increased callus formation at their bases, compared with shoots rooted in the presence of IBA or IAA.

Table 4. Root formation (> 1 cm) of shoots obtained from 6 months old BCT after two cycles of subculture on media supplemented with different auxins. Shoots were derived from media containing GA_3 (thin shoots) from the pea (*Pisum sativum* L.) variety Puget. Values are the mean of 3 replicates with 50-60 explants in total. *IBA* Indol-3-butyric acid

Auxin	# explants rooted (%)	
	First cycle of subculture	Second cycle of subculture
0.5 mg/l IBA	0	50
0.5 mg/l IAA	0	9
0.5 mg/l NAA	0	24

Acclimatisation of plants derived from BCT

All plants that rooted *in vitro* survived the transfer to the greenhouse. Within a period of 6–8 weeks, they flowered and subsequently formed fertile seeds. Of the plants that failed to form roots *in vitro*, 80% formed roots during acclimatisation and developed similarly as those that had rooted *in vitro*. Plants derived from BCT had the same ploidy level (2x), as control plants obtained from seeds.

Discussion

This study describes a novel regeneration protocol for pea (*Pisum sativum* L.). In a five-step procedure (Fig. 3), plants were regenerated from BCT-a meristematic/organogenic callus-like tissue. In step 1, BCT is initiated from one-node explants. In step 2, the BCT is isolated, purified and maintained. Regeneration of shoots from BCT and rooting are steps 3 and 4, respectively. Finally, the plants are

transferred to the greenhouse in step 5. All the varieties tested were able to produce BCT and regenerated plants, showing that this new regeneration system is applicable to a broad range of varieties. TDZ was essential for the induction and maintenance of BCT. When the TDZ concentration was 2.2–8.8 mg/l, about half of the BCT explants initiated only new BCT. At a lower concentration of TDZ all BCT explants initiated a mixture of new BCT and shoots. Only a few shoots were obtained from the low TDZ treatment and these shoots did not form roots on auxin-supplemented medium. Therefore other media were tested for shoot development. The survival of BCT cultured on medium supplemented with GA_3 was low compared to medium supplemented with BAP and NAA. However, because the number of shoots formed and their ability to form roots was higher in GA_3-supplemented medium, that medium was preferred for shoot development. It is well known that cytokinins such as TDZ suppress the growth of apical meristems and instead induce excess formation of lateral meristems, resulting in multiple shoots (Sanago et al. 1996). Popiers et al. (1997) reported the formation of hyperhydric tissue in pea together with the formation of multiple shoots after prolonged exposure to TDZ. In their system, only shoots isolated from multiple shoots developed into complete plants. Most probably, their medium for plant development was not optimal for formation of shoots from the hyperhydric tissue. We observed that multiplication and maintenance of BCT on TDZ-supplemented medium for a period exceeding 2 years reduces the capacity of the regenerated shoots to form adventitious roots. Madsen et al. (1998) and Bean et al. (1997) also observed that long exposure of pea to either TDZ or BAP inhibited the capacity of shoots to form adventitious roots. Bean et al. (1997) used grafting techniques to transfer rooted plants to the greenhouse. Here it is shown that non-rooted *in vitro* plants form *ex vitro* roots. The plants grew normally in the greenhouse, looked phenotypically like seed-derived plants, did not have altered ploidy levels, and formed viable seeds. Plants derived from these seeds were also similar to wild-type plants. However, more research is needed to determine if plants derived from BCT that has been maintained for a prolonged period of time are affected by somaclonal variation. The regeneration protocol has characteristics both of an adventitious and a non-adventitious system. The ability of BCT to be maintained in a cyclic manner confers adventitious characteristics. However, the BCT itself is differentiated; it is covered with small meristems, which are most probably the source of the formation of new buds in the next multiplication cycle, giving it non-adventitious characteristics.

The ability of the BCT to reproduce itself might be beneficial for obtaining genetically modified plants. For successful genetic modification it is necessary that genetically modified cells have the ability to develop independently from wild type cells, and that the growth of genetically modified cells is favoured. This is usually accomplished by coupling the gene(s) of interest to genes governing resistance to antibiotics or herbicides. The appropriate antibiotic or herbicide is added to the medium and consequently only transgenic cells are able to divide. In most crops, selection is applied during a callus phase (undifferentiated cells). After the selection phase, the callus is cultured for regeneration. In pea, the regeneration systems that have so far been used for transformation are based on cytokinin-induced direct formation of shoots either from existing meristems or from newly formed meristems (Schroeder et al. 1993; Grant et al. 1995; Bean et

al. 1997; Nadolska-Orczyk and Orczyk 2000; Polowick et al. 2000). The major drawbacks of these methods are low selection efficiency of the regenerating shoots and the often highly chimeric status of the transformed plants. Most probably this is caused by the fact that meristems are complex, multicellular structures in which it is difficult for individual cells to divide independently. The main difference between the regeneration system described here and the regeneration systems used previously (Schroeder et al. 1993; Grant et al. 1995; Bean et al. 1997; Nadolska-Orczyk and Orczyk 2000; Polowick et al. 2000) to obtain genetically modified plants is the cyclic multiplication of BCT versus the linear development of shoots. In preliminary experiments, BCT has been subjected to transformation experiments using *A. tumefaciens* carrying the luciferase reporter gene. Luciferase-positive BCT was obtained and cultured repeatedly on TDZ-supplemented medium. During this process selection was based only upon luciferase activity. This resulted in complete luciferase- positive BCT and subsequently in complete luciferase-positive plants (data not shown), indicating the potential for genetic modification of the regeneration system described here.

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4. Effect of TDZ on plant regeneration from mature seeds in pea (*Pisum sativum* L.)

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Abstract

Pea (*Pisum sativum* L. cv. Espace) seeds directly cultured on thidiazuron (TDZ)-containing medium formed large amounts of multiple shoots. The number of shoots per seedling depended on the concentration and duration of the TDZ treatment. The best treatment was 12 weeks incubation on MS medium supplemented with 4 mg/l TDZ followed by 4 weeks culture on MS medium supplemented with 0.5 mg/l benzylaminopurine (BA) and produced more than 400 shoots/seedling. Isolated shoots rooted at a high frequency on MS medium containing 3.0 mg/l indole-3-butyric acid and 2.0 mg/l α-naphtalene acetic acid. In addition to the formation of shoots, bud containing tissues (BCT) were formed at the cotyledonary nodes, shoot nodes, tendrils, stipules and stalks. The BCT from the cotyledonary nodes and the shoot nodes was maintained in its pure state on MS medium supplemented with 4 mg/l TDZ by repeated culture. Shoot development was accomplished when the BCT were left on MS medium supplemented with 4 mg/l TDZ without refreshment. Seven genotypes were tested in this regeneration system. The optimum TDZ concentrations for multiple shoot production and BCT formation varied with genotypes. All tested genotypes formed shoots from BCT.

Keywords: pea (*Pisum sativum*); seed culture; thidiazuron; TDZ; multiple shoots; bud containing tissues (BCT)

Introduction

An effective procedure for regeneration of plants is essential for crop improvement via genetic modification. Such a regeneration system requires large amounts of totipotent cells that are capable of receiving DNA, independent division of genetic modified cells, efficient regeneration of plants, easy to be manipulated under *in vitro* conditions and genotype independency.

Most transformation protocols use a regeneration system which starts with physical isolation of the explants followed by exposure of the explants to a suitable plant growth regulator regime to activate a new developmental pathway (Steward, 1964), either somatic embryogenesis or organogenesis. In many crops this strategy is very successful, usually accomplished by optimizing the type of explants used, growth regulators used, and the nutritional and physical conditions for its culture.

In pea, protocols for somatic embryogenesis (Loiseau et al. 1998; Griga 1998, 2002) and organogenesis (Kartha et al. 1974; Kallak and Koiveer 1990) have been described; however, none of them has been used successfully for the routine production of genetically modified plants. As it is in most other legume species, the regeneration system used to produce genetically modified plants in pea is based on multiple shoot formation (Jordan and Hobbs 1993; Nadolska and Orczyk 2000). The multiple shoots are formed essentially from existing meristems of the cotyledonary nodes when they are cultured on a benzylaminopurine (BA) containing medium (Schroeder et al. 1993; Grant et al. 1995; Bean et al. 1997; Nadolska and Orczyk 2000; Polowick et al. 2000). The multiple shoot clusters consist of about 4 to 15 shoots. The efficiency of the multiple shoot system for genetic modification is low. Most of the shoots produced are escapes. Only 0.1-5% of the explants produces transgenic shoots (Christou et al. 1990; Jordan and Hobbs 1993; Lulsdorf et al. 1991), however, not all of them are capable of transmitting the transgenes to the next generation. The reason for this low efficiency is that the proliferating meristems are highly complex tissues with many cells participating in the formation of a new plant and that the regenerating meristematic regions activated by cytokinins represent a very low proportion of cells in the explants, severely limiting the number of actual target cells for transformation capable of regeneration. It is expected that the efficiency of genetic modification will be improved if the proportion of meristematic cells in explants is increased or if the number of shoots regenerated from the existing meristems is increased. Malik and Saxena (1992a,b) found that 5-20 more shoots in a multiple shoot cluster were induced when intact seeds of pea, chickpea, and lentil were exposed to a medium with high concentrations of cytokinins such as BA or TDZ compared to isolated explants such as cotyledonary nodes. In Chapter 3 it is shown that a tissue covered with bud primordia was produced when shoot nodes were repeatedly cultured on TDZ-containing medium.

TDZ, a synthetic plant growth regulator with both auxin and cytokinin like properties, is more active than BA in altering the development of lateral meristems (Murthy et al. 1998). It can be stably maintained *in vitro* for a long period of time (Mok and Mok, 1983) and its metabolism is still unclear. Over-exposure to

TDZ can retard shoot and root development and thus limits the recovery of functional plants (Murthy et al. 1998).

The research described here shows the effect of TDZ on growing pea seedlings. An efficient genotype independent regeneration system was established and its possible application for genetic modification is discussed.

Materials and methods

Seeds of the pea (*Pisum sativum*) cultivar Espace were used in this research. Seeds were sterilized in 75% ethanol for 3 min, and then in 0.5% (w/v) sodium hypochlorite for 20 min, followed by rinsings four with sterile water. The basic culture medium contained Murashige and Skoog (1962) salts and vitamins, 3% sucrose and 0.8% agar (MS). pH was adjusted to 5.8 prior to autoclaving (121°C for 15 min). All growth regulators were added filter-sterile to the medium after autoclaving. Cultures were incubated at 25°C with a 16 h photoperiod using cool white fluorescent tubes and an irradiance of 40 μmol m^{-2} s^{-1}.

Culture of seed on media containing TDZ

In one experiment both the effect of the concentration of TDZ and the duration of the TDZ culture was tested. To test different concentrations, seeds were cultured in plastic boxes containing MS medium supplemented with 0, 2, 4 or 8 mg/l TDZ (MS0, MS2T, MS4T, and MS8T). Four weeks later shoots were divided into one node cuttings and cotyledonary nodes were divided into 2-5 pieces. The shoot nodes and cotyledonary node pieces were transferred to MS medium supplemented with 0.5 mg/l BA (MS0.5BA) for shoot development. The MS0.5BA medium was refreshed 2 times every 4 weeks.

To test different durations of TDZ preculture, seeds were cultured for 4, 8 and 12 weeks on MS2T, MS4T, and MS8T. After four weeks shoots were divided into one node cuttings and cotyledonary nodes were divided into 2-5 pieces and either transferred to the TDZ supplemented medium or to MS0.5BA. The MS0.5BA medium was, depending on the duration of culture on TDZ, refreshed once or twice times (every 4 weeks).

Every 4 weeks the total number of shoots (containing an apical meristem and at least one extra node) was counted and removed and the remaining shoot nodes and cotyledonary nodes were subcultured on fresh medium.

Four seeds were cultured in one plastic box (6 cm high and a diameter of 9 cm), and 12 seeds were used per treatment.

Formation of bud containing tissues, its multiplication and regeneration into plants

When the seedlings were cultured for more than 4 weeks on MS4T or MS8T, small sectors of bud containing tissues as described in (Chapter 3) were formed. The bud containing tissues (BCT) were isolated and recultured on MS4T. The medium was refreshed every 4 weeks. To test the optimal TDZ concentration for maintenance, BCT were cut into small pieces (about 10 mg/piece) and cultured in Petri-dishes containing MS2T, MS4T and MS8T medium. Ten pieces (in total ± 100 mg) were cultured per Petri-dish, with 9 replicates per treatment. Medium was refreshed every 3-4 weeks. The increase in fresh weight and morphogenesis of the tissues were recorded.

BCT, which had been maintained on MS4T for 3 months by repeated subculture, was used for plant regeneration. For this pieces of about 10-15 mm^2 were cultured in plastic boxes (6*9cm) containing MS4T medium for 16 weeks without subculture.

Rooting of shoots

Isolated single shoots either derived from multiple shoot cultures (treatment 12 weeks MS8T) or derived from BCT were cultured in plastic boxes (6*9cm) containing MS, MS supplemented with (1, 2 or 3 mg/l) IBA combined with (1 or 2 mg/l) NAA, or MS supplemented with 1.5 mg/l IAA and 0.5 mg/l kinetin. Root development and lateral root number were recorded after 4 weeks. Ten shoots were used for each treatment with two replications.

Results

Effect of TDZ concentration on seedlings development

Pea seeds germinated within 5-7 days on all tested media. The germination frequency was not significantly influenced by the different TDZ culture conditions. The axillary buds were visible with the naked eyes 4 day after seed culture.

The seedlings cultured on MS0 grew normally with one shoot, unfolded stipules and 3-5 green, finger per tendrils and 5-10 roots. The cotyledon remained unchanged in size and color. On TDZ supplemented medium elongation of the main shoot was inhibited, and axillary buds were formed flanking the cotyledonary node. The main root was short and thin without lateral roots. Seedlings formed multiple shoots with folded stipules and dark green, tendrils that have 8-20 fingers per tendril. Tendrils and stipules were completely folded on MS4T and MS8T medium.

The cotyledons remained unchanged in color and size compared to those on MS0. More axillary buds developed from the cotyledonary nodes and the shoot nodes. The highest amount of axillary buds was formed on the cotyledonary nodes, followed by the lower shoot nodes. The upper shoot nodes contained only a few axillary buds. The axillary buds developed into shoots while new axillary buds were formed continuously. Seedlings cultured on MS4T or MS8T medium, also formed axillary buds on tendrils and stalks. Up to 20 axillary buds, organized in clusters were formed on tendrils. The clusters of buds were small in size (3-10 mm^2) and were distributed on the base of tendrils and on the tendril fingers. The bud clusters on stalks were bigger in size (4-30 mm^2) and consisted of up to a few hundred buds, distributed continuously along the longitude of the stalk. There was no connection between the bud clusters and existing meristems. Only few bud clusters were formed on MS2T-cultured seedlings and none on MS0 cultured seedlings.

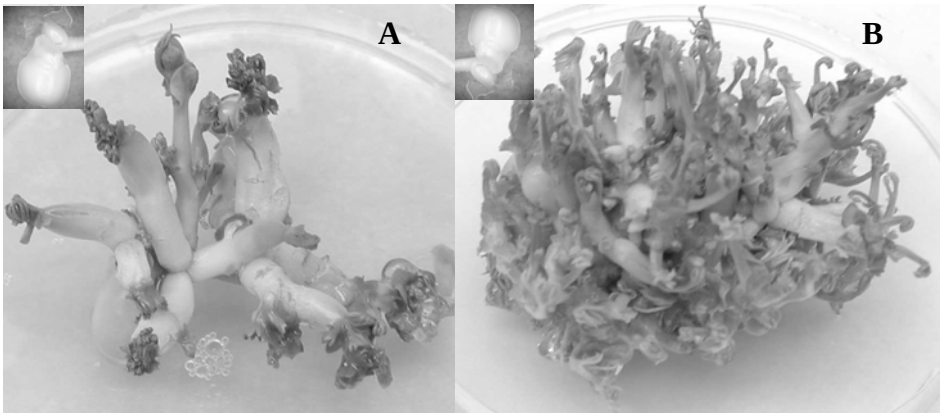

Figure 1. Effect of orientation of seed on medium on shoot formation (after one month of culture on MS4T). A. hilum up. B. hilum down

Seed orientation influenced both the formation of multiple shoots and bud clusters. When the seed hilum was down, in contact with the medium, much more multiple shoots and bud clusters developed than when the seed hilum was up, i.e. not in contact with the medium (**Table 1**, **Figures 1A,B**). The effect of different TDZ concentrations on seed culture became obvious during development of the seedlings. Four weeks after culture, the seedlings from MS0 developed 1 main shoot per seed with 2-3 nodes whereas those from TDZ-containing medium developed 12-15 shoots per seed, each shoot containing 1 to 2 nodes. There was no significant difference in number of shoots between the different TDZ treatments.

Table 1. Influence of the seed orientation on shoot number in multiple shoot clusters

Seed orientation	Shoot number/seedling at different duration	
	4 weeks on MS4T	8 weeks on MS4T
Hilum up	17[b]	48[b]
Hilum down	80[a]	343[a]

[a,b]: values with same superscript letter in a column are not statistically different at $p<0.05$; 10 seeds were used per treatment

More shoots developed when the seedlings were subcultured as single shoot nodes and cotyledonary node as pieces on MS0.5BA medium. During the first 4 weeks of culture on MS0.5BA, the nodes derived from MS0 culture produced only 1 shoot per node, while those from TDZ-cultured seedlings produced 2-4 shoots/node.

Table 2. Effect of TDZ concentration on number of shoots produced.

TDZ (mg/l)	number of shoots isolated			
	4wksT+0wks BA	4wksT+4wks BA	4wksT+8wks BA	4wksT+12wksBA
MS2T	13[a]	42[a]	66[b]	97[b]
MS4T	16[a]	52[a]	139[a]	248[a]
MS8T	14[a]	34[a]	156[a]	240[a]
MS0	1[b]	2[b]	2[c]	2[c]

[abc]:values with same superscript letter in a column are not statistically different (p<0.05 LSD); data mean of 3 replicates (4 seeds per replicate).

The shoots developed from the buds in the cotyledonary nodes, shoot nodes, tendrils, stalks and new buds were continuously initiated. The total number of shoots per seedling ranged from 34-52 with no significant differences between the TDZ concentrations. Significant differences were observed after 8 and 12 weeks (**Figure 2**). The total shoot number from MS4T- and MS8T-cultured seedlings increased much more than that from MS2T treatment. The seed culture on MS4T for 4 weeks resulted in 248 shoots per seed after 12 weeks of culture on MS0.5BA medium (**Table 2**).

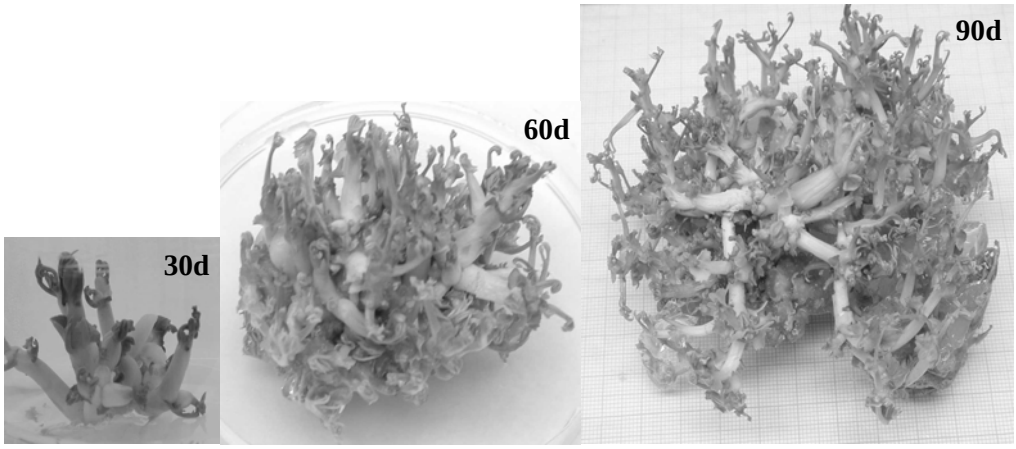

Figure 2. Effects of culture duration of pea seedling on medium supplemented with TDZ. 30d old seedling; 60d old seedling; 90 days old seedling

A) Effect of TDZ incubation duration on multiple shoot production

Also the duration of TDZ-culture influenced the number of shoots formed. Twelve weeks MS2T and MS4T resulted in higher numbers of shoots than 4 and 8 weeks preculture on TDZ. No significant differences between the different periods of preculture were observed on MS8T (**Table 3**).

Table 3. The effect of the concentration and duration of the TDZ treatment on the number of shoots produced per seedling after 16 weeks of culture.

duration of TDZ culture	number of shoots isolated		
	MS2T	MS4T	MS8T
4 weeksT+12 weeks BA	97[b]	248[b]	240[a]
8 weeksT+8 weeks BA	133[b]	277[b]	286[a]
12 weeksT+4 weeks BA	279[a]	417[a]	286[a]

[ab]:values with same superscript letter in a column are not statistically different (p<0.05 LSD); data mean of 3 replicates (4 seeds per replicate)

The buds initiated from the cotyledonary nodes and the shoot nodes developed into shoots, and more new buds were continuously produced. Multiple buds and shoots were also produced from secondary shoot nodes.

The 4 and 8 weeks seed culture gave the best results when TDZ in a concentration of 4 or 8 mg/l was used. In the 12 weeks seed culture the best results were obtained when 4 mg/l TDZ was used. In the latter treatment the highest number of shoots per seedling was obtained.

B) Bud containing tissue formation and multiplication

Highly active bud containing tissues (BCT) were observed at the cotyledonary nodes or the shoot nodes. Depending on the concentration and the duration of the TDZ treatment, the size of the BCT ranged from 2- 50mm^2. Larger BCT were formed on seedlings cultured on MS4T and MS8T compared to MS2T. Seed culture on MS4T or MS8T for 8 or 12 weeks resulted in larger BCT than seed culture on MS2T. The buds were mostly arranged in clusters, attached to a hard type of tissue, light green of color. The BCT was isolated and cultured for 16 weeks on MS4T. Then to test the optimal concentration for maintenance, small pieces of BCT (10 mm^2) were cultured on MS2T, 4T and 8T medium. On all three media the fresh weight increased 4-6 times every 3 weeks of culture. However, the morphology of the BCT varied with the concentration of TDZ used. On MS2T, the color remained unchanged and the buds developed into short shoots (about 0.2-1cm). The tissue was maintained as “shooty” when BCT were cultured on MS2T (**Figure 3A**). On MS4T, the tissue was maintained as pure BCT of which a few buds developed into shoots (**Figure 3B**). After 2-4 subcultures the buds were distributed more uniformly over the surface of the explants. On MS8T, the buds became smaller and some non-regenerable compact callus was formed on the base of the BCT. Based on the above results MS4T was selected as the best medium for BCT maintenance.

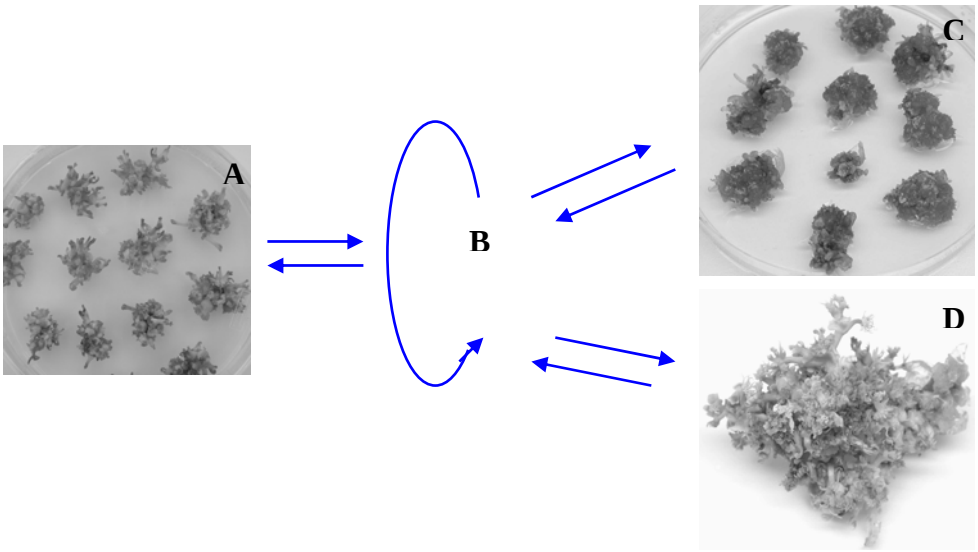

Figure 3. Effects of various concentration of TDZ on BCT culture A. BCT was maintained at 'shooty' stage culture on MS2T; B. BCT maintenance on MS4T; C. excess callus outgrowth in BCT when repeated cultured on MS8T; D. BCT was cultured on MS4T for 16 weeks without refreshing medium and subculture.

Regeneration of shoots from BCT was accomplished when the BCT was cultured without refreshment on MS4T medium. During the first 4 weeks the BCT proliferated. Then shoots were formed from the BCT. Every 3-4 weeks the shoots were isolated from the explants and either cultured on MS0.5B or used for rooting experiments. About 700 shoots/g fresh BCT with a length of 0.5-1 cm were obtained after 16 weeks of culture (**Figure 3D**).

C) Rooting of shoots derived from multiple shoot cultures or from BCT

Shoots of about 2 cm having 2-3 internodes, derived from multiple shoots cultures or BCT cultures, were transferred to different rooting media (**Table 4**). Most shoots started rooting within 2 weeks on all tested media. Combination of NAA and IBA resulted in better rooting efficiency than MS0 and MS supplemented with 1.5 mg/l IAA and 0.5 mg/l KN. 50-100% of the shoots developed roots within 4 weeks on medium supplemented with NAA and IBA. The rooted shoots also produced 1-8 lateral roots per shoot. There was less lateral root formation on MS supplemented with IAA+KN. The shoots derived from seed cultures rooted at higher frequencies than the shoots isolated from BCT (**Table 4**).

Table 4. Rooting efficiency of shoots derived from BCT (maintained on MS4T) or shoots derived from TDZ-cultured seedling (MS8T 12weeks seedlings) on different medium after 4 weeks of culture

Rooting media	Shoots from BCT		Shoots from TDZ-cultured seedlings	
	plants with roots	# lateral roots/shoot	plants with roots	# lateral roots/shoot
MS 2NAA+3IBA	70%	3.4	100%	8.5
MS 2NAA+2IBA	75%	3.4	100%	6.5
MS 1NAA+1IBA	60%	2.6	95%	5.4
MS1.5IAA+0.5KT	15%	1.0	30%	1.0
MS0	30%	2.5	5%	1.0

data mean of 2 replicates (10 shoots per replicate) for each treatment

Rooted plants derived from multiple shoot cultures and from BCT cultures have been transferred to greenhouse. The plants who survived the transfer to the greenhouse were very small, however, they all set seed. The plants of the S1 seeds were of normal size comparable with control plants derived from seeds.

Discussion

Culture of seed on medium containing TDZ or BA has been used in mungbean (Avenido and Hattori, 2001), common bean (Cruz et al. 2001), wild groundnut (Gagliardi and Pacheco 2000), chickpea (Jayanand 2003), cowpea (Le Bui 2002), barley (Ganeshan 2003) and switchgrass (Gupta and Conger 1998). In legumes such as chickpea (Jayanand 2003) and common bean (Malik and Saxena, 1992b), long periods of culture or culture on high concentrations of TDZ resulted in seedling deformation, necrosis, browning and subsequently death of the seed(ling)s. The optimal concentration of TDZ for multiple shoot induction in the above mentioned species ranged from 0.1-4 mg/l. In the case of pea culture for a long period of time on medium with a high TDZ concentration (12 weeks on 8 mg/l TDZ) does not affect the germination of seeds and the vitality of the seedlings. In the best treatment (culture of seeds on MS4T for 12 weeks) more than 400 shoots were isolated from one seed, together with about 0.5 gram of BCT/seedling. This BCT can produce about 350 when transferred for 12 weeks to MS0.5BA. Such high multiplication rates have not been reported before in pea.

Malik et al. (1992b) hypothesed that the high regeneration potential of seeds is caused by the physiological structural integrity of the explant. However, as it is shown for pea in Chapter 3 and soybean (Shan et al. 2005), huge amounts of shoots can be regenerated from BCT indicating that structural integrity of meristems rather than that of the intact explant might be essential for a high regeneration potential.

The effect of the seed orientation on the regeneration of multiple shoot clusters in pea has not been reported before. Much more shoots were formed when the hilum of the seeds was in contact with the medium than when the cotyledons were in contact with the medium. The effect of the explant orientation in organogenesis or embryogenesis has been reported before. In cassava (Stamp and Henshaw 1987) and soybean (Finer and Nagasawa 1988) somatic embryos are only formed if the abaxial side of the explants is in contact with the medium. In Venus fly-trap (*Dionaea muscipula*) and Troyer Citrange, the leaf or epicotyl

segment orientation was also found to influence the efficiency of organogenesis (Teng 1999; Garcia et al. 1999). The reason for this might be the fact that different hormone gradients are established in explants leading to different developmental programs.

TDZ is known to be associated with poor shoot and root development, especially when used in high concentrations (Murthy et al. 1998). To minimize these negative effects, protocols were developed with an as low as possible concentration of and an as short as possible exposure to TDZ (Fratini and Ruiz 2003). In pea, it was observed that shoot development can be simply achieved by continuous culture of explants without refreshment on TDZ-containing media. The shoots derived from seed cultures or BCT rooted well on the media that contained high levels of auxins.

An interesting observation was the development of clusters of buds on tendrils, stipules and stalks. This has not been described before in pea except for the formation of the multiple buds on stalk (Malik et al. 1992a). When attached to the plant, shoots develop from the buds and new buds were initiated. This phenomenon occurred frequently when seedlings were exposed to high levels of TDZ or long duration, implying that organogenesis can also be induced from tissues without meristems in pea. However, when tendrils, stipules and stalks were isolated from the seedlings and cultured separately on TDZ-containing media, no regeneration was observed. The reason for this difference requires further investigation.

Seven other pea genotypes (Solara, Classic, Puget, Finer, PCD2, AR6 and A12311) were also tested in the seed culture system described here. All of them gave 100% multiple shoot regeneration response. However, depending on the genotypes, the optimal TDZ concentrations for multiple shoot production varied from 2-14 mg/l (data not shown). The optimal TDZ concentration can easily be determined by the growth characteristics of the seedling. When TDZ concentration was below the optimal one, the shoots grew fast, the tendrils and stipules were unfolded and no BCT was formed at the cotyledonary node. When the TDZ concentration was near the optimum, tendrils and stipules were folded and BCT was formed at the cotyledonary node. When the TDZ was too high, only a few shoots developed, the cotyledonary node became swollen with abundant formation of non-regenerative compact callus. A similar relation between concentration of TDZ and morphogenesis has been observed in soybean multiple bud tissue culturing (Shan et al. 2005).

The current systems for genetic modification in pea are all based on using meristems of germinated seeds as starting material (Schroeder et al. 1993; Grant et al. 1995; Bean et al. 1997). After infection with *Agrobacterium tumefaciens* the germinated seeds are cultured on a medium with BA where they form multiple shoots clusters of 4-15 shoots. These systems are not very efficient in producing genetic modified plants which is caused by the fact that meristems are highly complex, multicellular structures. If in above systems BA is replaced by TDZ, much higher number of shoots can be obtained and this might increase the efficiency of transformation. Maybe more important than the high number of shoots is the BCT formation. The BCT obtained from seed-culture was identical to that from shoot-node culture (Chapter 3). In the culture system described here BCT is formed in a shorter period of time without laborious subculture regimes. After

isolation of BCT from TDZ-cultured seedlings and subculture them on TDZ-containing medium a pure BCT is obtained which behaves identical as the BCT cultures described (Chapter 3). The formation of multiple bud containing tissue has also been described in soybean (Shan et al. 2005) and observed in our laboratory in other legumes such as peanut and common bean (unpublished results). This might imply that BCT formation is a common response of legumes to TDZ. Most legumes use a genetic modification protocol similar to pea with the same problems as in pea. Part of these problems might be solved when the TDZ induced multiple shoot system of the BCT system is used as target tissue for genetic modification.

Acknowledgements

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5. Transformation of pea (*Pisum sativum* L.) using a cyclic organogenic system

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Abstract

Transgenic pea (*Pisum sativum* L.) plants have been obtained after co-cultivation of bud containing tissue (BCT) and shooty BCT with *Agrobacterium tumefaciens* strain AGL0(pG49A). The binary vector pG49A contained an interrupted inverted repeat of a *legumin* A gene, flanked by the promoter of the *trypsin/chymotrypsin inhibitor* gene, together with the *luciferase* gene for selection of transgenic tissue. Luciferase positive tissue was identified, isolated, and subcultured on TDZ-supplemented medium. On this medium, BCT can be multiplied, and the shooty BCT will become pure BCT again. The luciferase based selection procedure was repeated until (almost) completely luciferase positive BCT cultures were obtained. From 31 of these BCT lines, 25 yielded plants. Luciferase positive plants were obtained in all 25 lines. Plants (S_0) of 23 lines were grown in the greenhouse. The S_0 plants were smaller in size and produced less seeds than the control plants. All lines produced luciferase positive seeds. The transgenic nature of 5 S_0 plants was further confirmed using Southern blot analysis. Protein analysis of the seeds of 8 lines suggested that protein composition was altered. Seeds of 6 lines were grown to produce S_1. The S_1 plants were comparable in height to control plants. However, the number of seeds per plants was significantly lower.

Keywords: Pea; TDZ; transformation; BCT; organogenesis; luciferase

Introduction

Pea (*Pisum sativum* L.) is grown in the moderate climates of Northern Europe and America. Its economical importance remained stable in recent years, despite strong competition from other protein-rich crops such as soybean (Linnemann and Dijkstra 2002; Casey 2003). To increase its economical importance, pea has to be improved in, for example, resistance to pathogens, competitiveness against weeds, protein content and protein composition. Because the genetic variation of pea for these traits is limited, biotechnology is necessary to accomplish these improvements (Polowick et al. 2000). For this, a reliable and efficient transformation system is required. Puonti-Kaerlas et al. (1990) and De Kathen and Jacobsen (1990) were the first who transformed pea. They co-cultivated shoots and epicotyls of seedlings with *Agrobacterium tumefaciens*, followed by shoot regeneration on medium supplemented with cytokinins and (optionally) auxins. Problems, such as loss of transgenic traits in subsequent generations, polyploidisation and sterility were reported. In principle, the same method of regeneration was used by others, using immature seeds (Nadolska-Orczyk and Orczyk, 2000; Pniewski and Kapusta, 2005), embryonic axises (Schroeder et al. 1993; Polowick et al. 2000), cotyledons (Grant et al. 1995, 1998) or cotyledonary nodes (Davies et al. 1993; Bean et al. 1997). In general, 6-9 months were required from inoculation of the explants to seed set in primary transformed plants. The efficiency was between 1.5-7%, calculated as number of transgenic plants per number of initial explants used. Recently, Grant et al. (2003) reported that the use of hypervirulent *Agrobacterium* strains, as for example KYRT1 (Torisky et al. 1997), and a proper co-cultivation temperature increased the transformation efficiency fourfold compared to the commonly used strain for transformation AGL1.

However, in all cases the transformation procedure resulted in a high number of ‘escapes’ or only partly transformed plants, which did not transmit the transgene to the next seed generation (Davies et al. 1993; Bean et al. 1997; Grant et al. 2003). Grant et al. (2003) concluded that despite the progress that has been made in pea transformation, the available systems are still low in efficiency and not routinely reproducible. The limitations of the currently available transformation systems are associated with the explant source and the regeneration systems used. In all systems, most of the cells of the explants used as target tissue for genetic modification are not capable of regeneration, and in most cases the plants are regenerated from existing meristems. The existing meristems are multicellular structures and regeneration of plants is based on direct outgrowth of the existing meristems into plants. This explains why a large amount of the plants obtained in transformation experiments are chimeric or escapes. The low efficiency of the current genetic modification protocols for pea limits their use in commercial crop production (Welham and Domoney 2000). For commercialization, it is necessary to develop a reliable transformation protocol, which will allow the production of sufficient transgenic lines in a reasonable time span and with acceptable labor input.

In Chapter 3, a new regeneration system for pea is described. So-called bud containing tissue (BCT) is formed after repeated subculture of cuttings on Thidiazuron-supplemented medium. The BCT can be multiplied repeatedly, and it contains much higher numbers of meristems than the previously used explants

for genetic modification. Another difference is the fact that the meristems of BCT do not develop directly into plants, but instead initiate new meristems. This chapter describes the use of BCT for genetic modification of pea.

Pea seeds are rich in storage proteins, mainly the so-called globulins, of which legumin, vicilin, and convicilin are the representative classes (Casey et al. 2003). Globulins are used in the food industry in various applications. Peas with a specific globulin composition, for instance enriched in particular legumin and vicilin isoforms, or lacking convicilin, would be desirable as a raw material for the food industry (Casey and Domoney 1999; O'Kane et al. 2004b). In our investigation, we have not found pea lines lacking any particular globulin (Chapter 2). The absence of such lines can be explained by the fact that globulins are encoded by multigene families (Domoney and Casey 1985). It might be possible to obtain pea lines with a more desirable protein composition with genetic modification. In this study, we have chosen to down-regulate the amount of legumin A, the more genetically diverse isoform among pea legumins (Casey and Domoney 1999), in order to provide proof of concept for modifying pea seed storage protein composition by genetic modification. At the time that our investigation started, it was not clear which of the storage proteins of pea should be reduced in amount for specific technological applications. The choice for the *leg*A gene was directed by its availability to us, rather than by specific technological benefits. To achieve efficient down-regulation of the gene expression, we have based our gene silencing construct on the method of dsRNA-directed ssRNA-cleavage via interrupted inverted repeats (IIR) constructs (Mette et al. 2000; Baulcombe 2004). The IIR construct was driven by the TI promoter, which has a similar expression pattern as the legumin gene family. For plant transformation, the AGL0 *Agrobacterium tumefaciens* strain was used, as it is known to be efficient, due to its virulence, for species recalcitrant to transformation like pea.

Materials and methods

Media and growing conditions

Unless stated otherwise, the basis of the media contained B_5 (Gamborg et al. 1968) salts and vitamins, 20 g/l sucrose and 7.5 g/l micro agar ($B_5$2). The pH was adjusted to 5.7 prior to autoclaving (121 °C for 15 min). All growth regulators were added filter-sterilized to the autoclaved medium. Media were dispensed into 9-cm petri dishes (25 ml) or into 9-cm jars (310 ml). All cultures were incubated in a growth chamber with a temperature of 24 °C, a photoperiod of 16 h and an irradiance of 40 μmol m^{-2} s^{-1}.

Explants used for transformation

Bud containing tissue (BCT) was induced as described in Chapter 3 from the variety Espace (kindly provided by Cebeco Zaden B.V. (The Netherlands). Three different types of explants (A, B, and C) were used for genetic modification. Type A explants were obtained by multiplying, every 4 weeks, 2-5 mm slices of BCT in $B_5$2 medium, supplemented with 2.2 mg/l of TDZ. Type B and C explants were produced by culturing BCT for 3 weeks on a medium which stimulates the

development of shoots. B and C explants differ in the medium used for shoot development, i.e. $B_5$2 medium supplemented with 1 mg/l BAP, 1 mg/l GA_3, 1 mg/l IBA, and $B_5$2 medium supplemented with 1 mg/l GA_3, respectively.

Agrobacterium tumefaciens strain and construct

Standard molecular biology techniques were used for DNA manipulations (Sambrook et al. 1989). *Escherichia coli* stain DH5α (Invitrogen, Carlsbad, CA, USA.) served as a host for plasmid amplifications. *Agrobacterium tumefaciens* strain AGL0 (Lazo et al. 1991) containing the binary vector pG49A was used for transformation.

The pG49A contains the *luc* gene flanked by the 35S CaMV promoter and terminator, and the *TI*-*leg*AIIR-*nos* chimeric gene (**Figure 1**). The *TI*-*leg*AIIR-*nos* chimeric gene contains an interrupted inverted repeat (IIR) of a 438 bp and a 335 bp section of the *leg*A gene (obtained by PCR amplification), flanked by the promoter from the *TI* gene (clone *TI*1, AJ276900 encoding trypsin/chymotrypsin inhibitors (Welham and Domoney, 2000)) and a *nos* terminator. The *TI*-*leg*AIIR-*nos* fragment was made as follows. A *TI*1 trypsin inhibitor clone with promoter and gene (AJ276900) was kindly provided by Dr. Casey from the John Innes Center (Norwich, UK). *TI*1 was excised from the pUC19 plasmid with *Cla*I/*Hind*III, and cloned in a pBluescript® II SK cloning vector from Stratagene (La Jolla, CA, USA). The open reading frame of the *TI*1 gene was excised by *Bgl*II and *Eco*RV, and only the promoter fragment of about 1 kb remained in the resulting linearized plasmid. Subsequently, the *Bgl*II sticky end was blunded by Klenow polymerase treatment, after which the vector DNA was circularized by blunt-end ligation using T4 DNA ligase from Stratagene, resulting in the pTI plasmid. In this plasmid, a *nos* terminator was cloned in the *Spe*I and *Not*I restriction sites, resulting in the plasmid p*TI*-*nos*.

The constituent fragments of the IIR region were obtained by PCR amplification, using the pea RC924 *leg*A clone (AJ132614; kindly provided by Dr. Casey) as a template. Two fragments were obtained after PCR amplification, using the following primers: legAF1 (5' TAGGATCCGCACTTTCTCTTTCATTC 3') and legAR1 (5' ATAAGCTTCATCCAAAATACAATACC 3') for the sense part of the IIR, and legAF1 and legAR3 (5' ATAAGCTTGATTCTTGTGGCTCTTC 3') for the antisense part of the IIR. The two fragments are the same except for the extra 102 bp fragment in the longer one (which acts as the spacer in the construct). *BamH*I and *Hind*III restriction sites were inserted in both fragments during the PCR amplification for further use in the cloning procedure. Fragments obtained by PCR were subcloned into the pGEM®-T Easy and pGEM®-T cloning vectors, obtained from Promega (Madison, WI, USA). Subsequently, the two fragments were excised by *Hind*III/*EcoR*I (of the pGEM®-T Easy vector) and *BamH*I/*Hind*III (of the pGEM®-T vector) digestion, and subcloned into pUC19 (New England Biolabs, Inc., Beverly, MA, USA) in the *BamH*I/*EcoR*I restriction sites by three-point ligation, resulting in the plegAIIR plasmid. The *leg*AIIR fragment was excised with *BamH*I and subcloned in the *BamH*I site of the p*TI*-*nos* plasmid resulting in the plasmid p*TI*-*leg*AIIR-*nos*. Finally, the *TI*-*leg*AIIR-*nos* fragment was excised with *Kpn*I and *Not*I and cloned into pGreen49 (Hellens et al. 2000), resulting in the pG49A binary vector (**Figure 1**). The pG49A was introduced into *Agrobacterium tumefaciens* strain AGL0 by electroporation, which was grown in liquid Luria broth medium containing 20 mg/l tetracycline and 100 mg/l ampicilin at 30 °C. After two days of culture the bacterial suspension was centrifuged, and the bacterial pellet was dissolved in liquid Luria broth medium to a density of 0.3 ($OD_{550\ nm}$).

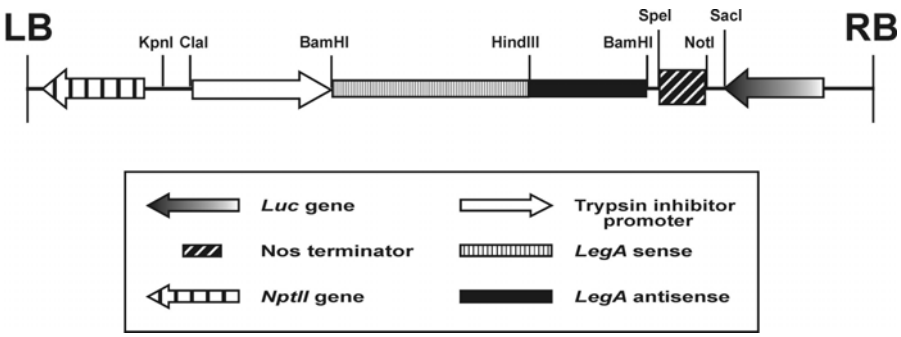

Figure 1. The pG49A plasmid carried the *TI-legA*IIR-*nos* sequence for silencing of the *legumin A* gene. *TI-legA*IIR-*nos* consisted of an inverted repeat, made by two PCR fragments of the *legumin* A gene (AJ132614), flanked by the promoter of the *TI1* gene (AJ276900) and the *nos* terminator. Selection of transgenic tissue was based on the activity of the enzyme of the *luciferase* gene. LB and RB indicate left and right border, respectively.

Transformation procedure

Type A, B and C explants were either sliced in pieces of 4-5 mm 1 day prior to transformation or left intact. Sliced and not sliced explants were dipped in the bacterial suspension for 10 seconds. Subsequently the explants were dried using filter paper and cultured on $B_5$2 medium containing 4.4 mg/l TDZ. After 2 days the explants were washed with liquid $B_5$2 medium supplemented with 200 mg/l claforan and 4.4 mg/l TDZ and cultured for 3 days in the same medium on a rotary shaker, after which they were transferred to solid $B_5$2 medium containing 4.4 mg/l TDZ and 200 mg/l claforan.

Measurement of luciferase activity in plant tissue

Petri dishes with explants were sprayed with 0.25 mg ml^{-1} luciferin (Promega, E160). Luciferase activity was determined using a VIM intensified CD camera and an Argus-50 photon counting image processor (Hamamatsu Photonic Systems).

Production of transgenic lines and plant regeneration

The first luciferase assay was done 4 weeks after transformation. Luciferase positive explants were identified, cut in pieces of 5–10 mm^2 and subcultured as a line for multiplication on $B_5$2 medium supplemented with 4.4 mg/l TDZ and 200 mg/l claforan. This procedure was repeated every 4 weeks until (almost) completely luciferase positive BCT lines were obtained. Subsequently the luciferase positive BCT explants were cultured on $B_5$2 supplemented with 1 mg/l GA_3 and BAP for regeneration of shoots. Luciferase positive shoots with a size of 2-6 cm were transferred to $B_5$2 supplemented with 0.5 mg/l NAA for rooting.

Transfer of luciferase positive plants to greenhouse and production of seeds

In vitro plants, with or without roots, produced as described above, were planted in pots containing standard potting soil and maintained under constant temperature of 18 °C and relative moisture of 100% for further development. Plants without roots were covered with plastic pots to reduce water evaporation prior to root formation. Plants produced

seeds which were harvested dry and were used for determination of luciferase activity or protein content or to produce S_1 generation plants.

Determination of the protein composition of the transgenic lines

S_1 seeds from the transgenic lines 1 to 8 were analyzed. From each line, three to four mature seeds were peeled and ground with a mortar and pestle at room temperature until a fine powder was obtained. Total pea protein extract was prepared by stirring 25 mg of ground pea seed in 1.5 mL of 0.1 M Tris HCl buffer pH 8 for 1 h at 30 r.p.m. at room temperature. Subsequently, the samples were centrifuged at 1,500 × *g* for 7 min at 20 °C to precipitate insoluble material. The protein composition of the supernatant, referred to as total pea protein extract (TPPE) was determined in triplicate by SDS-PAGE on a Mini-Protean II electrophoresis system from Bio-Rad Labs (Hercules, CA). Five hundred µL of TPPE was diluted 1+1 (v/v) in sample buffer, consisting of 0.02 M Tris-HCl buffer (pH 8), 2 mM EDTA, 20 % glycerol, 2 % SDS, and 0.002 % Broomphenol Blue. The samples, sealed in 1.5 mL tubes, were heated in boiling water for 5 min, and proteins were separated using 12% Tris-HCl polyacrylamide Ready Gels from Bio-Rad Labs (Hercules, CA). 20 µL of sample was loaded into each well. The gels were run at a constant voltage of 200 V in a buffer solution of 0.025 M tris, 0.19 M glycine, and 0.1% SDS (pH 8.3), and calibrated with a molecular mass marker (10-250 kDa from Bio-Rad Labs). Subsequently, the gels were stained as described in Chapter 2. The bands were assigned to legumin based on relative motilities on SDS-PAGE gels, with and with out β-mercaptoethanol, as described in Chapter 2.

Southern blot analysis

DNA manipulations were conducted using standard procedures as described by Sambrook et al. (1989), unless specified otherwise. Genomic DNA from young leaves of S_0 plants showing luciferase expression was extracted according to the CTAB method of Rogers and Bendich (1994). For Southern blot analysis, extracted DNA was digested with restriction endonouclease (*Kpn*I) the products were resolved by electrophoresis through 1% agarose gel (4 µg/lane) and transferred onto a nylon membrane (Hybond N^+, Amersham). The membrane was pre-hybridized for 3 h at 65 °C in modified Church buffer. For hybridization, the luciferase gene of plasmid pGreen49 excised by *Bgl*II-*Sac*I was used as probe. Hybridization was carried out for 16 h at 65 °C in modified Church buffer with a *luc* gene labeled with [α-^{32}P] dCTP by random primed labeling using the Rediprime II kit (Amersham Pharmacia Biotech). The blot was washed at 65 °C for 15 min each time, the two first times in 2× SSC, 0,1% SDS and finally in 1× SSC, 0,1% SDS. The radioactively labeled blots were incubated with a X OMAT S and AR films (Kodak) and after 2 days they were developed.

Results

Sequence analysis of legumin genes

Database searches at http://www.ncbi.nlm.nih.gov/ revealed 25 legumin accessions, of which one *leg*A (X02982), one *leg*J (X07014), one *leg*S (X67424) were full length, i.e. there was one full length representative of each legumin family (Casey and Domoney 1999). The sequence comparisons showed that the similarities are 42.4% between the *leg*A and *leg*J genes, and 42.6% between the *leg*A and *leg*J genes. **Figure 2** shows the alignment of these three genes for the part of the *leg*A that was used for the preparation of the IIR construct.

Figure 2. Sequence alignment showing the sequence divergence between representatives of the pea legumin gene subfamilies. The following nucleotide sequences were used: *leg*A (X02982), *leg*J (X07014), and *leg*S (X67424). The alignment only shows the part of *leg*A (nucleotide 16→350), which was used for the assembly of the IIR construct. The MegAlign program from the DNA-Star package was used to align the sequences according to the Clustal method using a PAM250 weight table, and gap penalties and gap-length penalties of 10.

The alignment showed that *leg*A is the most divergent of the three, and that *leg*J and *leg*S are more homologous. This suggests that the IIR construct is *leg*A-specific, and that it is unlikely that the level of *leg*J and *leg*S expression will be reduced, as there are no stretches of 23 identical nucleotides with the latter two, which are necessary to exert a gene silencing effect (Mette et al. 2000).

Production of transgenic lines

Type A, B, and C explants (**Figure 3**), either cut in pieces or left intact, were infected with the *Agrobacterium* strain AGL0(pG49A). Four weeks after infection, 25% to 100% of the explants were luciferase (*luc*) positive (**Table 1**). About 50% of the *luc* positive explants contained more than one luc

positive sector. Luc positive explants were fragmented, and subcultured on $B_5$2 medium, supplemented with 4.4 mg/l TDZ.

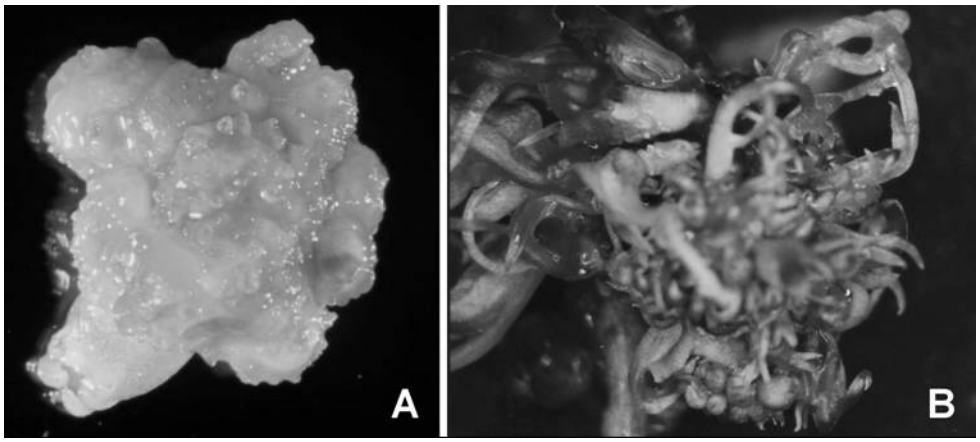

Figure 3. Three types of explants, were used for transformation of pea (*Pisum sativum* L.). **A**, explant type A (pure BCT); **B**, explant types B and C, which were phenotypically similar, where BCT covered with developing shoots.

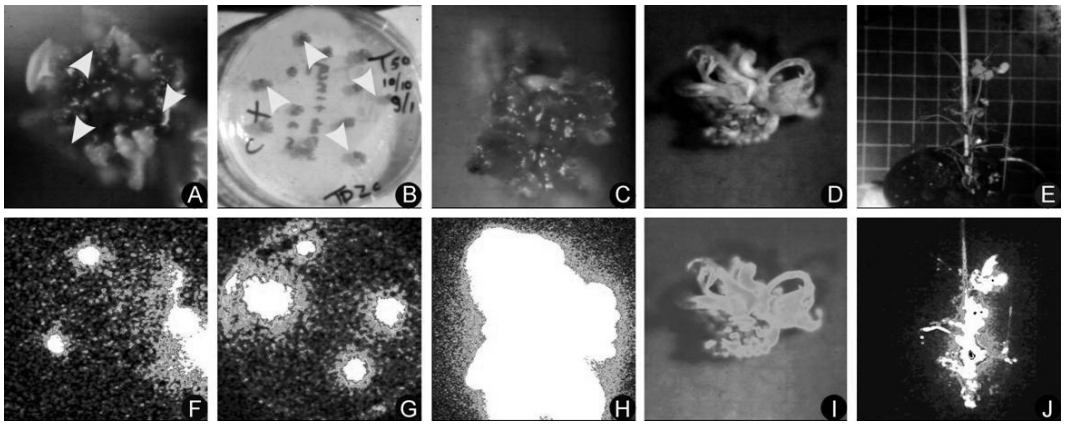

Figure 4. Transformation of bud containing callus (BCT) pea (*Pisum sativum* L.) by *Agrobacterium tumefaciens* using luciferase activity for selection of transgenic plants. The upper panel (A-E) shows photographic images of tissue subjected to transformation and regeneration of plants, whereas the lower panel (F-J) shows images of the luciferase activity of the same explants. **A/F**: partial luciferase positive BCT. **B/G**: excision and subculture of transgenic sectors of explants from A/F, on medium containing TDZ. This resulted in a number of BCT explants, a few of which are chimeric (indicated by the arrow heads), although they had larger transgenic sectors than the explants of which they were excised. **C**/**H**: repetition of the procedure from step B/G resulted in the production of fully transgenic BCT explants. **D/I**: explants obtained from C/H formed transgenic shoots after subculture on media containing GA_3. **E/J**: transgenic shoots were transferred to the greenhouse and formed roots, resulting in completely transgenic plants.

The pieces derived from one explant were cultured as one line, even if the original explant contained more than one luc positive sector (see **Figure 4**). Four weeks later the cultured lines were again assayed for

luc activity. This procedure was repeated until (almost) complete *luc* positive BCT was obtained (purification of transgenic sectors) (**Figures 4 C,H**). The number of *luc* positive lines decreased the first 4-6 subcultures, and after that remained stable (**Table 1**).

In about 75% of the cases *luc* activity disappeared because of loss of transgenic cells. In the other cases, the explants either became contaminated with residual *Agrobacterium*, or the BCT tissue died for unknown reasons. Significant differences were observed between explant type used, and intact explants versus explants cut one day before transformation.

Table 1. Production of luciferase positive lines in pea (*Pisum sativum* L.) via transformation of BCT and BCT with shoots with *Agrobacterium tumefaciens* strain AGL0(pG49A).

	Explant			**# of luc$^+$ lines** (% of luc$^+$ explants between brackets)					
Exp[a]	**Type**[b]	**#**	**Cut**[c]	**4 w**[d,e]	**7 w**	**10 w**	**14 w**	**18 w**	**22 w**
1	**A**	106	No	26 (25)	4 (4)	1 (1)	1 (1)	1 (1)	1 (1)
	A	150	Yes	63 (42)	16 (11)	9 (6)	5 (3)	4 (3)	1 (1)
	B	79	No	79 (100)	50 (63)	21 (27)	16 (20)	9 (11)	9 (11)
	B	156	Yes	125 (80)	40 (26)	14 (9)	7 (4)	2 (1)	2 (1)
	C	76	No	70 (92)	50 (66)	42 (55)	35 (46)	20 (26)	10 (13)
	C	136	Yes	39 (29)	19 (12)	6 (4)	5 (4)	3 (2)	3 (2)
2	C	627	No	226 (36)	128 (29)	100 (16)	85 (14)	32 (5)	32 (5)

[a] Experiment.
[b] Type of explant. A: pure BCT; B: shooty BCT obtained by culturing BCT for 3 weeks on $B_5$2 medium, supplemented with 1 mg/l BAP, 1 mg/l GA_3, and 1 mg/l IBA; C: shooty BCT obtained by culturing BCT for 3 weeks on $B_5$2 medium, supplemented with 1 mg/l GA_3.
[c] explants were cut in pieces of 4-5 mm, one day prior to transformation, or left intact.
[d] Weeks after infection.
[e] From this time point onwards, luciferase positive (luc$^+$) explants were cultured separately as independent lines.

Twenty-two weeks after transformation 0.6%, 1.2%, and 2.2% of the initially infected cut explants resulted in *luc* positive BCT lines (type A, B, and C, respectively). For the intact pieces this was 0.9%, 11.3% and 13.1%, respectively. In total, 58 *luc* positive BCT lines were obtained. The lines differed in the amount of *luc* positive tissue. In 45 lines between 50-90% of the tissue was *luc* positive. These lines were cultured for regeneration. In the other lines less of the tissue was *luc* positive and these lines were not used for plant regeneration.

Regeneration of plants from luciferase positive lines

The 45 luciferase positive BCT lines were cultured for plant regeneration on $B_5$2 medium supplemented with 1 mg/l GA_3 and 1 mg/l BAP. The lines which were not completely *luc* positive were still subjected to *luc* based selection, until the line was completely *luc* positive. The first shoots appeared after 6 weeks of culture. More than 700 shoots were isolated during a 5 months culture period. During the process of plant regeneration, 14 lines were lost, either because of bacterial infection, or because the tissue died. Plants were obtained from 25 of the 31 remaining lines. In first instance, the shoots were highly vitrified. However, when isolated shoots were cultured on $B_5$2 medium supplemented with 0.5 mg/l NAA, a large part of the

plants lost its vitrified character and continued growing. Plants higher than 2 cm, consisting of an apical meristem and one extra node, were assayed for luc activity. The number of plants assayed for luc activity varied from less than 5 for the lines 14 and 23 to more than 30 for the lines 3 and 12 (**Table 2**). Eighty eight percent of the 450 analyzed plants were luciferase positive, and all lines yielded at least one luciferase positive plant.

Twenty percent of the plants had formed roots. Most of the plants had less than 2 roots; mostly very small, sometimes longer than 5 cm. All the plants, including the luciferase negative ones and the ones without roots, were transferred to the greenhouse. The overall survival rate was 29%. Acclimatized plants were obtained from all lines, except from the lines 14 and 19. Rooted plants had a higher survival rate than non rooted plants, 19% versus 48%. The first flowers appeared 3-4 months after transfer to the greenhouse. The height of the plants varied from 10.5 cm for line 16 to 49 cm for line 22. The control had a length of 50 cm.

Almost all transgenic plants formed pods. The number of pods per plant varied from less than 1 for the lines 10 and 15 to more than 20 for line 21. The control had formed 2.6 pods per plants. The number of seeds per pot varied from 0.5 for line 21 to 1.8 for the lines 1 and 10. The control plants formed 4.1 seeds per pot. Most of the seeds were stored at 4ºC and measured for luciferase activity 5-8 months later. All the seeds of 1-3 pods of in total 57 plants derived from 17 luciferase positive lines were measured for luciferase activity. In most lines, all the plants had produced luciferase positive seeds. Only in the lines 16, 18 and 24 a few plants did not contain luciferase positive seeds. Because, not all the seeds became swollen when incubated in the luciferin solution and this is required for activation of the luciferase gene, it is difficult to say something about the segregation of the *luc* gene in the different independent transformed plants.

S_1 plants have been grown from luciferase positive seeds from the lines 1, 2, 3, 6, 7 and 12. The results are shown in **Table 2**. The length of the plants of all six lines was comparable with that of the control plants, and much higher than that of the corresponding S_0 plants. The number of branches per S_1 plant was in some lines higher, and in some lines lower, than in the corresponding S_0 plants. This was also the case for the number of pods per plants. In all the lines the number of seeds per pod was higher than in the corresponding S_0 plants, but lower than in the control plants.

Table 2. Regeneration of plants from luciferase positive BCT cultures and description of their growth in the greenhouse.

Plant line	# *in vitro* plants	# luc$^+$ plants	Surv[a] green house	S_0 plants in the greenhouse					S_1 plants in the greenhouse					
				Length (cm)	# bran[b]/ plant	# pods/ plant	# seeds/ pod	# plants with luc$^+$ S1 seeds	# of plants	Length (cm)	# bran/ plant	# pods/ plant	# seeds /pod	#plants with luc$^+$ S2 seeds
1	28	21	9	37.8	4.1	4.4	1.8	6/6	25	68.4	4.4	6.0	1.9	4/4
2	27	25	6	34.8	9.7	7.5	0.7	3/3	5	72.6	7.0	5.6	1.9	3/3
3	37	35	10	26.2	3.2	4.8	1.1	4/4	7	76.1	3.9	6.9	1.4	2/2
4	17	16	4	38.0	3.3	6.8	0.6	2/2						
5	19	19	9	nd	nd	2.2	0.2	2/2						
6	24	24	11	31.6	1.4	5.5	1.2	4/4	8	52.9	1.4	2.9	2.6	nd
7	15	10	2	14.0	1.5	2.5	1.4	nd	9	68.4	2.0	4.4	2.5	nd
8	7	5	3	31.6	2.0	4.7	1.6	2/2						
9	7	7	2	13.1	1.0	1.0	1.0	nd						
10	24	21	6	15.8	1.0	0.7	1.8	1/1						
11	15	12	1	20.0	4.0	4.0	0.9	nd						
12	32	31	16	36.7	2.8	12.5	0.7	4/4	20	62.3	2.7	5.9	1.1	2/3
13	13	13	2	21.0	3.0	2.0	1.5	nd						
14	4	2	0											
15	26	22	5	10.5	1.0	0.8	1.0	nd						
16	23	17	10	29.2	2.4	5.5	1.2	6/7						
17	18	12	2	22.0	1.0	5.5	1.2	nd						
18	19	19	3	20.5	2.3	2.0	1.1	1/2						
19	17	17	0											
20	23	18	10	20.0	1.0	6.7	0.7	4/4						
21	17	15	6	49.0	2.5	25.0	0.5	4/4						
22	20	17	4	38.3	6.0	17.7	0.7	4/4						
23	2	2	1	41.1	3.3	20.0	0.7	1/1						
24	8	6	4	33.0	3.5	7.2	1.4	3/5						
25	8	8	3	31.0	1.5	7.0	0.6	2/2						
Average	18	15.7	5.1	28.0	2.8	6.8	1.0	0.92	12.3	66.7	3.5	5.1	1.9	
Espace			3	50.0	1.3	2.6	4.1		6	58.3	4.0	2.0	3.3	0/6

[a] Surv. = survival.
[b] Bran. = branches

Southern blot analysis

Five lines of S_0 plants showing *luc* activity were used for Southern analysis using as a probe the *luc* gene from the pGreen49 binary vector. Pea transformation was confirmed by Southern blot analysis (**Figure 5**). As the *Bgl*II-*Sac*I restriction enzymes cut the binary vector pG49A only once, southern blot analysis revealed that all luciferase positive plants tested contained between 1-3 inserts of the *luc* gene.

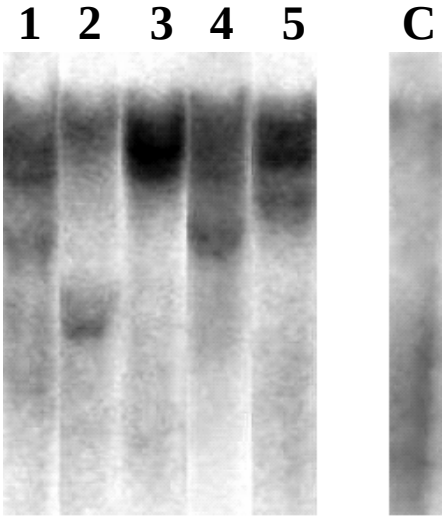

Figure 5. Southern blot analysis of transgenic pea (*Pisum sativum* L.) plants containing the *luc* gene. DNA from all the transformants was derived from shoots of *in vitro* plantlets. A total of 10 μg of DNA was applied per lane, blotted on to a hybond filter and hybridised with a ^{32}P-labelled 1.5-kb luciferase gene. C, control DNA from non-transgenic *in vitro* propagated Espace plants. Lanes 1–5 contain DNA from five different transformed lines.

Determination of the protein composition of the transgenic lines

Seeds of different transgenic pea lines were analyzed for modification in their protein composition. PAGE analysis without prior β-mercaptoethanol treatment did not reveal any differences between the seeds of transgenic and control lines (data not shown). **Figure 6** shows the protein compositional analysis of two representative samples after β-mercaptoethanol treatment. The acidic and basic subunits of legumin (before β-mercaptoethanol treatment connected by a disulfide bridge) are clearly visible at 35-43 kDa and 21-23 kDa, respectively.

The intensity of some bands in the gel differs between the transgenic and control seeds, whereas the intensity of other bands is unaffected. More in particular, the control seeds showed a band of low intensity around 22 kDa, which was more pronounced in the transgenic seeds (**Figure 6**: indicated by arrow head). Also, the transgenic seeds seem to have more of a ~37 kDa band. The other transgenic lines also showed more of the 22 and ~37 kDa bands, although in some lines this effect was not as pronounced. The various control samples were all identical to lane C. These results suggested that silencing of the *leg*A gene leads to

compositional changes in storage proteins, although it seems as if the total amount of legumin is up-regulated, rather than down-regulated, contrary to our expectations.

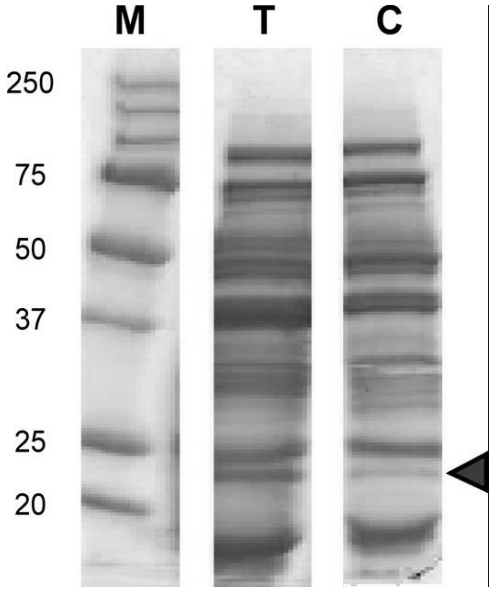

Figure 6. Polyacrylamide gel (12%) electrophoretic separation of selected protein extracts from (transgenic) pea lines. Prior to electrophoresis, the samples were subjected to treatment with β-mercaptoethanol, to cleave the disulfide bridges connecting the acidic and basic subunits of legumin. The triangle indicates one of the basic subunits of legumin. Lane M shows the marker, with the molecular weights indicated in kDa. Lane C represents the extract from an untransformed pea plant, whereas lane T represents the extract of a transgenic pea line.

Discussion

The results presented in this chapter show that the BCT system (Chapter 3) can be used for genetic modification of pea (*Pisum sativum* L.). BCT (explant type A) or shooty BCT (explant type B and C) were co-cultivated with *Agrobacterium tumefaciens* and cultured on medium with TDZ. On this medium BCT will be multiplied, whereas shooty BCT will revert gradually into pure BCT cultures. Transgenic BCT sectors were identified based on luciferase activity and subsequently physically excised from the explants and subcultured. This process was repeated until (almost) completely luciferase positive BCT was formed. After that the BCT was allowed to regenerate into plants using the regeneration system described in Chapter 3.

More transgenic lines were produced when shoot containing explants (B and C) were used as starting material than when type A explants were used. Fragmentation of the explants was expected to have a positive effect on transformation; however the transformation rate of cut explants was lower than of intact explants. This might be explained by the extra stress caused by the fragmentation of explants. However, if transformation efficiency is calculated as number of transgenic lines per amount of infected tissue instead of number of transgenic lines per number of infected explants, fragmentation seems to be as good as intact tissue.

In the here described procedure for genetic modification meristematic cells are the cells capable of plant regeneration and these cells should be targeted by the gene transfer vector. One can only speculate why type A explants are less efficient than type B and C explants in producing transgenic tissues. Previous studies

in petunia (Ulian et al. 1988), sunflower (Schrammeijer et al. 1990) and corn (Gould et al. 1991) have demonstrated that meristematic cells are not the optimal choice for transformation. This might be in agreement with the fact that BCT (type A) explants produce less transgenic plants than shooty BCT (type B and C) explants. BCT explants consist of small meristems, whereas shooty BCT consist of a mixture of shoots and buds. When shooty BCT are transferred to TDZ supplemented medium, they gradually convert to pure BCT cultures. As is shown in Chapter 3, BCT originated from the base of small shoots. The base of the small shoots contains axillary meristems and the BCT originated from cells surrounding the meristems. Maybe these surrounding cells, which are not yet organized in meristems, are responsible for the relatively high frequency of producing genetically modified plants.

The BCT system using explants B or C has an average transformation efficiency of 11%-13% of infected explants. This is higher than the average transformation efficiency of about 4% observed in previously published protocols, using seed derived explants (Bean et al. 1997; Polowick et al. 2000; Grant et al. 2003; Pniewski and Kapusta 2005). Another advantage is the fact that in the BCT system almost all transgenic shoots are completely transgenic, and are capable of transferring the transgenes through seeds. With the previous systems only a portion of transgenic explants are completely transgenic and the rest is chimeric of which a large part is sectorially transformed and not capable of transmitting the transgenes through seeds (Bean et al. 1997; Grant et al. 2003).

In the here described procedure of genetic modification, it takes 14 months from infection to primary transformants (S_0) with seeds. This is longer than in the previously described protocols, where it took 6-9 months (Bean et al. 1997; Polowick et al. 2000; Grant et al. 2003; Pniewski and Kapusta 2005). The time period needed to produce transgenic plants/seeds can be reduced significantly by shortening the luciferase based selection/purification period; however, as a consequence not all plants will be transgenic and most probably also some chimeric transformed plants will be formed. Most probably, also the use of selectable marker genes will reduce the period needed to produce transgenic plants.

In the future, a system, combining the reporter *luc* gene with a chemical selection gene, might prove to be more advantageous than the currently developed system, because it might combine the efficiency of the luciferase system with the reduced labor input of the chemical selection system.

Using a direct regeneration system from immature cotyledons Nadolska-Orczyk and Orczyk, (2000) produced transgenic peas with a efficiency ranking from 1% to 8.2% depending on the *Agrobacterium* strain used and high number of escapes. Nine months were needed to produce seed bearing transgenics plants. These authors mentioned that besides of the *Agrobacterium* strain, the addition in the growing media of the methylation inhibitor factor 5-azacytidine and the selection agent used were critical factors.)

The occurrence of somaclonal variation in genetically modified plants of pea has been reported before (Puonti-Kaerlas et al. 1990; De Kathen and Jacobsen 1990). The primary transformed plants (S_0) produced from BCT are very weak compared to control plants derived from seeds. The S_1 plants were more similar to the control plants than the S_0 plants, however, still not all completely identical to the control. All the S_1 plants

had a lower number of pods per seed than the control plants. At the moment it is not clear whether this is caused by physiological differences, somaclonal variation, or is a consequence of the introduced trait. More generations of selfing are needed, both strict selfings and selfings with non transgenic plants of the same genotype to answer this question.

PAGE analysis of the transgenic seeds indicated that the protein composition was altered, particularly with respect to legumins. Based on the divergence in nucleotide sequences of *leg*A, *leg*J, and *leg*S (**Figure 2**), it was expected that only the amount of LegA would be reduced by the introduction of the IIR construct. Our analysis does not allow us to conclude that indeed the accumulation of LegA is affected. We have not evidenced a reduced amount of the 23 kDa basic subunit of LegA, but instead an increased amount of a 22 kDa band, probably corresponding to the basic subunit of LegJ (LegS is unlikely, because it is probably present in small amounts in the seeds used in this study, as we have never evidenced its characteristic ~80 kDa precursor) (Casey and Domoney 1999). A more detailed analysis with antibodies raised against LegA and LegJ is necessary for establishing the alterations in protein composition more precisely. Alternatively, 2D-gel electrophoretic separation could be used for this purpose, preferably in combination with mass spectroscopy to unambiguously reveal the identity of the down- and up-regulated proteins. Furthermore, it will be necessary to link the protein compositional data to mRNA transcript levels. The observed changes in protein composition might be caused by changes induced by the tissue culture procedure. If this is the case than the same effect should be observed in not transgenic seeds. This will be done in the near future.

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6. General discussion

Seed storage proteins are a major component of pea (Chapter 2). The major seed storage proteins of pea are the globulins. They are subdivided into two major groups based on their sedimentation coefficient: the 11S fraction (legumin) and the 7S fraction (vicilin, convicilin). Each group of globulins consists of several isoforms. So far, peas lacking any specific class of globulin have not been found. In order to obtain pea seeds with a more defined, less heterogeneous protein composition, two approaches were followed. First, the natural variation in protein composition was explored to select for pea lines enriched in one of the protein classes. Second, genetic modification was used anticipating that the natural variation of pea might not provide for the desired specific protein composition, due to the fact that the pea proteins are encoded by multi-gene families. A specific gene-construct, to regulate expression of globulins, was constructed for introduction into pea. A problem is that pea is rather recalcitrant to transformation, and that the existing protocols are inefficient and laborious. We thus embarked on the development of new regeneration systems for pea. One of these systems was combined successfully with a transformation procedure to produce transgenic pea plants. The other led to the production of transgenic tissue, which is currently cultured for plant regeneration. A next step in the procedure was the successful transfer of *in vitro* plants to the greenhouse in order to produce viable seeds. The stable inheritance of the transgenes into the subsequent generations, as well as the alteration in their protein composition, was examined.

Identifying pea lines for industrial applications

Pea globulins have different physico-chemical properties. Vicilin has a significantly greater foaming capacity than legumin, and a slightly lower emulsifying capacity (Wright et al. 1984; Casey 2003). Both legumin and vicilin have good gelation abilities, but under different conditions. On the contrary, convicilin is not capable to form gels. These differences are reflected in the physico-chemical properties of their mixtures; protein-mixtures varying in globulin composition have different physico-chemical properties. For instance, the higher the convicilin content of globulin mixtures, the lower their gelation ability (O'Kane et al. 2004b). Similar results have been found for soy, where globulin-mixtures varying only in a specific globulin have different gelation behaviour (Renkema et al. 2001). The differences in the properties of the various mixtures are of importance for industrial applications of pea and soy globulins (Casey 1999 and references therein). Pea lines differing with respect to globulin composition (Chapter 2) were present in our collection, and they can be good protein resources for applications in food industry. Meal or isolated proteins from these pea lines might have improved textural properties, and can be used as a high-quality food ingredient or in the development of Novel Protein Foods (NPFs), which are potential meat replacers (Owusu-Ansah and McCurdy 1991; Dijkstra et al. 2003 and references therein).

One can assume that by knowing the physico-chemical properties of the individual globulins and the exact globulin composition of a mixture, one can predict the physico-chemical properties of globulin mixtures, and subsequently predict its potential use in food applications. However, results with both soy

(Renkema et al. 2001) and pea globulins (O'Kane et al. 2004a, b, c) indicated that the properties of a mixture can not be predicted by simply adding up the contributions of the constituent globulins. O'Kane et al. (2005) showed that already small differences in the content of a particular globulin can lead to disproportional differences in their gelation behavior, indicating that gelation is a process involving complicated mechanisms. Moreover, little variation between two globulin mixtures, even on the isoform level, can result in considerable differences in the mixture's behavior for both pea (O'Kane et al. 2005) and soy (Lakemond et al. 2002) globulins. These examples indicate that in order to be able to predict the properties of a globulin mixture, one must know the properties and the amounts of all isoforms composing the mixture, as well as the mechanisms determining their interactions. Although the structure-function relationships of the various pea proteins are still not well understood, and although protein compositional analysis, particularly at the isoform level, is difficult, we think that globulin composition, but also the protein-to-starch ration, might become important criteria for pea breeding and industrial applicability. Extrusion of pea meals originating from varieties varying in both starch content/quality (wild type, *r*, *rug3* and *sim32* mutants) and globulins content/composition gives different products (Hughes et al. 2001). Similarly, meals from lines of our collection, which have extensive variation in both starch content and quality (differences in starch content/quality appear from their different shape indicated in **Table 1** of Chapter 2) and globulin content and/or composition (like the lines 1 (R-D), 9 (R), and 49 (W), Chapter 2), can be suitable raw materials for producing various commercially interesting extrusion products. However, it should be realized that the differences in the extrusion products do not only depend on differences in starch and globulin content and composition, but also on other constituents, such as oil, which can interfere with the protein functionality (Casey 2003).

Pea seed proteins are relatively poor in the sulphur-containing amino acids methionine and cysteine, which limits their nutritional value. However, legumins and albumins are 2-fold and 10-fold, respectively, richer in these amino acids than vicilin. As a result, pea's nutritional value is increased when the amounts of legumin and albumins are increased at the expense of vicilin (Tabe et al. 1998). Thus, the vicilin/(albumins+legumin) ratio can be a criterion for conventional breeding programs, aiming to increase pea's nutritional value. Pea line 52 (Chapter 3), which contains higher levels of albumins and legumins might be interesting for breeding programs aiming at increasing the nutritional quality of pea. So far, the nutritional quality of pea was an obstacle for its use in comparison with other seeds. Its improvement can lead to increased use of pea seeds and their cultivation. Enrichment in sulphur-containing amino acids can not only be obtained with conventional breeding programs, but also with the use of biotechnology (Tabe et al. 1993; Tabe and Higgins 1998).

To this end, we have shown that there is considerable natural variation for vicilin processing. Although the relevance of this for technological applications needs to be further established, we think that post-translational processing may influence the aggregation behaviour of vicilin. Lines like pea line 50 and 30, which contained low and high amounts of processed vicilin, respectively, may have vicilin with different

gelling properties. Thus, in the future, *in planta* processing of proteins may be an important criterion for application of pea proteins as ingredients in the food industry and, when the importance of this is established, also for pea breeding.

Can soy proteins be replaced by pea proteins?

Soybean is an agricultural product that is imported into the EU in vast amounts. Only from the US, in the year 2004 5,687,900 tones of soy beans and 63.000 tones soybean cake and meal have been imported into the EU (US-FDA 2004). The major use of soybeans is for oil production. Their seed proteins are used as ingredients in foods, cattle feed, cosmetics and many other products. Many protein rich crops have been proposed for use instead of soy, and each of them has specific advantages and disadvantages (Linnemann and Dijkstra 2002; Dijkstra et al. 2003).

Pea globulins have similar foaming and emulsifying capacity as their soy analogs (Casey 2003 and references therein). Also, various pea legumin and vicilin fractions (under specific conditions) have a similar gelation behavior as their soybean analogs (O'Kane et al. 2004c). Depending on the gelation conditions used, pea legumin can form stronger gels than soy glycinin, and has a different reheating/re-cooling behavior (O'Kane et al. 2004c). These results show that, besides similarities, pea and soy proteins have also differences. Their similarities suggest that pea proteins can technically be used for certain applications instead of soy proteins, whereas their differences indicate that pea globulins might be used instead of soy for applications in which soy globulins are not suitable. However, soybeans and their proteins are available in vast amounts and at lower prices than pea, and thus the use of pea proteins instead of soy is economically less attractive. The lower price of soy proteins as opposed to that of pea proteins is partially a result of the lower protein content of pea compared to soybean (Casey 2003 and references therein). Our results on protein content showed a variation from 16.3% to 36.6% of the total seed dry matter, whereas the overall average content is 26.6%. This is significantly lower than the protein content of soy bean seeds (42-45%; Toda et al. 2003). However, pea's protein content is high in some lines like in the wild relatives of pea, which have a protein content of 30% to 36%. This shows that particularly the wild relatives of pea contain genetic material that could potentially be used in pea breeding to increase the protein content of commercial varieties. However, to achieve a protein content level comparable to soybean, genetic modification could be an option.

Improving pea protein composition by genetic modification of globulin composition

It is shown (Chapter 2) that natural variation of pea protein composition is large, but that lines lacking one of the protein classes (null-mutants) do not seem to exist. However, artificial null-mutants with a very low content of one of the globulins might be created via genetic modification, with the implementation of a

technique like antisense or dsRNA-directed ssRNA-cleavage. Because globulins are encoded by multigene families, the number of the genes that have to be down-regulated to create null-mutants is extensive. In our research, we have chosen the highly efficient method of dsRNA-directed ssRNA-cleavage via interrupted inverted repeats (IIR) constructs, instead of the less efficient normal antisense. We made 4 IIR constructs for decreasing the amount of vicilins, convicilins, LegA, and LegJ. The two legumins families have less than 50% sequence similarities, thus it was necessary to create different constructs for each family.

Constructs with *leg*J, *vicilin*47, and *convicilin*B genes were prepared in a similar way as described for *leg*A in Chapter 5. For the *leg*A and *leg*J constructs, the TI promoter from the trypsin inhibitor, which is a pea endogenous promoter, was used, because it has the same expression pattern as the genes of the legumin families. The UbiI promoter, which is a constitutive promoter, was used for the vicilin (aiming to down-regulate vicilin and convicilin) and convicilin construct (aiming to down-regulate convicilin only). The use of this promoter for the convicilin construct is necessary, since the convicilin genes are expressed during the whole period of seed development, and therefore they require a constitutive promoter that will be active for a long time span. We did not use the frequently applied *35S* constitutive promoter, since studies from others have shown that it is not efficiently expressed in seeds (Taylor personal communications). To this end, the *leg*A construct has been used in pea transformation (Chapter 5). The other constructs will be used for future pea transformation experiments.

Chapter 5 suggested that the transgenic plants containing inverted repeat of the *leg*A gene showed differences in protein composition of the seeds. It has to be determined whether this is caused by the action of the inverted repeat of the *leg*A gene or if this caused by somaclonal variation or a consequence of the fact that seeds were obtained from morphological different plants. The S_1 plants are morphologically more identical. In the near future the seeds, both transgenic and non-trangenic, of these plants will be analyzed, to determine whether the increased accumulation of other legumin isoforms is caused by the transgenes or other factors.

Further, the germination frequency of the seeds needs to be determined, since it is unknown whether modification of the seed reserves affects these characteristics. In the longer term, we also intend to generate pea lines which are down-regulated in the other globulins, and in which post-translational processing of vicilin is reduced. The various transgenic seeds should then be assessed for differences in functionality.

Regeneration of pea from nodular explants of shoots with the use of TDZ

In pea somatic embryogenesis has been described by Loiseau et al. (1998) and Griga (1998, 2002). Shoot organogenesis has been reported using hypocotyls (Ochatt et al. 2000) and immature cotyledons as explants (Grant et al. 1995). Both systems have been tested in our laboratory in a range of varieties but the results (not shown) were not satisfactory to be used for genetic modification. Therefore, we developed two new regeneration systems, which have both adventitious and non adventitious properties (Chapter 3 and 4). Both systems are based on Thidiazuron (TDZ). TDZ is a substituted phenylurea (Thomas and Kattermen,

1986), and has a cytokinin-like activity. It has been used commercially as a cotton defoliant, but when it is used for *in vitro* micropropagation, it results in plant regeneration through organogenesis (chickpea lentil (Malik and Saxena 1992)) or somatic embryogenesis (*Cayratia japonica* V. (Zhou et al. 1994)).

In pea, TDZ induces excess formation of lateral meristems resulting in multiple shoots (Popiers et al. 1997). These authors used cotyledonary nodes from embryogenic axis and obtained excess number of multiple shoots after subculture in medium containing both auxins and TDZ. The multiple shoots were multiplied in TDZ and this resulted in the formation of normal and hyperhydric tissue, which subsequently produced secondary nodular tissue. Normal tissue formed shoots, whereas the hyperhydric part of the tissue was not capable to regenerate plants and was therefore discarded. In this thesis, shoot nodes (Chapter 3) and seeds (Chapter 4) were cultured on a medium with only TDZ and this resulted in the formation of excess number of shoots.

When shoot nodes were cultured on TDZ supplemented medium this resulted in the formation of excess number of secondary shoots with hyperhydric tissue in their base. This hyperhydric tissue (BCT), which contains high number of meristems, was isolated, and contrary to Popiers et al. (1997) regenerated into shoots. Induction of BCT, was depended on the concentration of TDZ used in the media and the time period the tissue was exposed to it. Higher concentrations of TDZ and longer exposure time increased BCT induction. Multiplication of BCT does not always results in production of pure BCT but in a mixture of both BCT and shoots. Most probably, the surface of BCT contains meristems with different levels of development. Less developed meristems are reproduced, whereas more advanced meristems develop into shoots. This results in the formation of a mixed type of explants. Higher TDZ concentration favors BCT reproduction, whereas a lower concentration favors shoot development.

GA_3 plays a major role in the regeneration of plants from BCT. The number of regenerated shoots increased significantly when GA_3 was applied. Also the morphology of the shoots differs compared to a medium without GA_3. Shoots derived from medium supplemented with GA_3 are thinner and taller than shoots derived from a medium without GA_3.

When seeds were cultured on TDZ supplemented medium this resulted in the formation of multiple shoots. In addition to the formation of shoots, bud containing tissues (BCT) were formed at the cotyledonary nodes, shoot nodes, tendrils, stipules and stalks. This shows the etreme effects that TDZ has to pea. The number of shoots per seedling depended on the concentration and duration of the TDZ treatment. The best treatment (400 shoots per seedling) was 12 weeks incubation on MS medium supplemented with 4 mg/l TDZ followed by 4 weeks culture on MS medium supplemented with 0.5 mg/l benzylaminopurine (BA). Isolated shoots rooted at a high frequency on MS medium containing 3.0 mg/l indole-3-butyric acid and 2.0 mg/l α-naphtalene acetic acid.

The regeneration systems described in Chapter 3 and 4 has been tested successfully in a range of different varieties, suggesting its formation is not variety depended making the systems more attractive to be used for genetic modification. Both regeneration systems have also the potential to be used for *in-vitro*

multiplication of pea since higher numbers of plants are produced in a shorter period of time as compared to other multiplication methods.

The BCT regeneration system described here has been tested successfully in other legumes such as soybean (Shan et al. 2005), common bean (unpublished results) and peanut (unpublished results) which are also known to be recalcitrant to genetic modification. In soybean pretreatment of seeds with cytokinins resulted in a tissue similar to the one reported in this study. The tissue has a high regeneration capacity and has the ability to multiply itself under the application of TDZ, having a potential to be used for genetic modification (Shan et al. 2005). However, further research is needed to test whether or not it can be used for the production of transgenic soybeans.

Optimization of the current regeneration system(s)

The regeneration system described in Chapter 3 has two undesirable characteristics; a) browning/lethality of BCT and 2) inability of shoots to form roots. The browning/lethality of the explants during BCT multiplication and shoot regeneration decreased the number of transgenic lines recovered and reduced the number of regenerated shoots per line. Browning occurs in a very short period of time in all explants of a Petri dish. This might indicate the presence in the media of toxic amounts of phenolic compounds or phyto-hormones such as ethylene or cytokinins which induce senescence similar to what has been observed in cotton (Smith et al. 1977), papaya (McCubbin and Van Staden 2003) and *Alstroemeria* (Kim personal communications). Most probably not phenolic compounds but ethylene or another phyto-hormone is responsible for browning, because browning was restricted to the tissue and was not observed around the explants (Kim, personal communications). In papaya activated charcoal, which absorbs phenolic compounds and phyto-hormones reduced significantly browning and lethality (McCubbin and Van Staden 2003). It has to be seen whether addition of activated charcoal in pea growing media would have similar results.

Silver nitrate is a common natural substance. Addition of silver nitrate in the growing media results in Ag2+ ions which reduces the capacity of receptors to bind ethylene resulting in reduced ethylene action (Yang et al. 1985).

Lethality was reduced very significantly when BCT was cultured on medium supplemented with $AgNO_3$, indicating a possible role of ethylene in senescence. Unfortunately, culture of BCT on medium supplemented with $AgNO_3$ resulted also in abnormal development of shoots. Furthermore, it was shown by others that $AgNO_3$ reduces the rooting abilities of pea shoots (Madsen et al. 1998). More research is needed to study whether other methods for ethylene reduction such as gas permeable vessels (Park et al. 2004) have the same positive effect on the tissues without showing the negative effects. A potential source of ethylene could be the response of the explants to mechanical wounding and/or TDZ induced ethylene production as has been observed in cotton (Suttle 1988).

The second undesirable characteristic of the regeneration system described in Chapters 3 is the reduced ability of the shoots to form roots. In chapter 5 it is shown that rooting was improved if the auxins concentration was increased. However, it has to be seen whether is also applicable to plants derived from BCT maintained for a prolonged period of time. Shoots derived from young BCT root at higher frequencies than shoots derived from older BCT. Problems with rooting in pea were also observed by Bean et al. (1997) and Madsen et al. (1998) after prolonged exposure to cytokinins. Another solution is grafting of pea plants as has been reported by Bean et al. (1997). In fava bean (*Vicia faba* L.) Khalafalla and Hattori (1999) also observed that TDZ-induced shoots have a reduced ability to form roots. Use of ethylene inhibitors like silver nitrate ($AgNO_3$) and the ethylene precursor ACC, improved rooting of TDZ induced shoots considerably in fava bean Khalafalla and Hattori (1999). For pea more research is needed to study whether reducing ability of shoots is associated with ethylene presence.

Genetic modification of pea's using the BCT system

In this study a new and reliable transformation protocol for pea (*Pisum sativum* L.) has been developed. BCT or BCT with shoots are co-cultivated for two days with *Agrobacterium tumefaciens*. Transgenic sectors are identified using luciferase as selectable marker and subsequently are physically excised from the explants and subcultured on media supplemented with 4.4 mg/l TDZ. This procedure is repeated until the BCT becomes completely luciferase positive. After that the BCT is cultured for regeneration of plants. Transgenic plants were selected using only the activity of the luciferase gene. Luciferase has been used in various crops such as cassava (Raemakers et al. 1996; 2001), *Alstroemeria* (Lin et al. 2000) and in rice (Ignacimuthu et al. 1998). According to our knowledge this is the first time that luciferase has been used as selectable marker in pea. The system is highly reproducible and results in a high number of transgenic plants and can be applied in a large number of varieties. However, the system is labor intensive and requires a long period of time before transgenic plants are obtained. The transformation system as described in Chapter 5 has two labor intensive and time consuming steps: initiation of BCT and selection of (completely) luciferase positive cultures. The time period needed for initiation of BCT can be reduced significantly and without laborious subculture regimes if seeds are germinated on TDZ supplemented medium as described in Chapter 4. In the transformation procedure described in Chapter 5 it took 3-4 months before completely luciferase positive cultures were obtained. Most probably it is not necessary to regenerate plants from completely luciferase positive cultures and thus reducing the time period needed. Especially if it is combined with chemical selection. Chemical selection using PTT (Bean et al. 1997) and kanamycin (Grant et al. 1998; Nadolska-Orczyk and Orczyk 2000) have been used successfully for pea transformation. Probably in the future a system combining *luc* based selection with chemical selection will be more efficient and less time consuming than the system of genetic modification described in Chapter 5.

A regeneration system similar to pea has been developed for soybean (Shan et al. 2005), bean and peaunut (unpublished results). It will be interesting to see if the BCT system can be used in other legumes and if it also can be combined with genetic modification.

From luciferase selection to marker free transgenic peas (*Pisum sativum* L.) for marketable products

In a procedure for the production of genetically modified organisms only a small portion of the target cells receives the transgenes stably. This makes it necessary to have a selection phase in which the transgenic cells will divide independently from non transformed cells. Commonly, this is done by coupling the gene(s) of interest to genes conveying resistance to antibiotics (Fraley et al. 1983; Herrera-Estrella et al. 1983) or herbicides (De Block et al. 1989; Fromm et al. 1990) and subsequently the relevant antibiotics or herbicides are used as selectable agents. This procedure has proved to be successful in many crops including legumes. In soybean the most commonly used chemical agents are phosphinothricine (PTT) (Zhang et al. 1999) and hygromycine (Olhoft et al. 2003; Yan et al. 2000), whereas in pea kanamycin and PPT are used (Nadolska-Orczyk and Orczyk 2000; Polowick et al. 2000). In these systems the selectable agent is added into the growing media after transformation and (in theory) as a result only transformed shoots carrying the selectable marker gene and the gene(s) of interest develop. However, when it comes to commercial application of the transgenic crops there are concerns about the potential spread of the antibiotic or herbicide resistance genes through horizontal gene transfer to the nature (US-FDA 1998; Hall et al. 2000). Different strategies for the development of marker free plants have been developed to overcome the environmental concerns. De Vetten et al. (2003) developed a marker free method for potato which includes transformation and regeneration without the use of selectable marker genes, instead genetic modified plants are selected using PCR. In other strategies selectable marker genes are first used for the production of transgenic plants and subsequently removed by various methods (Honma et al. 1993; Ebinuma et al. 2001). The methods include co-transformation of non-selected genes with selectable marker genes, subsequently the marker genes are removed by by crossings, the use of recombinases, and transposable elements. Because pea is a homozygous crop cotransformation using the *luc* gene as reporter gene and a chemical selection gene to reduce labor followed by crossing to segregate the *luc* gene and marker gene from the gene(s) of interest, is the preferred method. Production of marker free GMO pea varieties will ease environmental fears and will make more feasible in the future their use for commercial applications.

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Summary

Pea (*Pisum sativum* L.) seeds are a rich and valuable source of proteins, which can have potential for food industrial applications. Pea storage proteins are classified into two major classes: the salt-soluble globulins, and the water-soluble albumins. The globulins are subdivided into two major groups based on their sedimentation coefficient: the 11S fraction (comprising the class of legumin with various isoforms) and the 7S fraction (comprising the classes of vicilin and convicilin, each with various isoforms).

Pea cultivars with extreme variation in globulin composition (i.e. lacking a particular class of proteins) might become important for the food industry, because they could provide new raw materials for specific applications, like the production of Novel Protein Foods (NPFs), which receive attention as possible meat replacers.

This thesis aimed at (i) to determine the existing natural variation in pea's globulin content and composition, in order to identify suitable cultivars for the production of NPFs, (ii) to develop a more efficient protocol for genetic modification of pea, and (iii) to modulate pea protein composition, based on dsRNA directed silencing.

An inventory of protein content and composition of pea was performed to characterize the genetic variation for these traits (Chapter 2). To include a wide range of natural genetic variation, cultivars from a wide geographic distribution, with differences in leaf and seed characteristics, were selected and characterized. Large variation was observed between the various lines. Results on protein content showed a variation from 16.3% to 36.6% of dry matter (DM), with an overall average content of 26.6%. Globulins content varied between 49.2% and 81.8% of the proteins of the total pea protein extract (TPPE). On individual globulins level, legumin content varied between 5.9% and 24.5%. Vicilin was the most abundant protein of pea, and its content varied between 26.3% and 52.0% of the TPPE. The processed vicilin was the predominant of the two, with values between 17.8% and 40.8%, whereas the non-processed ones constituted between 3.1% and 13.5% of the TPPE. Convicilin was the least abundant globulin having an average content of 6.1%. Its content ranged from 3.9% to 8.3%. Finally, the globulin-related proteins were present in amounts ranging from 2.8% to 17.3% of the TPPE. The globulins showed the largest relative variation of the four globulin classes.

It is known that a low vicilin/convicilin ratio can result in poor gelation. Based on our data (Chapter 2) and the literature, it is concluded that pea isolates have a more favourable protein composition for gelling applications as compared to those from soybean. Moreover, the genetic variation for this trait appears to be larger in pea than in soybean, which might offer opportunities to reduce the convicilin content further.

Our inventory did not show cultivars lacking a specific globulin. Such cultivars might be important, because they could have more favourable physical properties for the production of NPFs. To produce such lines genetic modification approaches were employed. To carry out genetic modification a reliable protocol is needed. At the time this study started, protocols for the production of genetically modified peas were available, but particularly the regeneration of plants from transgenic cells was very inefficient. Most of the plants obtained were either escapes or chimeric (not all cells of a plant are genetically modified). Therefore,

our study focused on obtaining a novel regeneration protocol, which in combination with the transformation procedure would result in an improved method for obtaining transgenic pea lines.

The novel regeneration protocol started with subculture of stem tissue with one node (Chapter 3) or whole seeds (Chapter 4) on TDZ supplemented medium. Repeated subculture of stem tissue with one node resulted in a green hyperhydric tissue in the swollen bases of the multiple shoots, which is fully covered with small buds [bud-containing tissue (BCT)] was formed. BCT fragments were isolated and subcultured in the same medium and, as a result, they were able to reproduce themselves in a cyclic fashion. Subculture of BCT on medium supplemented with a combination of GA_3, cytokinins and auxins resulted in the production of rooted shoots. *In-vitro* plants were transferred to the greenhouse for acclimatisation and further development. All tested pea cultivars ('Espace', 'Classic', 'Solara', and 'Puget') responded in the same way.

Culture of seeds for a relatively long time (Chapter 4) on TDZ supplemented medium resulted in the production of very high numbers of shoots together with BCT which was identical to the BCT described in Chapter 3. This protocol resulted in the faster production of BCT as compared to the protocol described in Chapter 3.

The regeneration protocol from Chapter 3 was combined with genetic modification (Chapter 5). Transgenic pea plants were obtained after co-cultivation of bud containing tissue (BCT) and shooty BCT with *Agrobacterium tumefaciens* strain AGL0(pG49A). The binary vector pG49A contained an interrupted inverted repeat of a *legumin A* gene, flanked by the promoter of the trypsin/chymotrypsin inhibitor gene, together with the *luciferase* gene for selection of transgenic tissue. Luciferase positive tissue was identified, isolated, and subcultured on TDZ-supplemented medium. On this medium, BCT can be multiplied, and the shooty BCT will become pure BCT again. The luciferase based selection procedure was repeated until (almost) complete luciferase positive BCT cultures were obtained. Plants (S_0) of 23 transgenic lines were grown in the greenhouse. The S_0 plants were smaller in size and produced less seeds than the control plants. All lines produced luciferase positive seeds. The transgenic nature of 5 S_0 plants was further confirmed using Southern blot analysis. Protein analysis with SDS PAGE electrophoresis of the seeds of 8 lines indicated differences in protein composition, although our data were not conclusive on whether the amount of legumin A or other legumins was affected. Further experiments should show whether the protein compositional changes resulted from silencing of the *leg*A gene, or other factors such as genetic or epigenetic changes in the genetically modified plants, caused by the tissue culture procedures.

Seeds of 6 lines were grown to produce S_1. The S_1 plants were comparable in height to control plants. However, the number of seeds per plant was significantly lower.

The developed transformation protocol is highly repeatable. Each experiment resulted in genetic modified plants, in contrast to other systems, which have low repeatability. However, our system is more time consuming than those developed by others. Therefore, the regeneration system, which produces BCT directly from the seeds without the need for production of *in-vitro* plants, should be combined with genetic modification in the future. Furthermore, the selection of transgenic tissue should be optimised using selectable marker genes such as *npt*II or *pat*.

Samenvatting

De zaden van de erwt (*Pisum sativum* L.) zijn een rijke en hoogwaardige bron van eiwitten, die mogelijk gebruikt kunnen worden voor toepassingen in de levensmiddelenindustrie. De eiwitten van de erwt kunnen in twee groepen geclassificeerd worden, nl. de globulinen (oplosbaar in zout water) en de albuminen (water-oplosbaar). De globulinen kunnen aan de hand van hun sedimentatiecoëfficient verder onderverdeeld worden in twee groepen: de 11S fractie (de leguminen met verschillende isovormen) en de 7S fractie (de vicilinen en de convicilinen, ieder met verschillende isovormen).

Erwten cultivars met een grote verscheidenheid in globuline compositie (dus cultivars die één groep van eiwitten, b.v. de leguminen, missen) zouden op termijn belangrijk kunnen zijn voor de levensmiddelenindustrie vanwege hun vermeende betere geschiktheid als grondstof voor specifieke toepassingen, zoals de productie van 'Novel Protein Foods (NPFs)', die momenteel in de belangstelling staan als mogelijke vleesvervangers.

Het onderzoek beschreven in dit proefschrift had tot doel om (i) de genetische variatie in globulinegehalte en –compositie van verschillende erwtencultivars in kaart te brengen, om zo geschikte cultivars voor de productie van NPFs te kunnen selecteren, (ii) een meer efficiënte procedure voor genetische modificatie van erwt te ontwikkelen, en (iii) de eiwitcompositie van erwtenzaad te moduleren voor legumine gehalte d.m.v. de zogenaamde dubbelstrengs RNA inhibitie.

Hoofdstuk 2 beschrijft de inventarisatie van eiwitgehalte en –compositie van 59 cultivars en wilde soorten, hetgeen inzicht biedt in de genetische variatie voor deze eigenschappen. Hiervoor werden cultivars van verschillende geografische origine, en met verschillende blad- en zaadmorfologie gebruikt, om een zo breed mogelijke spreiding in eigenschappen te verkrijgen. Grote verschillen werden gevonden tussen de verschillende cultivars. Het eiwitgehalte varieerde van 16.3% tot 36.6% van het drooggewicht, met een gemiddeld gehalte van 26.6%. Het globulinegehalte varieerde tussen 49.2% en 81.8% van het totaal geëxtraheerde eiwit. Ook voor de individuele klassen van globulinen werd een grote variatie gevonden. Het leguminegehalte varieerde van 5.9% tot 24.5% van het totaal geëxtraheerde eiwit. Viciline was het meest voorkomende eiwit in erwt, met een gehalte variërend tussen 26.3% en 52.0%. Het post-translationeel gemodificeerde viciline was het meest voorkomend, met waarden van 17.8% tot 40.8%, terwijl het ongemodificeerde viciline tussen 3.1% en 13.5% van het totaal geëxtraheerde eiwit representeerde. Conviciline was het minst voorkomende globuline, met een gemiddeld gehalte van 6.1% (in een range van 3.9% tot 8.3%). Tot slot werden ook zogenaamde globuline-gerelateerde eiwitten gevonden, in hoeveelheden tussen 2.8% en 17.3% van het totaal geëxtraheerde eiwit. Deze laatste lieten de grootste relatieve variatie zien van de vier globuline klassen.

Het is bekend dat een lage viciline/conviciline verhouding kan resulteren in slechte gelering van erwteneiwit. Op basis van onze data (Hoofdstuk 2) en de literatuur kan geconcludeerd worden dat isolaten van erwteneiwitten een betere eiwitsamenstelling hebben voor applicaties waarin gelering belangrijk is dan die van soja. Tevens blijkt de genetische variatie voor deze verhouding in erwt groter te zijn dan die in soja, hetgeen goede kansen biedt om het convicilinegehalte van erwt verder te verlagen.

Onze inventarisatie heeft geen cultivars geïdentificeerd, waarin één specifieke klasse globuline afwezig was. Zulke cultivars zijn belangrijk, omdat ze betere fysische eigenschappen voor de productie van NPFs zouden kunnen hebben. Om toch de beschikking over zulk plantmateriaal te krijgen, werd geprobeerd om een genetische modificatie benadering te gebruiken. Bij de start van onze studie waren een aantal procedures voor de genetische modificatie van erwt voorhanden, maar met name regeneratie van planten uit transgene cellen was erg inefficiënt. Vele van de vermeende genetisch gemodificeerde planten bleken niet of slechts gedeeltelijk transgeen (chimeer). Het was daarom nodig om een betrouwbaarder protocol te ontwikkelen. In eerste instantie hebben we ons gericht op het verkrijgen van nieuwe regeneratieprocedures, die in combinatie met een transformatieprocedure, zouden moeten leiden tot een verbeterde methode voor de productie van genetisch gemodificeerde erwtenplanten.

Het regeneratie protocol gebruikte als uitgangsmateriaal stengel segmenten (Hoofdstuk 3) of hele zaden (Hoofdstuk 4) die op TDZ bevattend medium werden geplaatst. Nadat de stengel stengelsegmenten een aantal keren waren overgezet op TDZ medium ontstond een “hyperhydric” weefsel in de basis van de scheutclusters. Dit “hyperhydric” weefsel is volledig bedekt met kleine meristemen die BCT genoemd werd (BCT; ”bud-containing tissue”). BCT fragmenten, geïsoleerd uit de scheutclusters, werden gereproduceerd wanneer ze uitgelegd werden op medium gesupplementeerd met TDZ. Wanneer BCT werd overgebracht naar een medium met GA_3, cytokininen en auxinen ontstonden bewortelde scheuten. Alle getoetste erwten cultivars (‘Espace’, ‘Classic’, ‘Solara’, en ‘Puget’) reageerden op dezelfde manier.

Het uitleggen van zaden (Hoofdstuk 4) gedurende een relatief lange periode op een medium gesupplementeerd met TDZ resulteerde in de vorming van een zeer groot aantal scheuten samen met de vorming van BCT. Dit BCT is identiek aan de BCT beschreven in Hoofdstuk 3. De tijdsduur nodig voor de vorming van BCT uit zaad is korter dan in het protocol beschreven in Hoofdstuk 3.

Het regeneratieprotocol uit Hoofdstuk 3 werd gecombineerd met een transformatieprocedure (Hoofdstuk 5). Genetisch gemodificeerde erwten planten werden verkregen na co-cultivatie van BCT of zogenaamd scheut-dragend BCT met de *Agrobacterium tumefaciens* stam AGL0(pG49A). De binaire vector pG49A bevatte een zogenaamde ‘interrupted inverted repeat’ van het *legumin A* gen, aangestuurd door de promoter van het trypsine/chymotrypsine inhibitor gen, als ook het *luciferase* gen voor selectie van transgeen weefsel. Luciferase positief weefsel werd geïdentificeerd, geïsoleerd en verder gekweekt op medium met TDZ. Op dit medium wordt BCT vermeerderd en scheut-dragend BCT wordt opnieuw puur BCT. De selectie gebaseerd op luciferase activiteit werd herhaald totdat volledig luciferase positieve cultures werden verkregen. Planten (S_0) van 23 transgene lijnen werden overgebracht naar de kas. De S_0 planten waren kleiner en vormden minder zaden dan de controle planten. Alle lijnen produceerden luciferase positief zaad. Het transgene karakter van 5 S_0 planten werd verder bevestigd door “Southern blot” analyse. Analyse van de zaden van acht transgene lijnen m.b.v. SDS polyacrylamide gelelectroforese liet verschillen in eiwitsamenstelling zien, maar het is niet mogelijk om op basis van deze data te concluderen dat het legumine A gehalte daadwerkelijk verlaagd is. Andere experimenten zijn noodzakelijk om aan te tonen dat de veranderingen in eiwitsamenstelling het gevolg waren van uitschakeling van het *leg*A gen, of van andere factoren zoals genetische of epigenetische veranderingen in de genetisch gemodificeerde planten, veroorzaakt door de weefselkweekprocedure.

Zaad van 6 lijnen werd uitgezaaid voor de vorming van S_1. De S_1 planten hadden dezelfde grootte als de controle planten. Echter het aantal zaden per plant was duidelijk lager.

Het ontwikkelde genetisch modificatie protocol is reproduceerbaar. Ieder experiment resulteerde in genetisch gemodificeerde planten, dit in tegenstelling tot de eerdere gepubliceerde systemen voor de genetische modificatie van erwt, waarvan de herhaalbaarheid slecht is.

Echter, het in dit proefschrift beschreven systeem van genetische modificatie kost meer tijd dan de eerder gepubliceerde systemen. De benodigde tijd kan worden bekort indien het regeneratie systeem waarbij BCT wordt gevormd uit zaden wordt gecombineerd met genetische modificatie. Daarnaast moet de selectie van transgeen weefsel worden geoptimaliseerd door gebruik te maken van selectie genen zoals *npt*II of *pat*.

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Curriculum Vitae

Emmanouil N. Tzitzikas was born on 19 August 1970 in Greece. He studied Greenhouse Horticulture and Floriculture in the Technological Educational Institute of Heraklion, Greece, graduating in 1995. During his studies he was also busy with vegetal and flower production working in greenhouses and fulfilled his military service obligations. In 1997, he started a Master degree course in Plant Breeding in Wageningen University (The Netherlands), graduating in 1999. Thereafter, he started his PhD at the Laboratory of Plant Breeding, in the Department of Plant Science of Wageningen University, working on a project of PROFETAS (PROtein Food Environment Technology And Society), and completed the research presented in this thesis.

List of Publications

Tzitzikas EN, Vincken JP, De Groot J, Gruppen H, Visser RGF Genetic variation in pea (*Pisum sativum* L.) seed globulin composition and their *in planta* processing (under revision for the Journal of Agriculture and Food Science)

Shan Z, Raemakers K, **Tzitzikas EN,** Ma Z, Visser RGF Development of a highly efficient, repetitive system of organogenesis in soybean (*Glycine max* (L.) Merr). Plant Cell Rep **2005** Jun 17; [Epub ahead of print] PMID: 16075225

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