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Seed dormancy as the key domestication trait in legumes

Ph.D. Thesis

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Declaration:

I declare that I wrote this Ph.D. thesis by myself, and that all used literary sources are included in the chapter References. Published results have been approved by mentioned co-authors.

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Abstract:

Seed dormancy is one of two key domestication traits including in legumes. Selected cultivated and wild pea genotypes (*Pisum sativum* L. and *P. sativum* subsp. *elatius* (M. Bieb.) Asch. & Graebn.) were used as a model system. Pea seeds as well as other wild progenitors of cultivated legumes display physical dormancy. This type of dormancy is caused by water impermeable cell layers in the seed coat. First part of the thesis focuses on the comparative anatomical, chemical and transcriptomic analysis between cultivated non-dormant *P. sativum* (JI92, cv. Cameor), wild dormant *P. elatius* (JI64, VIR320) and respective recombinant inbred lines (RILs) established by crosses of JI64 and JI92 parents. Testa thickness was analyzed by micrometric, light and scanning electron microscopy measurements. Considerable differences were found in texture of testa surface, length of macrosclereids and seed coat thickness. Comparative transcriptomic analysis resulted in identification of differentially expressed genes. Selected genes were studied by qRT-PCR analysis. Among others, there was number of genes belonging to phenylpropanoid pathway, identified also in metabolomics study of seed coat. The second objective of the study was found of association between the seed dormancy, seed properties and environmental factors in using the wild pea model. Two oscillating temperature regimes were experimentally tested as dormancy release clue. To assess the dormancy status, seed imbibition and germination was scored. Seed total germination ranged from 0 to 100%. Only one environmental variable - annual temperature range (BIO7) - was significantly related to germination responsivity. The higher content of total proanthocyanidins in dormant seed coat as compared to non-dormant or responsive accessions was found and this corresponded also to comparative metabolomics analysis of contrasting genotypes.

Keywords: adaptation, dormancy, germination, legumes, pea, seed coat and transcriptomics

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Abstrakt:

Předkládaná práce je zaměřena na získání nových poznatků o dormanci semen, která je jedním ze dvou klíčových domestikačních znaků bobovitých rostlin. Modelovou rostlinou disertační práce je hrách, jeho vybrané kulturní a plané genotypy (*Pisum sativum* L. a *P. sativum* subsp. *elatius* (M. Bieb.) Asch. & Graebn.). Pro hrách a řadu dalších bobovitých rostlin je typická fyzická dormance, která je zprostředkována vrstvami palisádových buněk v osemení, které jsou nepropustné pro vodu. První část je věnována srovnání anatomických, chemických a transkriptomických odlišností mezi kulturními nedormantními (JI92, cv. Cameor), planými dormantními (JI64, VIR320) genotypy hrachu a rekombinatními inbredními liniemi (RILs) vzniklými křížením JI64 a JI92. Tloušťka osemení byla analyzována mikrometricky a pomocí světelného i skenovacího elektronového mikroskopu. Byly zaznamenány významné rozdíly v povrchové struktuře, tloušťce osemení a délce makrosklereid. Dynamická povaha genové exprese byla stanovována pomocí kvantitativní RT-PCR analýzy u vybraných diferenciálně exprimovaných genů. Mezi nimi bylo několik genů patřících do fenylpropanoidní dráhy, které byly také identifikovány při metabolomickém studiu osemení. Druhá část měla za cíl zjistit vztahy mezi dormancí semen, vlastnostmi semen a faktory prostředí za využití modelu planého hrachu. Jako možný zdroj k uvolnění dormantního stavu byl testován vliv oscilace teplot. Dormance byla hodnocena na základě bobtnání a klíčení semen. Klíčivost semen se pohybovala v rozmezí od 0 do 100%. Dále bylo zjištěno, že pouze jedna proměnná prostředí – roční teplotní rozmezí (BIO7) – významně souvisí s klíčením. Celkový vyšší obsah proanthokyanidinů byl nalezen v osemení dormantních druhů ve srovnání s nedormantními nebo responsivními, což odpovídalo i srovnávací metabolomické analýze kontrastních genotypů.

Klíčová slova: adaptace, dormance, klíčení, luštěniny, hrách, osemení a transkriptomika

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Content

1. General introduction

1.1. Introduction to plant dormancy

Seed germination is a very important step in the plant life that ensures the survival of plant species in diverse habitats. Seeds of some plant species display the dormancy, that is, the period when a healthy seed does not enter the germination phase even under suitable ambient conditions. It is a natural phenomenon that prevents wild plants from extinction under unfavourable climatic conditions. In this case, the dormancy is advantageous adaptation trait. On the contrary, seed dormancy is undesirable for agricultural crops, which are expected to have a uniform and quick germination to establish the high yield. Because of this, seed dormancy has attracted attention of various researchers, including plant biologists, geneticists, food scientists, breeders etc.

1.2. Seed dormancy

By definition, dormancy is an inability of viable and healthy seed to germinate under otherwise favourable conditions for germination (Bewley, 1997; Baskin and Baskin, 2004; 2014). There are many factors that are responsible for seed dormancy, and depending on the taxon, genotype and also the environment, seeds require specific conditions to break from this stage and start germination. Several schemes for classifying seed dormancy have been published. The first written account of seed physiology and various timing of germination was proposed by Greek botanist Theophrastus in third the century BC (Evenari, 1984). One of the earlier systems of classification was defined by Crocker (1916). He described several dormancy classes according to the treatments that were used to overcome dormancy and established the terms of primary and secondary dormancy. Other scientists who described different kinds of dormancy were Harper (1957), Nikolaeva (1967 [1969], 1977), or later Vleeshouwers *et al.* (1995). Dormancy classification can be subjective and there is no unanimous view. Nowadays, the classification of Baskin and Baskin (2004) is widely used and accepted. This comprehensive schema consists of five classes of seed dormancy: physiological (PD), morphological (MD), morpho-physiological (MPD),

physical (PY) and combinational (PY + PD). In addition to these five classes, further division into levels and types are proposed.

If we combine aspects of all known classifications of seed dormancy, primary and secondary dormancy can be considered as the two main categories (Crocker, 1916; Vleeshouwers *et al.,* 1995). **Primary (inborn) dormancy** occurs during seed development in mother plant (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006) and these seeds do not germinate under any set of environmental conditions until dormancy is relieved seed. This category includes three groups in Baskin and Baskin (2004) classification: endogenous, exogenous and combinational dormancy. Non-dormant seeds may enter to **secondary dormancy** when environmental conditions (low or high temperatures, darkness or prolonged light, water stress) are not convenient for seedling growth. In nature, seeds can accede or leave secondary dormancy even several times according to seasonal cues (Bewley *et al.,* 2013; Vleeshouwers *et al.,* 1995).

Endogenous type of dormancy is associated with seeds that, fail to germinate because of factors related with the embryo. This group comprises the physiological (PD) and morphological (MD) classes of dormancy. PD is usually based on blocking the action of abscisic acid (ABA) and gibberellins (GA). It is the most common type of dormancy and it occurs for example in brassicas, such as *Arabidopsis thaliana*, *lettuce (Lactuca sativa)* and, several cereals (Finch-Savage and Leubner-Metzger, 2006). Seeds with MD have underdeveloped but differentiated embryos, which need only longer time to grow to full size before it can germinate. Under suitable conditions this period lasts from several days, up to two weeks. MD is typical for Apiaceae (*Apium graveolens*) or Papaveraceae (*Papaver*) species.

Exogenous dormancy is imposed by factors outside of the embryo. Under exogenous dormancy belong to physical dormancy (PY), which is described in more detail below.

Third group of dormancy is called **combinational dormancy** and it combines two types of primary dormancy. It includes seeds that have both exogenous and endogenous dormancy, therefore PD with PY according to Baskin and Baskin classes. For example, seeds of *Medicago truncatula* exhibit this type of dormancy (PY + PD). Second subgroup of combinational dormancy is morpho-physiological dormancy (MPD). MPD is a combination of PD and MD and it occurs in seeds that do not have fully completed development of embryo and have physiological component of dormancy (Baskin

and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). A representative of this class is, for example, *Fraxis excelsio*r (Oleaceae).

1.3. Physical dormancy

Physical dormancy (PY, also known as hardseededness) in seeds is characterized by one or more water-impermeable layers of palisade cells in seed coat (or fruit) that can be broken by various types of disruption depending on plant species (Baskin *et al.*, 2000; Baskin and Baskin, 2014). Under natural conditions, PY may be broken by factors such as high temperatures, or even fire, alternating high and low temperatures and humidity, abrasion by soil particles, by an action of fungi and microorganisms in the soil or by passage through digestive tract of animals. In the laboratory conditions, breaking of PY requires scarification, either mechanical (such as abrasion or cutting) or chemical by application of acid (for example concentrated sulphuric or nitric acid). Once the seed coats are permeable to water, seeds can mostly germinate over a wide range of temperatures. Furthermore, once impermeable seeds with PY become permeable, they cannot revert to dormancy unlike seeds with PD, which may re-enter the secondary dormancy after primary dormancy is broken (Baskin and Baskin, 2000). Physical dormancy has been reported in at least 18 plant families of angiosperms including Fabaceae (Gama-Arachchige, *et al.* 2013; Baskin and Baskin, 2014; Geneve *et al.*, 2018). Other families with PD are Malvaceae, Convolvulaceae, Geraniaceae or Cannaceae etc. It usually occurs in wild ancestors of cultivated legumes and is one of the main modified during domestication (Abbo *et al.*, 2011; Smýkal *et al.*, 2014).

Sometimes a loss of dormancy is linked to structural changes in a specialized region of seed coat called water-gap (Baskin *et al.*, 2000; Gama-Arachchige *et al.*, 2013; Geneve *et al.*, 2018). The anatomy, morphology, origin and position of water-gaps can differ in specific between families and even genera and the region of this structure is a morphologically distinct from the rest area of the seed or fruit coat (Baskin *et al.*, 2000). There were identified three types of water-gap complexes based on morpho-anatomical features. In type-I, specific kind of cells (modified elongated palisade cells) is pulled apart to form a surface opening. This type has hilar slit, let slit or micropyle slit as a primary water-gap structure. Type-II is created by circular or linear lid-like structures, which is separates from the seed surface. And finally, type-III is constituted by plug-like structure,

which is usually formed by water-impermeable sclerenchyma cells. As a primary water-gap structures, chalazal or hilar oculus and chalazal slit were found (Gama-Arachchige *et al.*, 2013; Geneve *et al.*, 2018).

Moreover, cracks that occur in the outer coat layers may play an additional role in water entry in some physically dormant seeds (Morrison *et al*., 1998; Ma *et al*., 2004) including pea (Janská *et al*., 2019).

1.4. Pea

Pea (*Pisum sativum* L.) is one of the oldest domesticated crops of the Fertile Crescent region (Smýkal *et al*., 2012, 2015) and is currently grown in the temperate regions of the world. Together with cereals and other grain legumes (lentil, chickpea and common bean), it belongs to the important crops in agriculture. Pea belongs to family *Fabaceae* (also called *Leguminosae*), tribe Fabeae, which contains: *Lathytus* (grass pea), *Lens* (lentils), *Pisum* (peas), *Vicia* (vetches) and monotypic genus *Vavilovia* (Smýkal *et al.*, 2011; Schaefer *et al.*, 2012). Beside the protein (from 22 to 28%) pea seeds contain slowly digestible starch (up to 50%), soluble sugars, fibers with demonstrated health benefits, a range of vitamins (vitamin B, C, provitamin A) and important minerals such as K, Mg, Ca, Fe, Zn and P (Bastianelli e*t al.*, 1998; Iqbal *et al.*, 2006; Amarakoon *et al.*, 2012; Dahl *et al.*, 2012). In addition to its benefits in human and animal diet pea has considerable benefits also in nature and agriculture. Due to its ability to fixing atmospheric nitrogen through symbiosis with bacteria from *Rhizobium* genus, naturally enrich soil by nitrogen for other crop and ensures reduction of fertilisers use (Alves-Carvalho *et al.*, 2015).

Experimental studies in pea have a long history, starting from hybridization studies of T. A. Knight in the 18th century and then especially of G. J. Mendel. Since Mendel's work in the discovery of universal laws of inheritance (1866), pea has been an important model plant (Smýkal 2011, 2014).

Taxonomically, one of the accepted versions is the division *Pisum* into two species: *P. fulvum* Sbth. & Sm. and *P. sativum* L., which has two subspecies *P. sativum* subsp. *sativum* (domesticated forms) and *P. sativu*m subst. *elatius* (M. Bieb.) Asch. & Graebn. (wild types) (Smýkal *et al.,* 2017). Wild pea can be found in nature of southern Europe, western Asia and parts of North Africa. Mediterranean and the Middle East are considered to be regions of origin and initial place of domestication. (Smýkal *et al.*,

2017). In process of domestication, two key traits have been modified, pod dehiscence and seed dormancy. Other traits comprise seed size, branching pattern and flowering time control (De Ron, 2015).

All *Pisum* species are self-pollinated diploid with 2n = 14. The standard pea karyotype includes seven chomosomes (two sub-metacentric and five acrocentric) (Hall *et al.*, 1997; Ellis and Poyser, 2002). For cultivated pea, nuclear genome size estimates to be 9.09 pg DNA/2C, corresponding to its haploid genome size of 4.45 Gbp (Doležel and Greilhuber, 2010). Large part of genome is made of repetitive sequences. Murray *et al.*, (1978) states that its forms approximately 85% of pea genome. Majority of pea repetitive DNA sequences consist of made of LTR retrotransposons (Macas *et al.*, 2007). Due to its size and content of repetitive sequence, the pea genome was not fully sequenced for a long time. It was published after long-term efforts of the International Pea Genome Sequencing Consortium only in September this year (Kreplak *et al.*, 2019).

1.5. Pea seed coat structure and development

Pea has a typical non-endospermic seed which is composed of an embryo and a seed coat. **The embryo** consists of cotyledons and an embryonic axis with plumula (primordia), hypocotyl and radicule, Figure 1 (Finch-Savage and Leubner-Metzger, 2006; Bewley *et al*., 2013). During embryo development, the cotyledons consume nutritional reserves from endosperm. Consequently, endosperm is almost degraded in the mature seed and cotyledons serve as sole storage organ (Finch-Savage and Leubner-Metzger, 2006). Pea seeds can be distinguished according to stock energy substrate in cotyledons to be either round (higher content of starch) typical of field pea and wrinkled (higher content of free sugar) as found in garden green peas.

The seed coat or testa is of considerable importance to seed because it is often the only protective barrier between embryo and external environment. Anatomical structure of seed coat (Figure 2) differs among species and varieties, but in general, seed coat is of maternal origin, and its development begins after fertilization from two ovule integuments (inner and outer) in legumes (De Souza and Marcos-Filho, 2001; Smýkal, 2014). The inner integument largely disappeares during development, while the outer one comprises of several distinct cell layers. The outermost layer is cuticle, which represents first barrier

to water entry. Latter layer is composed of tightly packed macrosclereids (also called Malpighian or epidermal cells), which have characteristic thickening of the cell walls and modifications on the outer end termed terminal caps. Furthermore, there is a light line termed *linea lucida* in the macrosclereid cell walls under terminal caps. Its name is derived based on differences in refraction of the light by top and bottom portion of macrosclereids, which differ in chemical composition (Baskin and Baskin, 2014). The light line in pea has already been discussed, for example, by Harris in 1983. Central part of the seed coat is consisted of osteosclereids (so-called bone-shaped cells or hourglass) with large intercellular spaces. The innermost layer is formed by parenchyma cells that lost the protoplast during maturation and are compressed at maturity. The parenchymatous region is sometimes named to as nutrient layer due to its function during embryo development. During pea seed coat development three parenchyma sub-layers can be distinguished - chlorenchyma, ground parenchyma and branched parenchyma (Van Dongen *et al*., 2003). On the surface of the seed there are other structural features. Upon detachment from parent plant, the testa bears a scar, referred to as hilum. It is a specialized area by which the seed was joined to the funiculus. At one end of the hilum there is a small depression, the micropyle. Specific region of strophiole (lens or raphe) can be found on the opposite side of hilum. The area around hilum is the only one part of the seed containing both palisade and counter-palisade layers of macrosclereids (Rolston, 1978; De Souza and Marcos-Filho, 2001; Bewley *et al*., 2013; Smýkal *et al*., 2014; Janská *et al*., 2019).

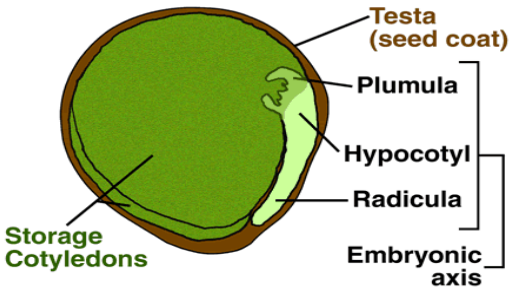

Figure 1: Structure of mature seeds of *Pisum sativum* L. Adapted from Finch-Savage and Leubner-Metzger (2006).

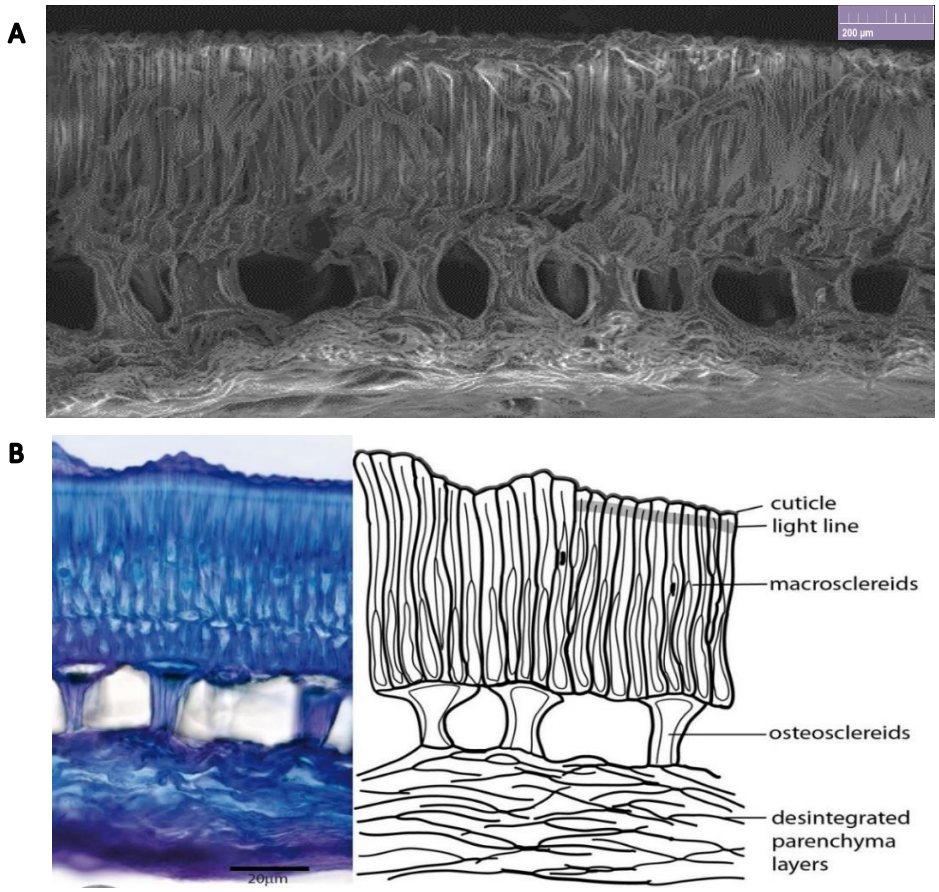

Figure 2: Structure of seed coat *Pisum sativum* L. (**A**) structure of cultivated seed coat from SEM analysis (**B**) transversal section of toluidine blue stain of wild *P. sativum* subsp. *elatius* (left) and schematic drawing (right). Adapted from Smýkal *et al.* (2014).

1.6. Seed coat and its role in dormancy

The seed coat (testa) is the outer covering of every mature seed and it functions as a physical barrier between the embryo and the environments. It plays an important role in embryo nutrition during seed development it protects the embryo against from mechanical injuries and attacks of microorganisms and other pests, and it promote seed dispersal. Besides all of this, the main role of seed coat is to control germination and dormancy through regulation of water and also gas permeability (Debeaujon *et al.,* 2000; De Souza and Marcos-Filho, 2001).

Structure and compositional of the seed can affect status of seed dormancy. For example, the histological analysis of seed coat in *Medicago truncatula* revealed changes

in cell wall thickness of macrosclereids and osteosclereids throughout seed development (Verdier *et al.,* 2013). Recently, structural features in pea seed coat affecting dormancy have been characterized, in dormant and non-dormant seeds (Janská *et al.,* 2019). Gillikin and Graham (1991) showed that seed coat gradually becomes impermeable during later stages of seed maturation in soybean as the result of peroxidase activity that causes the polymerization of soluble phenolics to insoluble polymers. Oxidation products of polyphenolic compounds formed by catechol oxidase are considered to contribute to impermeability in *Pisum sativum* (Marbach and Mayer, 1974; Werker *et al.,* 1979).

In *Arabidopsis*, it has been shown that in addition to seed coat structure also colour of testa affects dormancy status and germination, through the study of *transparent testa* (*tt*) seed mutants (Debeaujon *et al.,* 2000). Thus, another seed coad trait associated with water permeability and dormancy is a colour. Pigments in testa are generally phenolic compounds such as flavonoids. Flavonoids are secondary metabolites of plants and the main types of flavonoids found in seeds include flavonols, anthocyanins, isoflavones and tannins. The seed coat colour varies in different species, in plants with different genotypes within one spieces and it is vary in different development stages (Figure 3). Proanthocyanidins (PAs), chemical basis for condensed tannins, received attention due to their abundance (Dixon *et al.*, 2005; Zhao *et al.*, 2010) in seed coats including pea (Ferraro *et al.*, 2014). Isoflavone compounds were found in seed coat and embryo of soybean (Zhang *et al.*, 2011), procyanidines respectively condensed tannins can be found in seed coat of *Arabidopsis* (Lepiniec *et al*., 2006). In *Medicago truncatula* cells of the outer integument showed abundant accumulation of polyphenolic compounds which may impact seed permeability (Moïse *et al*., 2005). In legumes it was found that the colourless seeds imbibe faster than the seeds with pigmented testa and they subsequently undergo earlier germination. Moreover, seeds without pigment, crack more during swelling, which can affect their lifetime (Debeaujon *et al*., 2000).

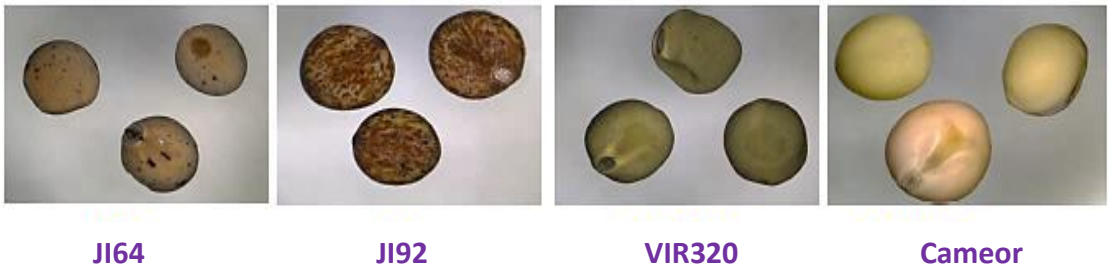

Figure 3: Variability of seed coat colour. Author of photos P. Kopecký.

1.7. Germination

Seed development, dormancy and germination are three important phases in the life cycle of plants. The right timing of seed germination determines whether, or not new seedlings survive and the next generation establishes. Seed germination is a complex process, which frequently involves recovery from maturation drying, re-establishment of basal metabolism and in some species overcoming of dormancy (Nonogaki *et al*., 2010). All these processes can be affected both by genetic and external influences. Mature dry seeds usually contain 5–15% water on a fresh weight basis (Bewley *et al*., 2013). Under these conditions, metabolic activity is close to zero. Metabolic activity increases upon the uptake of water. Germination starts with absorption (imbibition) of water by dry seed and terminates as radicle protrudes through tissues surrounding embryo. Water uptake by seeds is a triphasic process. It begins with a rapid initial imbibition (phase I). During a plateau phase (phase II), water absorption is minimal, embryo expansion occurs and metabolic activities increase. This second phase remains unchanged in seeds that do not complete germination, such as in dormant or unviable seeds. In dormant seeds, some limited metabolic activity can observed, however, the germination is blocked unless the dormancy is broken. Finally, the whole process of germination is terminated by a further increase in water uptake and elongation of embryonic axis (phase III) (Bewley, 1997; Manz *et al*., 2005). Primary pathway for water entry into pea seeds remains unresolved. According to the results of Janská *et al*. (2019) it seems that cuticle layer plays an essential role in non-dormant seeds, while the light line represents the key barrier in dormant seeds.

Except for water, other important external factors for seed germination are temperature, oxygen, sometimes light or darkness, soil disturbance, vegetation shading and others.

Internal mechanisms and hormone signalling inside seeds also play a significant role in regulation of seed germination. Major regulators of both germination and dormancy are two famous antagonistic phytohormones, abscisic acid (ABA) and gibberellins (GA). ABA is a positive regulator of induction and maintenance of seed dormancy, but it is a negative regulator of germination. While GA release dormancy, promote seed germination and counteract inhibitory effects of ABA (Kucera *et al.*, 2005; Finch-Savage and Leubner-Metzger, 2006).

1.8. Evolution of seed dormancy in terms of adaptation

The evolution of dormancy is remains unknown and it is unclear which type of dormancy was the default from which the other classes were derived. Because PD is the most phylogenetically spread dormancy class and it is distributed over the entire phylogenetic tree, it seems that this could be the oldest one. However, according to Baskin and Baskin (2004) and Finch-Savage and Leubner-Metzger (2006) MD is the most primitive class of dormancy and other classes of dormancy and non-dormant seeds were derived from MD later. On the contrary, PY and combinational type PY + PD are the most phylogenetically restricted and derived classes. PY and combinational type PY + PD are the only types of dormancy what are not found in gymnosperms (Baskin and Baskin, 2004). These results are supported by a study that evaluates and compares the ratio of calculated embryo size to the seed size (E:S value) by generalized least square method in 179 angiosperm families. This comparison confirmed that dormancy with underdeveloped embryo is an ancestral that of seed plants and other dormancy classes probably evolved from it, because E:S values in the phylogenetic tree shows an increase from low E:S values to high E:S values (Forbis *et al*., 2002). According to a newer study, MPD is the most probable ancestral trait for seed plants. But the claim that, physical dormancy (imposed by impermeable seed coats) and non-dormancy status appears to be highly derived states, present primarily at the tips of the phylogeny, still hold true (Willis *et al*., 2014).

1.9. Pea domestication

Plant domestication process is an interesting phenomenon which began about 12,000– 10,000 years ago in the transition of people from hunter-gatherer to settled

agriculture (Sakuma *et al.*, 2011; Meyer *et al.*, 2012). The transformation of wild plant forms into modern crop plants is a result of both human and natural selection. Domesticated plants vary from their wild ancestors in several morphological and physiological traits. Set of these traits were named domestication syndrome (Hammer, 1984; Le Thierry D'Ennequin *et al.*, 1999; Zohary and Hopf, 2000). Most of the domestication syndrome traits are associated with loss of seed dispersal mechanisms (shattering), reduced seed dormancy, trends towards increasing number of seeds and fruits or its larger number, change of colouration, more compact growth habit, synchronous tillering and ripening (Fuller, 2007; Gepts, 2014). Biological diversity is not evenly geographically distributed. The origin centers of species diversity was initially suggested by Swiss botanist Alfonse de Candolle (1890), who was inspirated by Charles Darwin, and later redefined and expanded by Russian geneticist Nikolai I. Vavilov (1926). Vavilov proposed eight independent centers (Chinese, Indian, Ethiopian, Central Asiatic, Middle East, Mediterranean, South American, South Mexican and Central American) of origin world's cultivated plants (Abbo and Gopher, 2017).

Members of Fabaceae family have been domesticated simultaneously with cereals (Zohary and Hopf, 2000) and formed important dietary components of early civilizations. Pea, lentil, chickpea, faba bean and grass pea belong among the first domesticated legumes in the Fertile Crescent of Mesopotamian agriculture. Domestication of pea has been experimentally tested, both in order to determine the genetic basis which led to the cultivated crop from the wild plant (Weeden, 2007), as well as wild pea harvesting (Abbo *et al*., 2010). The most important domestication traits in pea relates to seed dormancy and pod dehiscence. Weeden (2007) has identified two to three loci involved in pea seed dormancy, mediated by testa thickness and structure of testa surface, while there are two loci in case of pod dehiscence. Total at least 11 loci involved in domestication-relared traits in pea have been identified (Weeden, 2007).

1.10. Dormancy in terms of adaptation

Seed dormancy is an adaptive trait, which is usually associated with geographical distribution of plants and fluctuating (i.e. seasonal) climate. The prevention of germination of certain proportion of seeds can reduce the risk of extinction once conditions turned

to unfavourable. The evolution of non-dormant seeds in climates with long growing seasons was shown recently indicating that the seed dormancy is linked with environment seasonality (Rubio de Casas *et al.*, 2017). Seasonal and diurnal oscilations in temperature are considered the main regulatory factor of annual dormancy cycling (Bewley *et al*., 2013), while in semiarid and arid regions with high inter annual rainfall variability, soil moisture is a major determinant for seedling emergence (Holst *et al*., 2007). Even though physical dormancy has been relatively well studied in agricultural conditions, much remains unknown about the mechanisms and reasons for interspecific and intraspecific variations of PY in natural ecosystems (Hudson *et al*., 2015). Studies considering both sources of variability together are rare. One example is the work in which were compared two tropical Fabaceae tree species (Lacerda *et al*., 2004). Seed banks are also very important for the conservation of endangered plant species as a life-history trait modulating habitat fragmentation.

Dormancy can also relate to seed size as alternative adaptive strategy. Study of many legume species showed that smaller dormant seeds are favoured in the environments with short growing season, whereas in predominate an aseasonal environments, large non dormant seeds dominate (Rubio de Casas *et al*., 2017). Large seeds usually contain enough storage substances and able to give rise to larger, stronger germinating plants that will perform better even in unsuitable conditions and therefore do not need to induce dormancy to avoid these conditions. However, on other hand seed size is critical in terms of predation. The smaller and dry seeds are difficult to find in the soil, while large seeds easily become animal feed (Goméz, 2004). Moreover, plants, which have bigger seeds, are able to produce smaller the number seeds.

2. Aims of the Thesis

The presented work focuses on study of pea seed dormancy, displaying typical physical dormancy type. At the centre of interest are the following aims:

- To compare seed dormancy in wild and cultivated pea genotypes (*Pisum sativum* subsp. *elatius* and subsp. *sativum).*
    a) Analyze structure of seed coat and other phenotypic characteristic of pea seeds.
    b) Detect differences in gene expression between wild and domesticated pea.
- To study seed dormancy in an ecological context.
    a) Describe germination of seeds in various experimental temperature settings.
    b) Determine which ecological factors may drive seed dormancy level (and germination response) in wild pea (*Pisum sativum* subsp. *elatius*).
    c) Test if there is an association between seed dormancy, seed coat anatomy and proanthocyanidin content.

3. Materials and methods

This section contains methods done by me and there were only mentioned in brief in published articles or those directly relevant to my own experimental work.

3.1. Plant material

For study comparing wild and domesticated pea four parental genotypes (Table 1), plus 126 recombinant inbred lines (RILs) of F6 generation of JI64 x JI92 and their reciprocal cross (North *et al*., 1989) were used. Set of 97 wild peas (*Pisum sativum* subsp. *elatius*) was selected for the study of seed dormancy in the context of ecological conditions. This material spans from the western Mediterranean, through the southeastern Europe to the Middle East (Table 2). These genotypes were chosen based on the passport data including GPS information and assessed for genetic diversity relationship (Smýkal *et al.*, 2017; Smýkal *et al*., 2018; Trněný *et al*., 2018).

Plants were grown in 5 litre pots with sand peat substrate (1:9) mixture (Florcom Profi, BB Com Ltd.), in glasshouse from January to May. Conditions in glasshouse were day/night temperature within the ranges 35-20/15-12 °C, and a natural photoperiod increasing from 6 to 14 h without supplementary light. After harvest, fully mature seeds were cleaned from pods, packed in paper bags and stored in room temperature. The germination testing was performed within the month from harvest. Seeds for transcriptomic analysis were harvested at several developmental stages (2, 3, 4 weeks and older) in case of parental genotypes, and in case of RIL lines, there were approximately one month old. The seed coat was separated from embryo, immediately frozen in liquid nitrogen and stored at – 70 °C until analysis.

Table 1: List of parental genotypes used for study comparing wild and domesticated pea.

Genotype	Taxonomy	Origin	Source	Status	Flower colour	Testa colouration
JI64	*Pisum elatius*	Turkey	JIC	wild	violet	pigmented
JI92	*Pisum sativum*	Afghanistan	JIC	cultivated	white	pigmented
VIR320	*Pisum elatius*	Israel	VIR	wild	violet	none
Cameor	*Pisum sativum*	France	INRA	cultivated	white	none

***JIC**...John Innes Centre Germplasm Resources Unit , Norwich, UK; **VIR**...Vavilov Institute Research of Plant Industry, St. Petersburg, Russia; **INRA**…National Institute for Agricultural Research, France.*

Table 2: Seed collection of wild peas.

Accession	Origin	Source - collector	Accession	Origin	Source - collector
711	Israel	VIR	**P013**	Turkey	VIR
713	Israel	VIR	**P014**	Turkey	VIR
714	Israel	VIR	**P016**	Turkey	VIR
721	Israel	VIR	**P017**	Turkey	VIR
722	Israel	VIR	**PI343973**	Turkey	USDA
IG140897	Armenia	ICARDA	**PI343974**	Turkey	USDA
IG108291	Tunisia	ICARDA	**PI343975**	Turkey	USDA
IG112136	Morocco	ICARDA	**PI343977**	Turkey	USDA
IG140562	Armenia	ICARDA	**PI343979**	Turkey	USDA
IG140971	Armenia	ICARDA	**PI343992**	Turkey	USDA
IG52414	Syria	ICARDA	**PI343994**	Turkey	USDA
IG52442	Syria	ICARDA	**PI344007**	Greece	USDA
IG52443	Syria	ICARDA	**PI344011**	Greece	USDA
IG52496	Turkey	ICARDA	**PI344537**	Italy	USDA
IG52508	Turkey	ICARDA	**PI344538**	Italy	USDA
IG52518	Turkey	ICARDA	**PI344539**	Italia	USDA
IG52520	Turkey	ICARDA	**PI639961**	Turkey	USDA
IG52532	Turkey	ICARDA	**PI560058**	Israel	USDA
IG52560	Jordane	ICARDA	**PI560059**	Israel	USDA
IG52565	Jordane	ICARDA	**PI560072**	Israel	USDA
IG52595	Algeria	ICARDA	**PI639955**	Israel	USDA
IG52596	Algeria	ICARDA	**PIS2845**	Italy	IPK
IG64350	Algeria	ICARDA	**PIS2850**	Italy	IPK
IS18911	Israel	IGB	**PIS2853**	Hungary	IPK
IS18912	Israel	IGB	**PIS7388**	France	IPK
IS18914	Israel	IGB	**PIS7475**	Macedonia	IPK
IS18915	Israel	IGB	**UPOL-Srb**	Serbia	P. Smýkal
IS19864	Israel	IGB	**T-1**	Turkey	P. Smýkal
IS22287	Israel	IGB	**T14/8**	Turkey	P. Smýkal
IS24324	Israel	IGB	**T15/2**	Turkey	P. Smýkal
JI1075	Turkey	JIC	**T15/5**	Turkey	P. Smýkal
JI1089	Turkey	JIC	**TUR-15-18-5**	Turkey	P. Smýkal
JI1092	Greece	JIC	**UPOL_ARM1**	Armenia	P. Smýkal
JI1794	Israel	JIC	**UPOL_ARM2**	Armenia	P. Smýkal
JI2055	Italy	JIC	**UPOL_ARM3**	Armenia	P. Smýkal
JI2105	Iran	JIC	**W6 2044**	Turkey	USDA
JI2201	Russia	JIC	**W6 26109**	Georgia	USDA
JI2546	Georgia	JIC	**W6 26112**	Georgia	USDA
JI262	Turkey	JIC	**W6 26127**	Georgia	USDA
JI2630	Russia	JIC	**T14/3**	Turkey	P. Smýkal
JI2724	Spain	JIC	**T14/4**	Turkey	P. Smýkal
JI3271	Italy	JIC	**T14/5**	Turkey	P. Smýkal
JI3276	Israel	JIC	**T14/6**	Turkey	P. Smýkal
JI3511	Georgia	JIC	**T14/9**	Turkey	P. Smýkal
JI3553	France	JIC	**T15/3**	Turkey	P. Smýkal
JI3557	Portugal	JIC	**T15/4**	Turkey	P. Smýkal
JI3558	Spain	JIC	**T15/6**	Turkey	P. Smýkal
JI64	Israel	JIC	**T15/7**	Turkey	P. Smýkal
			T-2	Turkey	P. Smýkal

VIR*…Vavilov Institute Research of Plant Industry, St. Petersburg, Russia;* ***ICARDA****…International Center for Agricultural Research in the Dry Areas, Beirut - Lebanon;* ***IGB****…Israel Plant Gene Bank, Bet Dagan,*

Israel; ***JIC****…John Innes Centre Germplasm Resources Unit, Norwich, UK;* ***USDA****…United States Department of Agriculture, Pullman, USA;* ***IPK****…Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany;* ***P. Smýkal****…P. Smýkal, UPOL, CZ.*

3.2. Transcriptomic part

RNA isolation

The total RNA was isolated from separated seed coats by BioTeke Plant Total RNA Extraction Kit (China) according to the manufacturer's protocol. Yield and purity of RNA was determined by using NanoDrop 2000 spectrophotometer (Thermo Scientific) and diluted in DEPC-H_2O to the concentration of 100 ng/µl. Isolated RNA was stored at – 70 °C. Before MACE analysis and cDNA synthesis, residual DNA was removed from samples by Baseline-ZERO^{TM} DNase kit (Epicenter) according to manufacturer's instruction.

Massive Analysis of cDNA Ends (MACE)

Since the developmental stage when relevant genes are expressed is unknown, the bulk of four developmental stages (2, 3, 4 weeks and older) for every parental genotype was created. In addition, two bulks of contrasting RILs (dormant and non-dormant) were made, each containing 7 independent lines. Each sample of bulk contained 5 µg of DNase treated RNA. The samples were sent to GenXPro GmbH (Frankfurt, Germany) which carried out MACE analysis. The procedure was described in Zawada *et al*. (2014) or briefly in Hradilová *et al*., 2017. In total, 6 cDNA libraries were prepared and sequenced, each providing around 10 million Illumina reads.

cDNA synthesis and quantitative real time PCR

Reverse transcription was carried out with Oligo$(dT)_{15}$ primer (Promega) using a two-step. In first step, 15 µl premix I, including 0.5 µM primer and 1 µg RNA and DEPC-H_2O, were incubated 5 minutes at 70 °C. This was followed by cooling on ice. In the second step, 25 µl premix II, containing 1x Reverse Transcription buffer (Promega), 500 µM dNTP mix, 40 U RNasin® Plus Ribonuclease Inhibitor (Promega), 10 U AMV Reverse Transcriptase (Promega) and DEPC-H2O, was added to premix I. All was incubated 60 minutes at 42 °C.

The gene expression analysis was conducted accurately as described in Hradilová *et al*., 2017. The expression of selected genes was studied at 2 developmental stages in 4 contrasting parental genotypes (JI64, JI92, Cameor and VIR320). The analysis was run on CFX96^{TM} Real-Time Detection System (Bio-Rad) using SensiFast SYBR® No-ROX kit (Bioline). The specific primers were designed by FastPCR program (Kalendar *et al*., 2014) and are shown in Table 3. Primers specificity was verified by observing melting curve (65-94 °C, recovered every 0.5 °C held for 0.5 s) and in addition on GelRed (Biotinum)

stained 2% agarose gel. Every PCR reaction included 2 µl cDNA (1:10 diluted cDNA), 5 µl 2x SensiFast SYBR and 400 nM of each primer mix in final volume 10 µl. Quantification of transcript level was determined by CFX Manager Software (Bio-Rad). All samples were investigated in triplicates and normalized to the expression of actin (Ferraro *et al.*, 2014) reference gene. Changes in transcript were estimated as fold change relative to the expression in the genotype Cameor (younger stage).

Table 3: List of primers used in qRT-PCR analysis.

MACE	Forward primer	Reverse primer	Lenght (bp)	Homologue
MACE-S019	ctgtaaaaggcttacctagtcctagcgacg	agtcctttgccttttcacccga	99	PsCam043335
MACE-S066	ggacttaatgatgagaagcccttcac	tgctaattcacctctgacgcaa	144	PsCam034740
MACE-S069	ttctgaagtcacttggcctcg	ggatacttaatgttgtcaggcgct	81	PsCam023145
MACE-S070	ttaccggaaggatatgcctcg	ccttggtgatgaagccatgcta	138	PsCam014274
MACE-S082	acagtggctgccgaacttgtt	actggaaggcggctttgccat	133	PsCam054796
MACE-S101	tcgacaatgaagatggcacttcc	atgcaactaaagcaacctttcgtgg	132	PsCam049497
MACE-S108	tggaagggcaagaagatctccga	tgatctccctggacgcaagtc	95	PsCam042835
MACE-S110	gtgaaagagcttgcattccctgga	ggttgagcatctgcaaagtgg	89	PsCam049778
MACE-S111	ctgtgaaaggaaatgttaaggcagagc	ttcaggctccacccagaatgc	86	PsCam060235
MACE-S131	ccaacaagatcaggctcatgtg	gaaagaccagtttcagcgtcaa	77	PsCam016941
MACE-S132	tatcttccatttggtggaggtcca	ccgtcgaacaagcattgcaagagct	93	PsCam016688
MACE-S135	ggcatcctgctaaacctagtgaa	aaacgcacaaccgtgctcctt	87	PsCam028250
MACE-S139	tagtacccgcttactcatgggct	attgtagcgcgagacaagac	114	PsCam042999
MACE-S141	tcttgccagcagaatgtcac	gtcaaagaatagaacttgtatacatatc	220	PsCam021134

3.3. Seed germination tests

Study of seed germination variation in wild pea

Seed were tested the in two oscillating temperatures of 25/15 °C and 35/15 °C in dark at 14h/10h (day/night) regimes. Intact seeds (25 seeds per experiment in 2 replicates per treatment) were placed on two pieces of water saturated filter papers (Whatman Grade 1, Sigma) in 90 mm Petri dishes (P-Lab) in temperature controlled chambers (Laboratory Incubator ST4, BioTech CZ). Due to prolong testing (28 days), there was necessity to apply fungicide (Maxim XL 035 FS; containing metalaxyl 10 g and fludioxonil 25 g) to prevent fungal growth (we cannot sterilize seeds to avoid alteration of seed coat properties). Seeds were monitored at 24h intervals for 28 days and scored number of imbibing and also germinating seeds. During the scoring, the plates were randomly changed in positions

and water with fungicide was added as needed usually every 3rd day. Seeds were considered imbibed, when there was visible swelling and germinated when radicle protruded from testa. Germinated seeds were removed from the Petri dishes. After 28 days seed coat of non-imbibing seeds was mechanically disrupted by sandpaper to test for seed viability. Before real experiment the comparison of control and fungicide containing samples was carried out and this did not show significant difference.

The data (daily counts of germinated seeds) were analysed using spline function conducted by the colleagues from Department of Mathematical Analysis and Applications of Mathematics, UP Olomouc as described in Hradilová *et al*. (2019).

Comparative study of wild and domesticated pea seed germination

The seeds of four parental genotypes (wild JI64 and VIR320; cultivated JI92 and Cameor) and 126 RILs of F_6 generation of JI64 x JI92 and reciprocal cross, were tested. In this case, Petri dishes with samples (25 seeds per line or with minimum 10 seeds in event some RIL lines) were incubated at 25 °C for 7 days in dark and filter papers was saturated with water. Seed imbibition and germination was scored daily.

After testing, percentage of germination, coefficient of velocity, Timpson index and mean germination time were calculated according to (Kader, 2005; Ranal and Santana, 2006).

3.4. Weight of seeds and seed coat thickness measurements

The weight of 25 seeds per accession with three replicas was recorded and converted to hundred seeds weight (HSW).

Testa was dissected/peeled off from dry seed and thickness was measured by micrometer (0–25 mm, precision 0.01 mm, Hommel Hercules, Germany) and scanning electron microscope (VEGA3 LMU, Tescan) without the need for metal coating.

3.5. Quantification of proanthocyanidins in seed coat

Seed coats were separated from dry seeds and freeze before use. There were analysed both soluble proanthocyanidins (PAs, in acetone and 4-dimethylaminocinnamaldehyde [DMACA] reagent) and insoluble PAs (in butanol-HCl) using the method described by Pang *et al.* (2008). The procedure was exactly same as described in Hradilová *et al*. (2019).

4. Results

This section contains only brief results in the form of abstracts, complete results/articles are embedded in appendixes.

The key results of this dissertation are included in the three attached articles. First two papers (**Paper I and Paper II**) summarize differences in gene expression, seed coat structure, and metabolites composition between wild and cultivated pea seeds coats in relation to one of the crucial domestication traits: seed dormancy. This study was conducted within the Grant Agency of Czech Republic project named: *Seed dormancy and pod dehiscence as the key domestication traits in legumes* (Project number 14-11782S, 2014-2016). Third article (**Paper III**) focuses to test association between the seed dormancy, seed properties and environmental factors. Finding the information about dormancy in terms of adaptation was the target of project named: *Ecological genomic approaches to uncovering the adaptive significance of seed dormancy in legumes* (Project number 16-21053S, 2016-2018).

4.1. Paper I (Appendix I)

HRADILOVÁ I., TRNĚNÝ O., VÁLKOVÁ M., CECHOVÁ M., JANSKÁ A., PROKEŠOVÁ L., AAMIR K., KREZDON N., ROTTER B., WINTER P., VARSHNEY R. K., SOUKUP A., BEDNÁŘ P., HANÁČEK P. AND SMÝKAL P. (2017): A combined comparative transcriptomic, metabolomic and anatomical analyses of two key domestication traits: pod dehiscence and seed dormancy in Pea (*Pisum sp*.). Frontiers in Plant Sciences 8: 542.

Abstract

The origin of the agriculture was one of the turning points in human history, and a central part of this was the evolution of new plant forms, domesticated crops. Seed dispersal and germination are two key traits which have been selected to facilitate cultivation and harvesting of crops. The objective of this study was to analyze anatomical structure of seed coat and pod, identify metabolic compounds associated with water-impermeable seed coat and differentially expressed genes involved in pea seed dormancy and pod dehiscence. Comparative anatomical, metabolomics, and transcriptomic analyses were carried out on wild dormant, dehiscent *Pisum elatius* (JI64, VIR320) and cultivated, indehiscent *Pisum sativum* non-dormant (JI92, Cameor) and recombinant inbred lines (RILs). Considerable differences were found in texture of testa surface, length of macrosclereids, and seed coat thickness. Histochemical and biochemical analyses indicated genotype related variation in composition and heterogeneity of seed coat cell walls within macrosclereids. Liquid chromatography–electrospray ionization/mass spectrometry and Laser desorption/ionization–mass spectrometry of separated seed coats revealed significantly higher contents of proanthocyanidins (dimer and trimer of gallocatechin), quercetin, and myricetin rhamnosides and hydroxylated fatty acids in dormant compared to non-dormant genotypes. Bulk Segregant Analysis coupled to high throughput RNA sequencing resulted in identification of 770 and 148 differentially expressed genes between dormant and non-dormant seeds or dehiscent and indehiscent pods, respectively. The expression of 14 selected dormancy-related genes was studied by qRT-PCR. Of these, expression pattern of four genes: porin (MACE-S082), peroxisomal membrane PEX14-like

protein (MACE-S108), 4-coumarate CoA ligase (MACE-S131), and UDP-glucosyl transferase (MACE-S139) was in agreement in all four genotypes with Massive analysis of cDNA Ends (MACE) data. In case of pod dehiscence, the analysis of two candidate genes (*SHATTERING* and *SHATTERPROOF*) and three out of 20 MACE identified genes (MACE-P004, MACE-P013, MACE-P015) showed down-expression in dorsal and ventral pod suture of indehiscent genotypes. Moreover, MACE-P015, the homolog of peptidoglycan-binding domain or proline-rich extensin-like protein mapped correctly to predicted *Dpo1* locus on PsLGIII. This integrated analysis of the seed coat in wild and cultivated pea provides new insight as well as raises new questions associated with domestication and seed dormancy and pod dehiscence.

Keywords: domestication, legumes, pea (*Pisum sativum*), metabolites, pod dehiscence, seed dormancy, seed coat, transcriptomics

4.2. Paper II (Appendix II)

CECHOVÁ M., VÁLKOVÁ M., **HRADILOVÁ I.**, JANSKÁ A., SOUKUP A., SMÝKAL P., BEDNÁŘ P. (2017): Towards better understanding of pea seed dormancy using laser desorption/ ionization mass spectrometry. International Journal of Molecular Sciences 18: 2196.

Abstract

Seed coats of six pea genotypes contrasting in dormancy were studied by laser desorption/ionization mass spectrometry (LDI-MS). Multivariate statistical analysis discriminated dormant and non-dormant seeds in mature dry state. Separation between dormant and non-dormant types was observed despite important markers of particular dormant genotypes differ from each other. Normalized signals of long-chain hydroxylated fatty acids (HLFA) in dormant JI64 genotype seed coats were significantly higher than in other genotypes. These compounds seem to be important markers likely influencing JI64 seed imbibition and germination. HLFA importance was supported by study of recombinant inbred lines (JI64xJI92) contrasting in dormancy but similar in other seed properties. Furthermore, HLFA distribution in seed coat was studied by mass spectrometry imaging. HLFA contents in strophiole and hilum are significantly lower compared to other parts indicating their role in water uptake. Results from LDI-MS experiments are useful in understanding (physical) dormancy (first phases of germination) mechanism and properties related to food processing technologies (e.g., seed treatment by cooking).

Keywords: pea, fatty acid, seed coat, seed dormancy, seed hardness, laser desorption-ionization mass spectrometry, imaging mass spectrometry, multivariate statistics

4.3. Paper III (Appendix III)

HRADILOVÁ I., DUCHOSLAV M., BRUS J., PECHANEC V., HÝBL M., SMRŽOVÁ L., ŠTEFELOVÁ N., VÁCLAVEK T., BARIOTAKIS M., MACHALOVÁ J., HRON K., PIRINTHOS S., SMÝKAL P. (2019): Variation in wild pea (*Pisum sativum* subsp. *elatius*) seed dormancy and its relationship to the environment and seed coat traits. PeerJ 7: e6263.

Abstract

Seed germination is one of the earliest key events in the plant life cycle. The timing of transition from seed to seedling is an important developmental stage determining the survival of individuals that influences the status of populations and species. Because of wide geographical distribution and occurrence in diverse habitats, wild pea (*Pisum sativum* subsp. *elatius*) offers an excellent model to study physical type of seed dormancy in an ecological context. This study addresses the gap in knowledge of association between the seed dormancy, seed properties and environmental factors, experimentally testing oscillating temperature as dormancy release clue.

Seed total germination ranged from 0% to 100%. Cluster analysis of germination patterns of seeds under two temperature treatments differentiated the accessions into three groups: (1) non-dormant (28 accessions, mean germination of 92%), (2) dormant at both treatments (29 acc., 15%) and (3) responsive to increasing temperature range (41 acc., with germination change from 15 to 80%). Seed coat thickness differed between groups with dormant and responsive accessions having thicker testa (median 138 and 140 μm) than non-dormant ones (median 84 mm). The total PA content showed to be higher in the seed coat of dormant (mean 2.18 $mg.g^{-1}$) than those of non-dormant (mean 1.77 $mg.g^{-1}$) and responsive accessions (mean 1.87 $mg.g^{-1}$). Each soil and bioclimatic variable and also germination responsivity (representing synthetic variable characterizing germination pattern of seeds) was spatially clustered. However, only one environmental variable (BIO7, i.e., annual temperature range) was significantly related to germination responsivity. Non-dormant and responsive accessions covered almost whole range of BIO7 while dormant accessions are found in the environment with higher annual temperature, smaller temperature variation, seasonality and milder winter. Ecological niche modelling showed a more localized potential distribution of dormant group. Seed dormancy in the wild pea might be part of a bet-hedging mechanism for areas of the Mediterranean basin with more unpredictable water availability in an otherwise seasonal environment. This study provides the framework for analysis of environmental aspects of physical seed dormancy.

Keywords: dormancy, seed coat, proanthocyanidins, testa, pea, niche-modelling, pea, temperature oscillations, germination, legumes

5. Conclusions

5.1. Differences in seed coat structure, metabolites composition and gene expression between wild and cultivated pea seeds coats

Comparative anatomical, metabolomic and transcriptomic analyses were carried out on wild dormant *Pisum elatius* (JI64 and VIR320) and domesticated non-dormant *Pisum sativum* (JI92 and Cameor) and 126 pieces of recombinant inbred lines (RILs). Significant differences were found in the texture of testa surface, length of macrosclereids and seed coat thickness. Seed coat thickness was analysed by two independent methods: by micrometer and by microscopic measurement (by light microscope and scan electron microscope). Both methods established, dormant genotypes had considerable thicker testa compare to non-dormant, which might contribute to water impermeability of seed coat. Notable is the absence of gritty (rough) surface in wild VIR320 despite that this genotype proves strong seed dormancy. Similarly, recombinant inbred lines established from crosses of wild JI64 and domesticated JI92 do not have any statisticall relationship between seed coat surface structure and thickness and dormancy status. All together, this point out that there is no direct relationship between this trait and dormancy as previously postulated (Weeden, 2007). Seeds of cultivated genotypes (JI92 and Cameor) germinated very quickly (within 24 h), while wild pea seeds remain highly dormant and imbibe and germinate only to a certain level (in case of JI64 at 8% and VIR320 at 30%) after 7 days. Likewise, mean germination time, Timpson index and coefficient of velocity were different in parental genotypes. RIL lines displayed more variability in each of the respective measures. Percentage of germinated had wide range from 4 to 100 %, similarly coefficient of velocity from 0.61 to 4.44 or mean germination time from 1 to 7 (Figure S1). All these parameters indicate complexity of germination process as none single is sufficient to fully describe any line. As shown in Table S1, dormant bulk with average testa thickness 135 μm had on average 22% germination over 7 days, contrast to non-dormant bulk with average testa thickness 107 μm having 56%.

However, thickness or length of macrosclereids alone does not necessarily imply for water impermeability. Different chemical composition may also play a certain role. New knowledge about the chemical composition seed coat of wild and cultivated peas was provided by chemical analysis based on combination of ultra-performance liquid

chromatography with high resolution tandem mass spectrometry, laser desorption-ionization mass spectrometry. Dimer and trimer of gallocatechin, quercetin, myricetin rhamnosides and hydroxylated long chain fatty acids ranging from C26 to C28 (such as mono and dihydroxylated hexacosanoate, heptacosanoate and octacosanoate) were found in higher concentration in dormant peas compared to non-dormant. Similarly, higher signals of hydroxylated fatty acids with long chain were found in dormant RIL lines.

Bulk Segregant Analysis (BSA) coupled to high throughput RNA sequencing using Massive Analysis of cDNA Ends (MACE) approaches resulted in finding of altogether 770 differentially expressed genes (DEGs) between wild and cultivated genotypes. To valid MACE results, expression levels of selected genes was analysed by qRT-PCR. From fourteen selected genes, expression pattern of only four genes were in agreement with MACE data. One of the reasons for disagreement between MACE and qRT-PCR analysis is the bulking of different developmental stages or from various genotypes in case of RILs, which could also result in masking of DEGs. In addition, some imprecision in phenotypic classification and low number of RIL lines used for bulking, could contribute to the results. The most of DEGs belonged to phenylpropanoid and flavonoid biosynthetic pathways. For example, enzyme 4-coumarate CoA ligase (MACE-S131), which catalyzes conversion 4-coumarate and other derivatives to corresponding esters serving to create precursors for lignin, suberin and flavonoids which were shown to contribute to impregnation of the cell wall.

Future research facilitated by progress in sequencing technologies and genome availability focusing on differences in gene expression and metabolites content in seed coat during its development compare is needed to elucidate the mechanisms contributing to seed dormancy.

5.2. Wild pea seed dormancy as adaptation to environment

Testing of association between seed dormancy, seed properties and environmental factors was studied. Seed dormancy, which is manifested by impermeabile seed coat, is typical of wild legume species, including wild pea. This trait has been lost during domestication (Hradilová *et al.,* 2017; Janská *et al*., 2019) but it is variable also within wild pea. Ninety-seven pea accessions were monitored over the period of 28 days, the final percentage of germinated seeds ranged from 0% to 100%. The responses at two temperature

regimes (25/15 °C and 35/15 °C) were used to cluster samples into three main groups. One was dormant (D) with 27 accessions (mean germination up to 15%), second was non-dormant (N) with 29 representatives (mean germination of 92%) and third was responsive group (R) which reacted to increasing temperature range, with change of germination from 15% to 80%. The later group was further divided two subgroups R1 (8 accessions) and R2 (33 accessions).

Furthermore, relationships between germination and environmental conditions (bioclimatic and soil variables) at the site of source were studied using principal component analysis (PCA) and ecological niche modelling. As result of these analysis, only one environmental variable was was found to be in relation to the dormancy level, annual temperature range (BIO7). The non-dormant and, also responsive genotypes were found within broader range of BIO7 value, whereas dormant accessions occurred in the environment with higher annual temperature, milder winter, smaller temperature variation and seasonality.

There was dependence of seed coat thickness on seed dormancy using a statistical analysis. Dormant and responsive accessions had thicker testa (median 138 and 140 μm) than non-dormant ones (median 84 mm). In the case of seed pigmentation, it also appears that there is a correlation between the seed dormancy and quantity of pigments present in the seed coat (proanthocynidins, PAs). The total PA content showed to be lower in the seed coat of non-dormant (mean 1.77 $mg.g^{-1}$) than those of dormant (mean 2.18 $mg.g^{-1}$). The amount of proanthocynidins in responsive accessions was between the content of dormant and non-dormant (mean 1.87 $mg.g^{-1}$), with value a closer to the non-dormant samples. Nevertheless, in analysis Hradilová *et al.* (2019) only total content of PAs was measured and it remains to be tested if there are any specific PA compounds related to dormancy levels in wild pea. There were no relationship between seed size and the level of seed dormancy.

In this study we used new approach based on functional data analysis and this can be recommended as a new analytical tool in seed dormancy/germination studies. Although it was established, that temperature is the main driving force of dormancy release, a mechanistic and molecular mechanism are still lacking.

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7. Supplements

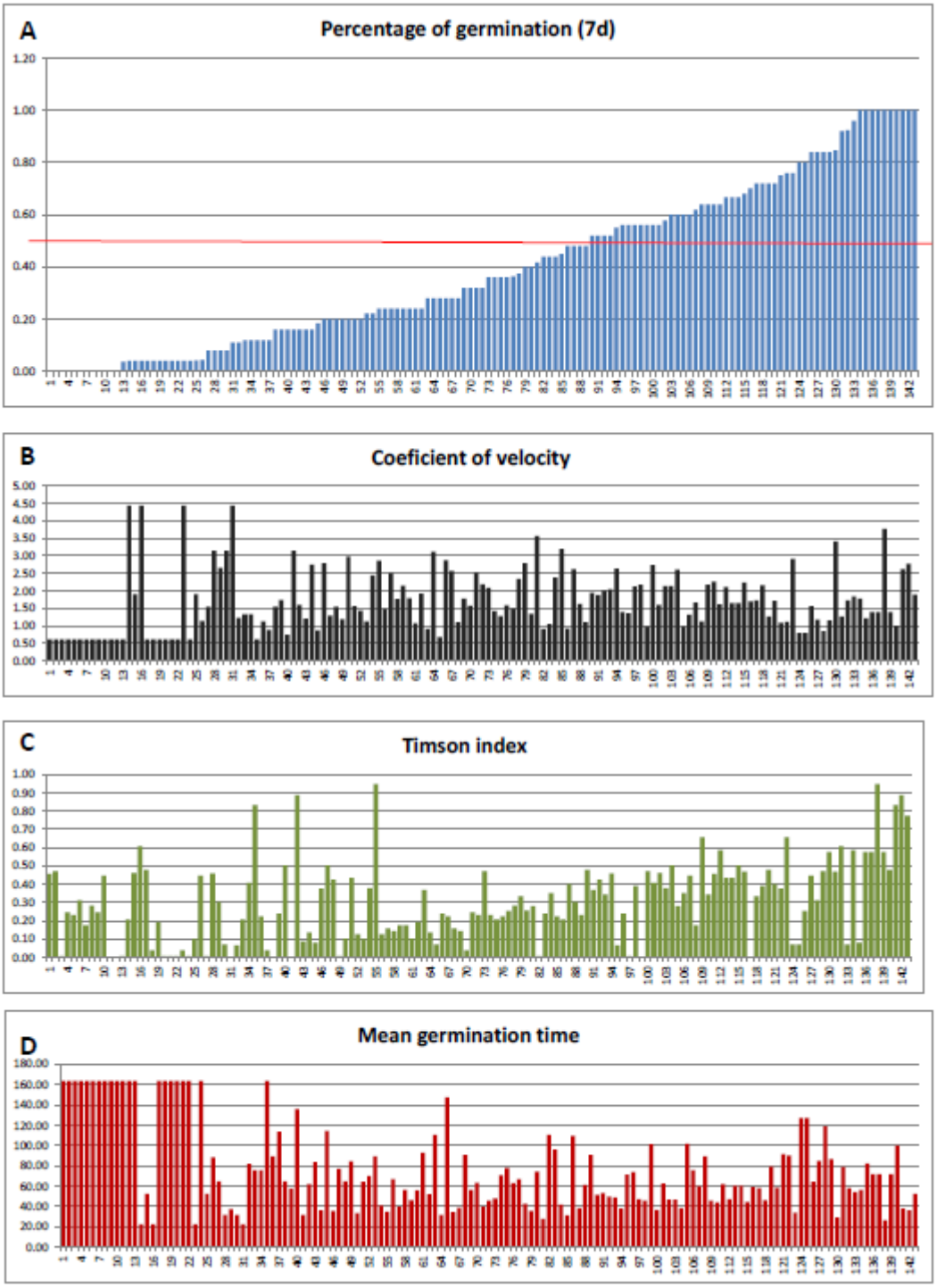

Figure S1: Germination indexes of 126 RIL lines (tested 25 °C over the period of 7 days). Ordered by cumulative germination percentages **(A)** with shown coefficient of velocity **(B)**, Timson index **(C)** and mean germination time **(D)**.

Table S1: Germination characteristics of parental and RIL lines.

DORMANT BULK			Germination percentage								
RIL lines	Gritty trait	testa thickness (mm)	0h	24h	48h	52h	88h	163h	Coeficient of velocity	Timson index	Mean germination time
42 AC	0	140	0	20	32	52	52	56	2,12	0,01	47,25
21 C	0	120	0	8	16	20	20	20	2,79	0,38	35,90
3 C	1	150	0	0	0	8	16	28	0,91	0,37	110,36
48 C	0	150	0	0	0	0	0	0	0,61	0,23	163,50
11 C	1	120	0	0	0	0	0	0	0,61	0,46	163,50
87 C	1	150	0	0	0	0	0	0	0,61	0,18	163,50
56 AC	1	120	0	0	16	52	52	52	2,04	0,34	48,96
Mean		**136**	**0**	**4**	**9**	**19**	**20**	**22**	**1,38**	**0,28**	**104,71**

NON-DORMANT BULK			Germination percentage								
RIL lines	Gritty trait	testa thickness (mm)	0h	24h	48h	52h	88h	163h	Coeficient of velocity	Timson index	Mean germination time
32 C	0	100	0	16	24	40	40	56	1,35	0,24	74,00
30 BC	0	100	0	24	40	40	44	56	1,59	0,41	62,71
40 C	0	110	0	12	12	28	32	32	2,19	0,23	45,75
54 AC	0	120	0	20	25	60	60	70	1,70	0,47	58,96
49 C	1	110	0	19	35	73	85	92	1,72	0,61	58,08
10 C	0	100	0	32	36	44	52	64	1,61	0,46	62,09
57 BC	1	110	0	0	7	7	7	19	0,87	0,08	114,50
Mean		**107**	**0**	**18**	**26**	**42**	**46**	**56**	**1,58**	**0,36**	**68,02**

PARENTAL GENOTYPES			Germination percentage								
Genotypes	Gritty trait	testa thickness (mm)	0h	24h	48h	52h	88h	163h	Coeficient of velocity	Timson index	Mean germination time
JI64	1	180	0	0	0	0,04	10	20	0,7	0,0	112,3
JI92	0	100	0	100	100	100	100	100	3,8	1,0	24,0

8. Appendixes

Appendix I (Paper I)

HRADILOVÁ I., TRNĚNÝ O., VÁLKOVÁ M., CECHOVÁ M., JANSKÁ A., PROKEŠOVÁ L., AAMIR K., KREZDON N., ROTTER B., WINTER P., VARSHNEY R. K., SOUKUP A., BEDNÁŘ P., HANÁČEK P. AND SMÝKAL P. (2017): A combined comparative transcriptomic, metabolomic and anatomical analyses of two key domestication traits: pod dehiscence and seed dormancy in Pea (*Pisum sp.*). Frontiers in Plant Sciences 8: 542.

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A Combined Comparative Transcriptomic, Metabolomic, and Anatomical Analyses of Two Key Domestication Traits: Pod Dehiscence and Seed Dormancy in Pea (*Pisum* sp.)

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The origin of the agriculture was one of the turning points in human history, and a central part of this was the evolution of new plant forms, domesticated crops. Seed dispersal and germination are two key traits which have been selected to facilitate cultivation and harvesting of crops. The objective of this study was to analyze anatomical structure of seed coat and pod, identify metabolic compounds associated with water-impermeable seed coat and differentially expressed genes involved in pea seed dormancy and pod dehiscence. Comparative anatomical, metabolomics, and transcriptomic analyses were carried out on wild dormant, dehiscent *Pisum elatius* (JI64, VIR320) and cultivated, indehiscent *Pisum sativum* non-dormant (JI92, Cameor) and recombinant inbred lines (RILs). Considerable differences were found in texture of testa surface, length of macrosclereids, and seed coat thickness. Histochemical and biochemical analyses indicated genotype related variation in composition and heterogeneity of seed coat cell walls within macrosclereids. Liquid chromatography–electrospray ionization/mass spectrometry and Laser desorption/ionization–mass spectrometry of separated seed coats revealed significantly higher contents of proanthocyanidins (dimer and trimer of gallocatechin), quercetin, and myricetin rhamnosides and hydroxylated fatty acids in dormant compared to non-dormant genotypes. Bulk Segregant Analysis coupled to high throughput RNA sequencing resulted in identification of 770 and 148 differentially expressed genes between dormant and non-dormant seeds or dehiscent and indehiscent pods, respectively. The expression of 14 selected dormancy-related genes was studied by qRT-PCR. Of these, expression pattern of four genes: porin (MACE-S082), peroxisomal membrane PEX14-like protein (MACE-S108), 4-coumarate CoA ligase (MACE-S131), and UDP-glucosyl transferase (MACE-S139) was in

agreement in all four genotypes with Massive analysis of cDNA Ends (MACE) data. In case of pod dehiscence, the analysis of two candidate genes (*SHATTERING* and *SHATTERPROOF*) and three out of 20 MACE identified genes (MACE-P004, MACE-P013, MACE-P015) showed down-expression in dorsal and ventral pod suture of indehiscent genotypes. Moreover, MACE-P015, the homolog of peptidoglycan-binding domain or proline-rich extensin-like protein mapped correctly to predicted *Dpo1* locus on PsLGIII. This integrated analysis of the seed coat in wild and cultivated pea provides new insight as well as raises new questions associated with domestication and seed dormancy and pod dehiscence.

Keywords: domestication, legumes, pea (*Pisum sativum*), metabolites, pod dehiscence, seed dormancy, seed coat, transcriptomics

INTRODUCTION

The origin of the agriculture was one of key points in human history, and a central part of this was the evolution of new plant forms, domesticated crops (Meyer et al., 2012; Fuller et al., 2014). The transformation of wild plants into crop plants can be viewed as an accelerated evolution, representing adaptations to cultivation and human harvesting, accompanied by genetic changes (Lenser and Theißen, 2013; Olsen and Wendel, 2013; Shi and Lai, 2015). Common set of traits have been recorded for unrelated crops (Hammer, 1984; Zohary and Hopf, 2000; Lenser and Theißen, 2013). These include loss of germination inhibition and loss of natural seed dispersal (Fuller and Allaby, 2009). The identity of some responsible genes has been revealed (reviewed in Meyer and Purugganan, 2013) through association mapping and genome sequencing, for example in soybean (Zhou et al., 2015), chickpea (Bajaj et al., 2015; Kujur et al., 2015), and common bean (Schmutz et al., 2014).

Members of the *Fabaceae* family have been domesticated in parallel with cereals (Smartt, 1990; Zohary and Hopf, 2000) or possibly even earlier (Kislev and Bar-Yosef, 1988) resulting in largest number of domesticates per plant family (Smýkal et al., 2015). Despite of crucial position of legumes, as protein crops, in human diet as well as crop rotation systems (Foyer et al., 2016), comparably little is known on their domestication. Pea (*Pisum sativum* L.) is one of the world's oldest domesticated crops and is still globally important grain legume crop (Smýkal et al., 2012, 2015). Experimental cultivation of wild peas have demonstrated that both seed dormancy and pod dehiscence cause poor crop establishment via reduced germination as well as dramatic yield losses via seed shattering (Abbo et al., 2011). The loss of fruit shattering has been under selection in the most seed crops, to facilitate seed harvest (Fuller and Allaby, 2009; Purugganan and Fuller, 2009), while in wild plants, shattering is a fundamental trait to assure seed dispersal (Bennett et al., 2011). Orthologous genes and functions were found to be conserved for seed shattering mechanisms between mono and dicotyledonous plants (Konishi et al., 2006). Recently, two genes have been identified to be involved in pod dehiscence in soybean. One of them is the dirigent-like protein (*Pdh1*) promoting pod dehiscence by increasing the torsion of dried pod walls, which serves as a driving force for pod dehiscence under low humidity (Funatsuki et al., 2014). The functional gene *Pdh1* was highly expressed in the lignin-rich inner sclerenchyma of pod walls. Yet, another NAC family gene SHATTERING1-5 (Dong et al., 2014) activates secondary wall biosynthesis and promotes the significant thickening of fiber cap cells of the pod ventral suture secondary walls. The differences between wild and cultivated soybean is within promoter region and subsequently expression level (Dong et al., 2014).

Timing of seed germination is one of the key steps in plant life. Seed dormancy is considered as a block to the completion of germination of an intact viable seed under favorable conditions (Baskin and Baskin, 2004; Weitbrecht et al., 2011). In the wild, many seeds will only germinate after certain conditions have passed, or after the seed coat is physically disrupted (Bewley, 1997; Baskin et al., 2000; Finch-Savage and Leubner-Metzger, 2006; Bewley et al., 2013). In contrast, crops were selected to germinate as soon as they are wet and planted (Weitbrecht et al., 2011). Moreover, easy seed imbibition has crucial role in cooking ability of most grain legumes. Hence, reducing seed coat thickness led to a concurrent reduction of seed coat impermeability during the domestication (Smýkal et al., 2014). Seed dormancy had played a significant role in evolution and adaptation of plants, as it determines the outset of a new generation (Nonogaki, 2014; Smýkal et al., 2014). A diverse dormancy mechanisms has evolved in keeping with the diversity of climates and habitats (Nikolaeva, 1969; Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). In contrast to hormone mediated seed dormancy extensively studied in *Arabidopsis* or cereals, we have very little knowledge on physical dormancy, as found in legumes (Baskin and Baskin, 2004; Graeber et al., 2012; Radchuk and Borisjuk, 2014). Although hard-seededness was largely overcome in all domesticated grain legumes except of fodder legumes (Werker et al., 1979; Smartt, 1990; Weeden, 2007), it appears in lentil or soybean depending on the cultivation conditions. Physical seed dormancy is caused by one or more water-impermeable cell layers in seed coat (Baskin et al., 2000; Koizumi et al., 2008; Weitbrecht et al., 2011; Radchuk and Borisjuk, 2014; Smýkal et al., 2014). Numerous *transparent testa* (*tt*) and *tannin deficient seed* (*tds*) mutants (Appelhagen et al., 2014) indicates the important role of proanthocyanidins and flavonoid pigments in *Arabidopsis* (Graeber et al., 2012) and *Medicago* (Liu et al., 2014) testa development. In *Arabidopsis*

and *Melilotus*, seed permeability is altered due to in mutation in extracellular lipid biosynthesis (Beisson et al., 2007). Similarly, in the *M. truncatula* transcriptomic data set (Verdier et al., 2013a), four of 12 Glycerol-3-phosphate acyltransferases (GPAT) genes were identified as putative orthologs of those reported in soybean (Ranathunge et al., 2010). Furthermore, cells of the outer integument in *M. truncatula* and pea showed abundant accumulation of polyphenolic compounds; which upon oxidation may impact seed permeability (Marbach and Mayer, 1974; Werker et al., 1979; Moïse et al., 2005). Seed dormancy was identified as monogenic trait in mungbean (Isemura et al., 2012); while six QTLs were detected in yardlong and rice bean (Kongjaimun et al., 2012). In pea, Weeden (2007) has identified two to three loci involved in seed dormancy, via testa thickness and structure of testa surface. Two genes involved in seed coat water permeability were recently identified in soybean. One of them, *GmHs1-1*, encodes a calcineurin-like metallophosphoesterase transmembrane protein, which is primarily expressed in the Malpighian layer (macrosclereids) of the seed coat and is associated with calcium content (Sun et al., 2015). Independently of this, *qHS1*, a quantitative trait locus for hardseededness in soybean, was identified as endo-1,4-β-glucanase (Jang et al., 2015). This genes seems to be involved in the accumulation of β-1,4-glucan derivatives that reinforce the impermeability of seed coats in soybean. Interestingly, both genes are positioned closely to each other of soybean chromosome 2.

Development of pea and particularly model legume *Medicago truncatula* seeds have been well-characterized at anatomical (Hedley et al., 1986; Wang and Grusak, 2005) and also transcriptomic and proteomic levels (Gallardo et al., 2007; Verdier et al., 2013a). RNA sequencing (RNA-seq) was used to study changes in gene expression, including *M. truncatula* (Benedito et al., 2008), *Medicago sativa* (Zhang et al., 2015), soybean (Severin et al., 2010; Patil et al., 2015), faba bean (Kaur et al., 2012), *Lotus japonicus* (Verdier et al., 2013b) and chickpea (Pradhan et al., 2014). In pea, transcriptome studies involved vegetative tissues (Franssen et al., 2011), including pods and seeds (Kaur et al., 2012; Duarte et al., 2014; Liu et al., 2015; Sudheesh et al., 2015), and nodules (Zhukov et al., 2015). Seed coat transcriptome of pea cultivars was analyzed in relation to proanthocyanidin pathway (Ferraro et al., 2014) and seed aging (Chen et al., 2013). Moreover, there is pea RNA-seq gene atlas for 20 cDNA libraries including different developmental stages and nutritive conditions (Alves-Carvalho et al., 2015). Comparative transcriptomics study in relation to domestication trait was conducted recently by Zou et al. (2015) in relation to glume and threshing in wheat. Some of the down-regulated genes in domesticated wheat were related to the biosynthetic pathways that apparently define the mechanical strength of the glumes, such as cell wall, lignin, pectin, and wax biosynthesis. Several of so far identified genes underlying key domestication traits (reviewed in Meyer and Purugganan, 2013) are regulated at transcriptional level with altered spatial and temporal expression, such as seed-shattering (*qSH1*) locus disrupting the development of the abscission zone between grains and pedicles in rice (Konishi et al., 2006) or *teosinte branched* (*tb1*) gene causing single stem growth in maize crop (Doebley et al., 1997).

In the present study, we used comparative transcriptomic, anatomical, and metabolite analysis to detect the differences in gene expression, seed coat structure, and metabolites composition between wild and domesticated pea seed coats in relation to one of the two key domestication traits: seed coat and transcriptomic and anatomical analyses of pod dehiscence.

MATERIALS AND METHODS

Plant Material

Four parental genotypes included wild *P. elatius* JI64 from Turkey and cultivated Afghan landrace *P. sativum* JI92 both from John Innes Pisum Collection (Norwich, UK); wild *P. elatius* VIR320 (Bogdanova et al., 2012) from Vavilov Institute Research of Plant Industry (St. Petersburg, Russia) and cultivated *P. sativum* cv. Cameor from INRA France. Furthermore, 126 $F_{5:6}$ recombinant inbred lines (RILs) derived from JI64 and JI92 cross (North et al., 1989) were used to establish respective phenotypically contrasting (dormant vs. non-dormant, dehiscent vs. indehiscent) bulks. *P. elatius* VIR320 differs from other wild peas in relation to the absence of gritty and testa pigmentation, possibly as the result of being either semi-domesticate or hybrid between wild and cultivated pea with unknown origin (Bogdanova et al., 2012).

Seed Water Uptake and Germination Assays

The seeds of four parental and 126 RILs of F_6 generation of JI64 (wild) × JI92 (cultivated) and reciprocal (RILs) were harvested from glasshouse grown plants (February–May 2015). Twenty five seeds per line were incubated in petri dishes (9 cm diameter) over two layers of medium speed qualitative filter papers (Whatman, grade 1) wetted with 3 ml of tap water and incubated in a 25°C incubator with darkness. Imbibition was scored at 24 h intervals based on changes in seed swelling and germination was determined based on the radicle breaking through seed coat. The percentage, Mean germination time (MGT), Timson index (TI), and Coefficient of Velocity (CV) were calculated over 7 days period. We have used these various mathematical measurements in order to more precisely describe germination process as shown by Ranal and Santana (2006).

Determination of Pod Dehiscence

Pod dehiscence was measured either by direct observation of the pods on the plant or by drying harvested pods at room temperature (Weeden et al., 2002; Weeden, 2007). In case of parental lines JI92 (domesticated, indehiscent pod) and JI64 (wild, dehiscent pod) are dehiscence/indehiscence obvious after pod maturing. On the other hand evaluation of RILs was difficult in some cases, as slight pressure on pods by fingers is necessary for opening. If slight pressure was enough to complete fruit opening the line was evaluated as dehiscent, if not this line was evaluated as indehiscent.

Anatomical Analyses

Samples of seed coat (JI64, VIR320, Cameor and JI92; at least five seeds per genotype) were dissected from dry seed and

saturated with 2% sucrose solution under vacuum. Equal volume of cryo-gel (Cryomatrix Shandon) was added to samples and shake overnight. Saturated samples were mounted into cryo-gel on the alum chuck, frozen down to −25°C and cut in cryotome (Shandon SME, Astmoor, UK) into 12 μm transversal section (Soukup and Tylová, 2014). Sections were stained with toluidine blue (0.01%, w/v in water), alcian blue (0.1%, w/v in 3% acetic acid), aniline blue fluorochrome (Sirofluor; 0.01%, w/v in 100 mM K_2HPO_4 with pH 9), or Sudan Red 7B (0.01%, w/v) according to Soukup (2014). The presence of proanthocyanidins was evaluated by staining with vanillin (Gardner, 1975) and DMACA (Li et al., 1996). Callose immunodetection was performed according to Soukup (2014) using primary antibody toward (1,3)-β-glucan (1:100; Biosupplies Australia PTY Ltd) and anti-mouse IgG Alexa Fluor 488 secondary antibody (1:500; Invitrogen). Control samples were processed without the primary antibody. Sections were observed with an Olympus BX 51 microscope (Olympus Corp., Tokyo, Japan) in bright field, blue (Olympus WB filter—callose immunodetection) or UV (Olympus WU filter—aniline blue fluorochrome and DMACA) excitation. Unstained control sections were surveyed in bright field or UV-excited autofluorescence. Figures were documented with an Apogee U4000 digital camera (Apogee Imaging Systems, Inc., Roseville, CA, USA). Dry intact seeds were vaccuum dried and gold coated (Sputter Coater SCD 050, Bal-Tec) before imaging with scanning electron microscope (JSM-6380LV; JEOL, Tokyo, Japan). Twenty days (after flowering) old pods of parental lines (JI64 and JI92) as well as RILs of F6 were fixed in 2% formaldehyde and stored in 4°C for latter observation of pod suture. Samples were cut on hand microtones at thickness of 100 μm, and the resulting segments were stained 1% phloroglucinol (Sigma, USA) in 12% HCl (Soukup, 2014).

Liquid Chromatography–Electrospray Ionization/Mass Spectrometry (LC/ESI-MS) Analysis

Testa was separated from the rest of the seed, crushed, and extracted using mixture of acetone:water (70:30, v/v) with addition of 0.1% ascorbic acid to achieve efficient extraction of polyphenolic compounds in wide range of polarity and structural diversity (adapted from Amarowicz et al., 2009). 0.5 ml of extract was dried under a stream of nitrogen and solid residue was dissolved in 0.5 ml of methanol. The samples were then analyzed by ultra-performance liquid chromatograph Acquity UPLC I-Class coupled to high resolution tandem mass spectrometer Synapt G2-S with ion mobility separation capability (Waters, Milford, USA). Chromatographic column Raptor ARC-18 (100 × 2.1 mm, dp = 2.7 μm, Restek) and mobile phases (MP) A: water + 0.1% formic acid, B: acetonitrile + 0.1% formic acid was used for separation of components present in seed coat extracts. Flow rate of mobile phase 0.2 ml/min was applied. Electrospray was used as ion source. Spray voltage 2.5 kV in positive and 1.5 kV in negative ion mode, were used, respectively. Process of the LC/ESI-MS method optimization and detailed setup of mass spectrometer will be provided in Válková et al. (in preparation).

Laser Desorption/Ionization–Mass Spectrometry (LDI-MS) Analysis

Seeds of each genotype/line were mechanically disrupted and the seed coats were separated and pooled (four seeds per genotype). Description of the studied RILs is given in **Table S1**. Small pieces ∼2 mm were fixed on MALDI plate using a common double sided adhesive tape. The samples were analyzed directly without application of a matrix. The prepared samples were analyzed using high resolution tandem mass spectrometer Synapt G2-S (Waters) equipped with vacuum MALDI ion source. For desorption/ionization a 350 nm 1 kHz Nd:YAG solid state laser was used. Details of LDI-MS setup and analytical parameters of hydroxylated fatty acids can be found in Cechová et al. (in preparation).

Metabolite Data Treatment

The obtained LC/ESI-MS and LDI-MS data were processed by MarkerLynx XS a software extension of MassLynx platform (Waters). The processed data matrix, i.e., after extraction, normalization and alignment of retention times (in case of LC/ESI-MS data), m/z-values and intensities of signals, were transferred to Extended Statistics (XS) module, EZinfo (Umetrics, Malmo, Sweden), and studied by principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA). Both PCA and OPLS-DA were used for reduction of data dimensionality. OPLS-DA is a multivariate statistical method employing latent variable regression developed as an extension of more frequently used partial least squares method (Trygg and Wold, 2002). Coordinates of particular samples and RT_m/z pairs (or m/z-values in the case of LDI-MS data) in appropriate biplots and S-plots were used for evaluation of dormant and non-dormant genotypes mutual segregation and significance of detected signals of metabolites. The procedure was adopted and modified from Kučera et al. (2017). The most significant markers were further studied by targeted MS/MS experiments to reveal their identity (Cechová et al., in preparation; Válková et al., in preparation).

RNA Isolation

For Massive analysis of cDNA Ends (MACE) each sample of parental genotype was composed by several pooled developmental stages of seed coat (2, 3, 4 weeks and older) as it is unknown at which stage putative candidate genes are expressed. Seven selected RILs forming each of dormant resp. non-dormant bulk were previously (at F_6 generation) and after mature seed harvest (of F_7 generation used for RNA isolation) tested for germination behavior (**Table S1**). Seed coat were dissected under stereomicroscope, immediately frozen in liquid nitrogen and stored at −70°C until use. Frozen seed coats or dorsal and ventral sutures of pods were ground to a fine powder with liquid nitrogen using sterile mortars and pestles. Total RNA was isolated from seed coat (∼100 mg) using the BioTeke Plant Total RNA Extraction Kit (China) or NucleoSpin RNA Plant kit (Macherey Nagel) for pods, according to the manufacturer's instructions. Yield/quantity and purity was determined by using NanoDrop 2000 spectrophotometer (Thermo Scientific) and diluted in DEPC-H_2O to 100 ng/μl. Isolated RNAs were treated

with DNase according to Baseline-ZERO™ DNase protocol (Epicenter). In case of parental genotypes (JI64, JI92, Cameor, and VIR320) four consecutive developmental stages of seeds (14–37 DPA) were taken each represented by 1.25 μg of total RNA. The RIL bulks were made of 1.425 μg of total RNA of each of seven lines. In case of RNA samples used for pod dehiscence study, two parental lines (JI64 and JI92) and two bulks of contrasting RILs (with dehiscent or indehiscent pods) were used. The bulk of dehiscent RILs was established from eight and bulk of indehiscent RILs from five lines using excised pod sutures of 10 and 20 days after flowering. Each of these four final samples contained ∼1 μg of total RNA each. The integrity of the RNA samples was examined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA).

Massive Analysis of cDNA Ends (MACE)

MACE libraries were generated using GenXPro's MACE kit (GenXPro GmbH, Frankfurt, Germany) as described in Zawada et al. (2014). Briefly, cDNA from 5 μg of total RNA was randomly fragmented and biotinylated 3′ ends were captured after binding to a streptavidin matrix. A library ready for high-throughput sequencing was prepared using TrueQuant adapters included in the kit. The library consisted of 50–700 bp-long fragments derived from the 3′-end of the cDNAs. The 5′-ends of the libraries were sequenced on a HiSeq 2000 machine (Illumina) with 100 cycles to generate the MACE tags, each tag representing one single transcript molecule. In total, 6 cDNA libraries were prepared and sequenced for seed dormancy, while 4 libraries for parents and two contrasting bulks for pod dehiscence, each providing over 10 million reads (**Table S2**).

Bioinformatics

After sequencing the reads are in 5′–3′ orientation. To remove PCR-bias, all duplicate reads detected by the TrueQuant technology were removed from the raw datasets. Low quality sequence-bases were removed by the software Cutadapt (https://github.com/marcelm/cutadapt/) and poly(A)-tails were clipped by an in-house python-script. The reads were aligned to reference sequences using Novoalign (http://www.novocraft.com). This tool maps reads to reference sequences depending on certain parameters (i.e., quality) and calculates thresholds for each assignment. The reference sequences consisted of all *Pisum* mRNA sequences from NCBI. We annotated these sequences to all Fabaceae proteins from Uniprot "http://www.uniprot.org/" by BLASTX to Swissprot ("sp|..," good annotation) and afterwards to Trembl ("tr|...," less good annotation) protein sequences. All reads that could not be mapped to Pisum mRNA sequences from NCBI were used for a *de novo* assembly to generate contigs denoted as "noHitAssembly_xxx" and annotated in the same way as the Pisum mRNA sequences from NCBI. Normalization and test for differential gene expression between the bulks were calculated using the DEGSeq R/Bioconductor package (Wang et al., 2010). Differential gene expression was quantified as the $\log_2$ ratio of the normalized values between two libraries ($\log_2$ FC). The *p*-value and correction for multiple testing with the Benjamini–Hochberg false discovery rate (FDR) were computed due to determining significance of gene expression differences in pairwise comparisons of libraries. Lists of Differentially Expressed Genes (DEGs) for three comparisons of contrasting phenotype (dormancy: wild × cultivated, RILs and their parents, dehiscence: RILs and their parents) were made based on combination of pairwise comparisons of $\log_2$ FC ratio of the normalized values ($\log_2$ FC > 2, $\log_2$ FC < -2) and FDR (FDR < 0.01) between all libraries of these groups.

GO and KEGG Annotation

Gene Ontology (GO) enrichment analysis and normalized gene expression data were used to identify function and relationships of differentially expressed genes (Young et al., 2010). The results of the GO analysis were then exported into the Blast2go for the final annotations. The annotations provided the fragments with blast hit with the appropriate gene ontology terms which were classified into three categories: biological process (BP), cellular components (CC), and molecular function (MF). The DEGs were subjected for their presence in the different Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The various enzyme activities and the DEGs involved in the KEGG pathways were revealed for each of the combinations.

Genetic Mapping of Dehiscence Specific SNPs-Methodology

Transcripts containing SNP with at least five reads in both samples that are homozygous distributed in dehiscent vs. indehiscent RILs bulks with only one false allele read in 100 in either bulks were considered dehiscence specific. SNPs were discovered using Joint-SNV-Mix (Roth et al., 2012). The output given by Joint-SNV-Mix was furthermore processed by GenXPro in-house software to filter the SNPs. A minimum coverage of 10 bp was needed to be identified as an SNP. To identify the genomic localization of the SNP the surrounding region of the SNP was assigned per blastn to the genome of *M. truncatula* "JCVI.Medtr.v4.20130313" from http://jcvi.org/medicago. The "snpviewer" a webtool from the "http://tools.genxpro.net" was used to visualize the data.

Real-Time Quantitative Reverse Transcription PCR

Gene-specific oligonucleotide primers were designed (**Table S3**) based on MACE consensus sequences using the FastPCR software (Kalendar et al., 2014). The expression of selected candidate genes was validated by quantitative real time PCR (qRT-PCR). RNA samples (treated with DNase) were reverse-transcribed with Oligo$(dT)_{15}$ primer (Promega) in a two steps reaction in final volume 40 μl. The qRT-PCR analysis was run on the CFX96™ Real-Time Detection System (Bio-Rad) using the SensiFast SYBR® No-ROX kit (Bioline) or LightCycler® 480 SYBR Green I Master kit (Roche) in case of pod dehiscence study. Primers were designed using FastPCR or Oligo Primer Analysis Software (Molecular Biology Insights, USA) and produced amplicons ranging from 77 to 220 bp (**Table S3**). Every PCR reaction included 2 μl cDNA (1:10 diluted cDNA), 5 μl 2× SensiFAST SYBR mix or LightCycler® 480 SYBR and 400 nM of each primer in final volume 10 μl. The expression was studied at two developmental stages in four contrasting parental genotypes (JI64, JI92, Cameor, and VIR320) and in case of dehiscence study also contrasting RILs with dehiscent or indehiscent pods. The

conditions for PCR were: 95°C for 2 min; 45 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 20 s; followed by a melting curve of 65–94°C (recovered every 0.5°C held for 0.5 s). The specificity of primers was confirmed by melting curve and gel analysis of products. Quantification of transcript level was determined by CXF Manager Software (Bio-Rad). Actin or β-tubulin gene was used as a reference to normalize relative quantification (Ferraro et al., 2014) using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Changes in transcript were estimated as fold change relative to the expression in the genotype Cameor (younger stage) in case of seed dormancy study and genotype JI92 (younger stage) in case of pod dehiscence study.

RESULTS

Seed Coat Mediated Dormancy of Wild Pea Seeds

Anatomical and Germination Differences between Seed Coat of Wild and Cultivated Peas

Wild pea seeds display high level of dormancy mediated by seed coat permeability. Two contrasting parental pairs of wild (*P. elatius*) JI64 and VIR320 and cultivated (*P. sativum*) cv. Cameor and JI92 peas were selected as they differ in testa pigmentation, thickness, and dormancy levels as well as pod dehiscence trait. Moreover, RILs were generated from cross of JI64 and JI92 to facilitate mapping. While cultivated pea seeds imbibe readily and germinate within 24 h (JI92, cv. Cameor), wild pea seeds remain highly dormant and imbibe and germinate at 8% (JI64) or 30% levels (VIR320) after 7 days (**Figure 1**). Mean germination time, Timson index, and Coefficient of Velocity are also very different between respective parental genotypes, 3.4 (MGT), 1 (TI), and 0.29 (CV) for JI92 while being 7, 0.008, and 0.14 for JI64, respectively. RILs displayed more variability in each of the respective measures (**Figure S1**), with wide range of percentage of germination (at 7 days) from 4 to 100%, 0.61 to 4.44 CV, 1 to 7 MGT, and 0 to 0.95 TI. All these parameters indicate complexity of imbibition and germination processes as none single is sufficient to fully describe any given line. As shown in **Table S1**, non-dormant bulk had on average 56% germination over 7 days period, 1.58 CV, 0.36 TI, and 68.02 MGT respective, contrast to dormant bulk lines having 22%, 1.38 CV, 0.28 TI, and 104.71 MGT, respectively. Similarly testa thickness was 107 vs. 135 μm, respectively.

Testa thickness was analyzed by micrometric, light microscopy or SEM measurements. Especially JI64 and JI92 differ substantially in palisade cells length, which contributes to overall testa thickness (**Figure 2**). Dormant genotype JI64 has significantly thicker testa, which might contribute to the water impermeability of seed coat of dormant pea genotypes. There are considerable differences in surface pigmentation and texture of individual lines (**Figures 3a,d,g,j**). While Cameor is not pigmented visible pigmentation is present in other genotypes with different intensity and localization. The texture of surface is variable among genotypes, particularly in details of macrosclereid tips arrangement defining the surface shape being covered by thin cuticle (**Figures 3b,c,e,f,h,i,k,l** and **Figure S2**). The most obvious is gritty surface of JI64 which was absent in all the other genotypes included in this study. The continuity of surface (cuticle integrity) was interrupted locally by minor fissures in all genotypes. Large fissures developed in seed coat of the non-dormant genotypes Cameor and JI92, mostly in the hilar and strophiolar region later during the imbibition (data not presented). The cytological arrangement of seed coat of tested genotypes varies particularly in macrosclereids. The surface is covered with thin cuticle (**Figure S2**) which copies the outer extremities of palisade macrosclereids. Based on light microscopy we did not see any apparent difference in cuticle properties among genotypes as revealed by autofluorescence or Sudan staining (**Figure S2**). Interestingly, cuticle is not the only lipidic material localized close to the seed coat surface. Non-cuticular lipidic extracellular material was present also in the very tips of the macrosclereids (**Figures S2e–h**) containing autofluorescent material (**Figures S2a–d**). The analysis of seed coat surface was complemented with histochemical analysis of selected compounds of cell wall. Metachromatic staining with toluidine blue of non-dormant genotype Cameor exhibited high level of polyanionic cell wall components, while in contrary non-dormant, but well-pigmented JI92 showed lower abundance of polyanionic compounds similarly to dormant genotypes where metachromasy was mostly limited to macrosclereid tips bellow the cuticle (see **Figures S2a–d**). Staining with Alcian blue further supports such conclusion (data not shown). Interestingly, metachromatic staining was enhanced with 3% acetic acid pretreatment (**Figures S3e–h**). Clear connection between presence of tannins and toluidine blue stainability is well-documented in JI92, where deeper staining is present out of pigmented spots (proanthocyanidin positive; **Figure S3f**). The presence of proanthocyanidin within cell walls of macrosclereids was not detected in non-dormant genotype Cameor but was obvious in JI92 as well as in the dormant genotypes (**Figure 4**) using both HCl-Vanilin and DMACA tests. Condensed tannin presence was never recorded within the light line (**Figure 4**) of macrosclereids of any genotype. Macrosclereids of dormant type genotypes seems to be enriched with proanthocyanidins in the cell walls of the entire macrosclereids up to the light line. Seed coat of JI92 is highly enriched with proanthocyanidins only in the dark pigmented spots. Interestingly, the abundance of condensed tannins negatively correlates with toluidine blue stainability and sirofluor staining of cell walls indicating tannin linkages to other compounds within the cell wall. Detected tannins are not extractable with ethanol, or 1M HCl. Alkaline hydrolysis of cell wall bound tannins by 1M sodium hydroxide resulted in loss of tannins stainability (**Figures 4c,f**). There was intense aniline blue staining of macrosclereids in all genotypes, particularly in the light line of macrosclereids (**Figures S2i–l**) and their outer part composed in majority of the secondary cell walls. The strongest signal was observed in dormant genotypes, especially in the light line. However, the signal for callose specific antibody did not correspond with aniline blue fluorophore and in general was found rather week and discretely localized (**Figure S2i**—inlay). Phloroglucinol staining indicative of lignin provided no response in non-dormant nor in dormant pea genotypes indicating the absence

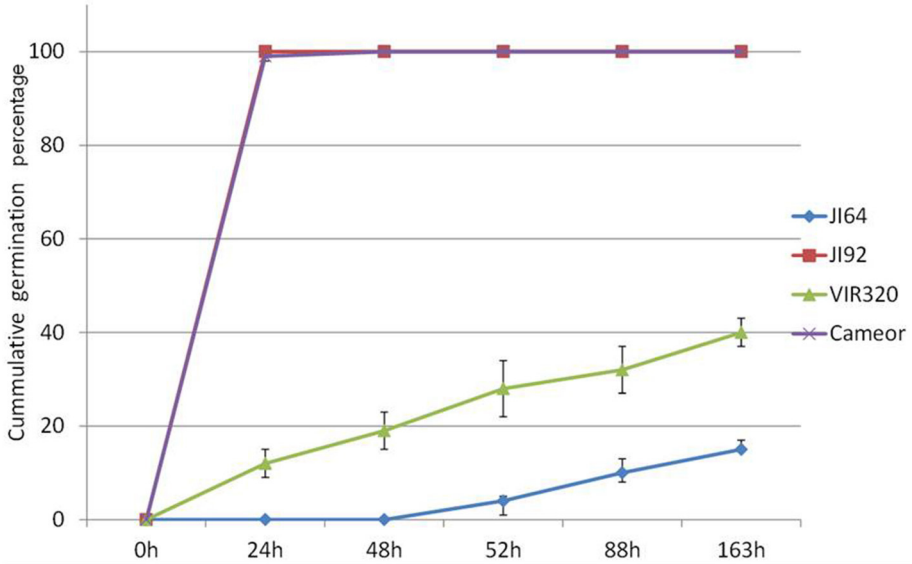

FIGURE 1 | Cumulative germination percentage of wild *P. elatius* (JI64, VIR320) and cultivated *P. sativum* (JI92 and cv. Cameor) seeds tested at 25°C over the period of 163 h.

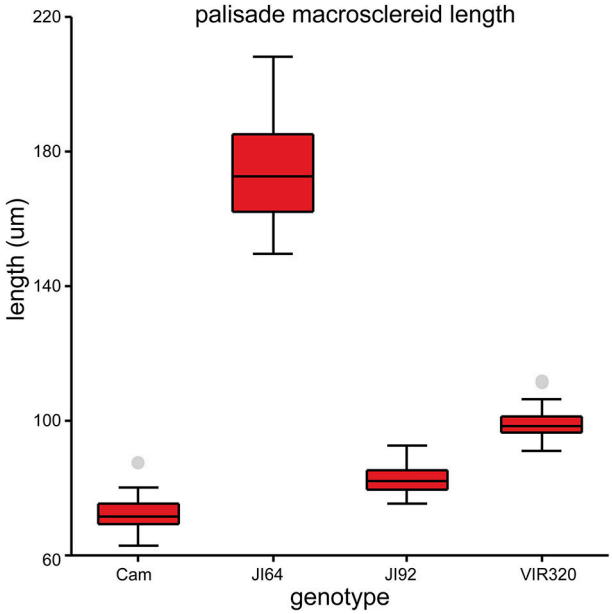

FIGURE 2 | Length of the seed coat palisade cells of selected pea genotypes (Cameor, JI92, JI64, and VIR320). Box plot of median with 25th and 75th percentile, whiskers are 5th and 95th percentile; $n = 75$; each genotype is different from the other (ANOVA $p < 0.001$).

of significant amount of lignins in the testa of analyzed pea genotypes.

Chemical Analysis of Seed Coat Composition

Detection of metabolites present in seed coat related to dormancy was based on comparison of LC/ESI-MS and LDI-MS data of dormant and non-dormant pea genotypes using principal component analysis and orthogonal projection to latent structures. **Figure 5** reflects the differences in coordinates of particular genotype samples in corresponding Score plot obtained by Principal Component Analysis of LC/ESI-MS data. Although, individual coordinates do not exhibit statistically significant differences among all the genotypes (e.g., t[2] coordinates of Cameor, Terno, and VIR320), location of each genotypes given by combination of both coordinates provided resolution among particular genotypes. The differences in the coordinates (mutual orientations and values) clearly show the separation of dormant (i.e., L100, JI64, and VIR320) from non-dormant genotypes (i.e., Terno, Cameor, and JI 92) using the acetone:water extract. Separation of L100 and JI64 from non-dormant genotypes is much more significant compared to the separation of VIR 320. This can be explained by possible semi-domesticated status of this genotype. Based on the achieved separation of dormant and non-dormant genotypes by unsupervised Principal Component Analysis (PCA), supervised Orthogonal Projection to Latent Structures (OPLS-DA) was used to find signals mostly responsible for the chemical differences in dormant and non-dormant samples. Those signals (m/z-values of markers with increased intensity in dormant genotypes compared to non-dormant ones) were studied in detail by targeted tandem mass spectrometry (study of their fragmentation after collision induced dissociation in collision cell of mass spectrometer). Details of analytical interpretation can be found in Válková et al. (in preparation). Attention was especially focused on the chemical differences between morphologically the most similar pair of genotypes, i.e., JI 64 and JI 92. Combination of information about retention time, exact mass measurement and fragmentation revealed the

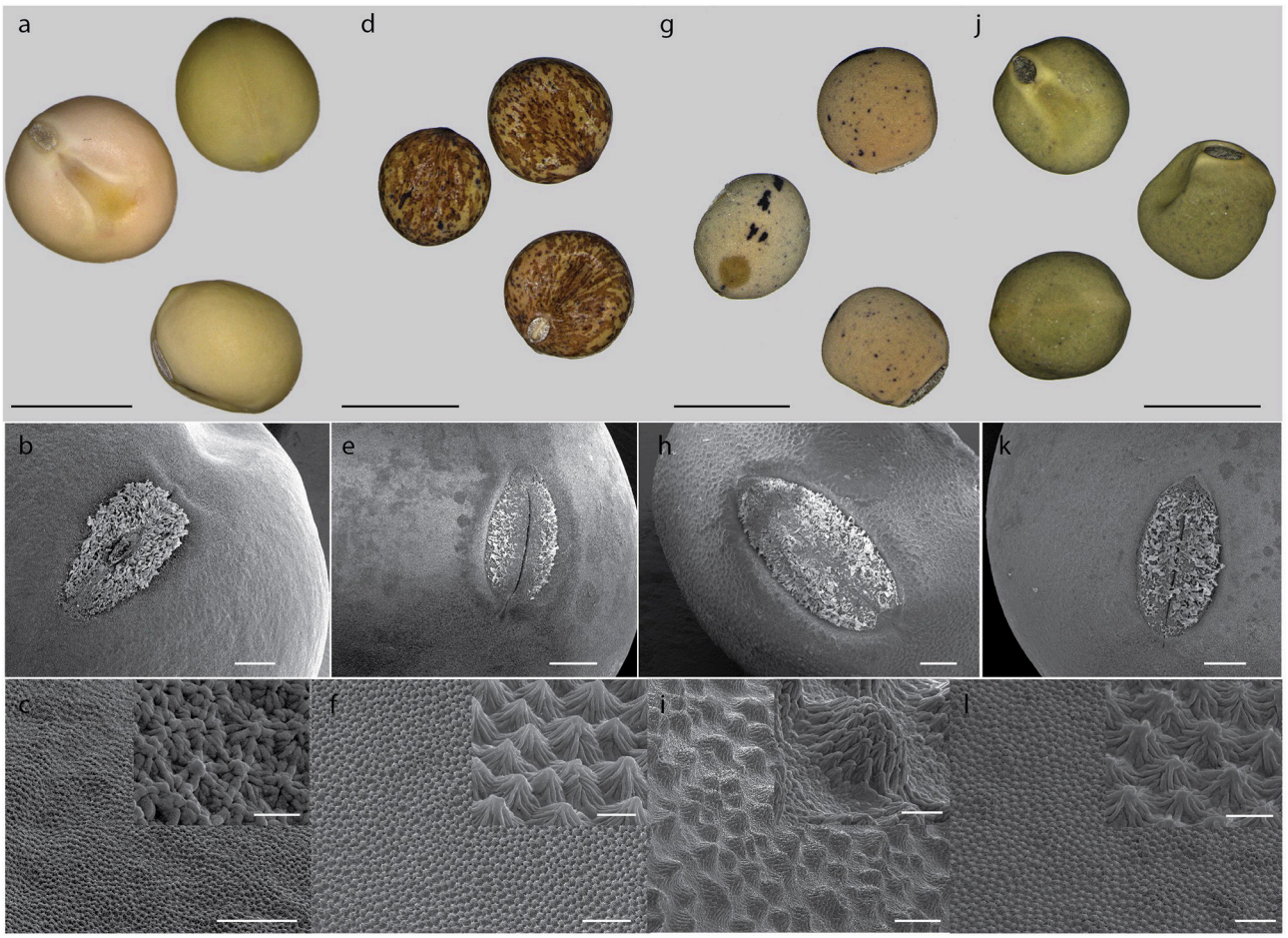

FIGURE 3 | Seed coat surface of selected pea genotypes. Pigmentation of seed coat: Cameor **(a)**, JI92 **(d)**, JI64 **(g)**, and VIR320 **(j)**; scale bar = 5 mm. Surface texture (SEM): Cameor **(b,c)**, JI92 **(e,f)**, JI64 **(h,i)**, and VIR320 **(k,l)** overall view, scale bar = 500 μm; details of extrahilar region **(c,f,i,l)** with inlay of high magnification, scale bars = 100 μm **(c,i)** and 50 μm **(k,l)**, inlay scale bars = 10 μm **(c)** 20 μm **(l)**.

identity of the most significant dormancy markers found in acetone-water extracts—dimer and trimer of gallocatechin (m/z 611.1387 and 915.1945, deviation of measured from theoretical m/z-value of parent ion, dtm, –0.8 and –3.3 mDa), respectively, quercetin-3-rhamnoside (m/z 449.1045, dtm –3.3 mDa) and myricetin-3-rhamnoside (m/z 465.1112, dtm 7.9 mDa). Analogously, the chemical differences between dormant JI64 and JI92 genotypes were studied by laser desorption-ionization mass spectrometry (LDI-MS). Measurement in negative ion mode in combination with PCA a OPLS-DA revealed marked differences in the profile of particular hydroxylated long chain fatty acids [i.e., m/z 411.3865, hydroxyhexacosanoate (dtm 2.7 mDa); m/z 425.3927, hydroxyheptacosanoate (dtm −6.8 mDa); m/z 427.3875, dihydroxyhexacosanoate (8.8 mDa); m/z 437.4033, hydroxyoctacosanoate (dtm 3.8 mDa); m/z 441.3973, dihydroxyheptacosanoate (2.9 mDa) and m/z 455.4180, dihydroxyoctacosanoate (dtm 8.0 mDa)]. Two orders of magnitude higher normalized signals of dihydroxyheptacosanoate and dihydroxyoctacosanoate were measured in JI 64 compared to JI 92, i.e., $(1.21 \pm 0.92).10^{-2}$ and $(2.50 \pm 1.54).10^{-2}$ vs. $(1.34 \pm 0.02).10^{-4}$ and $(2.19 \pm 0.58).10^{-4}$, respectively. **Figure 6** shows the normalized signals of both dihydroxylated long chain fatty acids in seed coats of JI64, JI92, and RILs with respect to dormancy. The majority of dormant RILs exhibit higher content of those fatty acids compared to non-dormant ones.

Seed Coat Transcriptome Differences between Wild and Cultivated Pea

In order to understand the mechanism of testa mediated dormancy we selected seed coat derived from two contrasting parental pairs and two bulks of RILs differing in dormancy level to extract RNA and to identify candidate genes using the next-generation sequencing method of Massive Amplification of cDNA Ends (MACE). The isolation of RNA from wild pea seed coat tissue proved to be very difficult. It is likely that high due to content of free metabolites (oligosaccharides and proanthocyanidins). We assessed the expression patterns in domesticated (cv. Cameor and JI92 landrace) vs. wild *P. elatius* (JI64, VIR320) pea. Each sample has yielded between 8 and 15

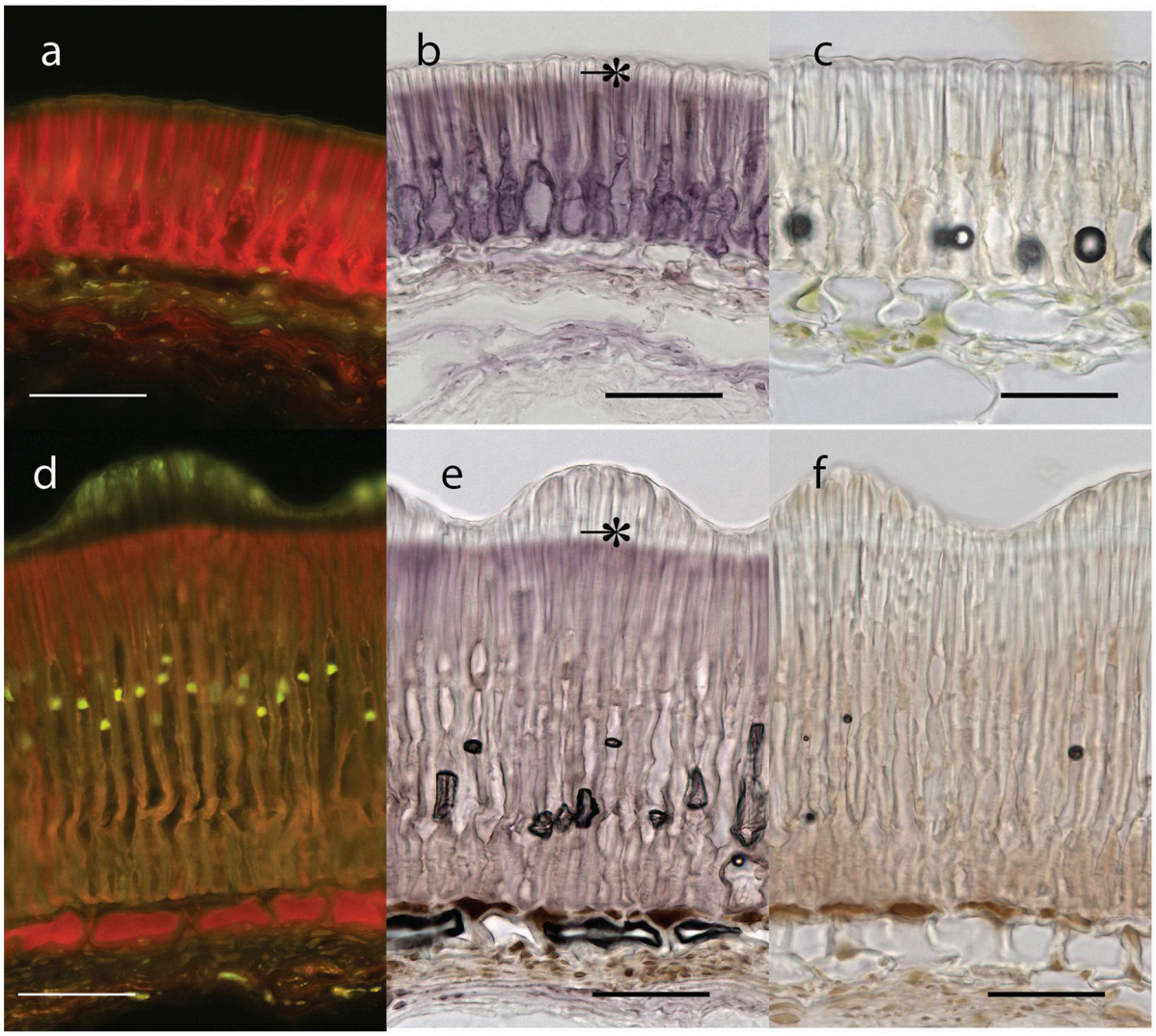

FIGURE 4 | Seed coat transverse sections from extrahilar region stained with DMACA for proanthocyanidins polyphenolics (bar = 50 μm): Upper row—JI92, Lower row—JI64. Blue excited red fluorescence of DMACA **(a,d)** and violet coloration in bright field **(b,e)**. Similar sections as in **(c,f)** but stained with DMACA after mild alkaline hydrolysis; scale bars = 50 μm. *, Light line.

million clean reads (**Table S2**). Bioinformatics analysis resulted in identification of 144,000 transcripts (e.g., MACE annotated fragments) expressed in immature seed coat tissue. We have used stringent values of false discovery rate (FDR) ≤ 0.01 and fold-change ($\log_2$ FC) ≥ 2 as a threshold to identify the significant differences in the gene expression. Applying these criteria, a total of 10,132–11,808 transcripts were found differentially expressed between cultivated (JI92, Cameor) and wild (JI64, VIR320) parents (**Table S2**). Of these 770 were differentially expressed between all wild vs. cultivated genotypes, of these 374 were up-regulated in cultivated genotypes, and 396 down-regulated (**Figure 7A**, **Table S4**), when annotated to pea transcriptome, respectively.

A heat-map of 1,000 genes with the highest variance among normalized expression between cultivated and wild pea samples (**Figure 8A**) further illustrates differences between domesticated and wild pea seed coat expressed genes. This comparison shows the differences between respective pairs (cultivated vs. wild). In addition to contrasting parental genotypes, two bulks of seed coats at several pooled developmental stages of seven dormant and seven non-dormant RILs (**Table S1**) were analyzed. The bulks were included to minimalize identification of genes associated with respective genetic background rather than dormancy trait. This is clearly shown when DEGs are compared between parents and RIL bulks (having largest number of specific DEGs between parents e.g., 6,204 up-/5,604 down-regulated transcripts, while only 869 and 1,014 down-regulated transcripts in RIL non-dormant vs. dormant bulks. In case of gene expression profile of dormancy and non-dormancy RILs and their parents, 299 DEGs were found. When comparison

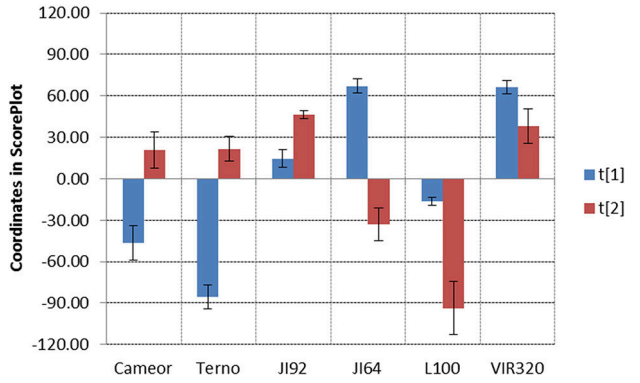

FIGURE 5 | Score Plot of LC/ESI-MS data of dormant and non-dormant pea genotypes. Data filtration threshold 1000 counts in MS spectrum; replicated analyses, three repetitions of each genotype, level of significance α = 0.05, UV scaling, no transformation.

included JI64 and JI92 parents and two respective RIL bulks, there were 83 up- and 216 down-regulated genes (**Figure 7B**). In order to visualize the expression pattern of RILs and theirs parents, heatmaps were constructed for 11,808 genes from libraries of dormancy and 6,259 genes from libraries of dehiscence (**Figure 8B**).

Verification of Differentially Expressed Genes during Seed Coat Development

We selected DEGs based on expression level difference and gene annotation (**Table S4**), regarded as candidate genes for seed coat mediated dormancy in two possible directions of evolutionary changes (i.e., up- or down-regulated in the domesticated pea compared to its wild progenitor). To validate MACE results, expression levels of 14 selected DEGs was analyzed by qRT-PCR. According to MACE results, the selected genes comprised of five up-regulated genes (direction dormant to non-dormant): porin (MACE-S082), NADPH-cytochrome P450 reductase (MACE-S101), peroxisomal membrane PEX14-like protein (MACE-S108), UDP-glucose flavonoid 3-O-glucosyltransferase (MACE-S139), xyloglucan:xyloglucosyl transferase (MACE-S141), and 9 down-regulated genes: NADH-cytochrome b5 reductase (MACE-S019), divergent CRAL/TRIO domain protein (MACE-S066), 1-deoxy-D-xylulose, 5-phosphate, reductoisomerase (MACE-S069), probable aldo-keto reductase 1 (MACE-S070), cupin RmlC-type (MACE-S110), heavy metal transport/detoxification protein (MACE-S111), 4-coumarate CoA ligase (MACE-S131), cytochrome P450 monooxygenase CYP97A10 (MACE-S132), β-amyrin synthase (MACE-S135). Of these tested 14 genes, 4 genes (MACE-S082, MACE-S108, MACE-S131, and MACE-S139) were in agreement in all four genotypes with data obtained with MACE method (**Figure 9**). Relative expression level by MACE-S019 and MACE-S135 was completely contrary to MACE results, where higher values were in wild dormant genotypes. The qRT-PCR expression pattern of MACE-S066 and MACE-S101 were different in two genotypes (Cameor and VIR320) compared to MACE data, with MACE-S066 higher expressed in wild dormant (VIR320) and MACE-S101 higher expressed in cultivated non-dormant genotype (Cameor). Discrepancy between qRT-PCR and MACE methods in JI64 and JI92 was found in case of MACE-S069, MACE-S070, MACE-S110, MACE-S111, MACE-S132, and MACE-S141.

Enrichment Analysis of DEGs Functional Classes between Wild and Domesticated Pea Seed Coats

In order to investigate transcriptome changes in seed coat associated with evolution under domestication, we assessed the expression patterns of the DEGs in domesticated (cv. Cameor and JI92 landrace) vs. wild *P. elatius* (JI64, VIR320) pea. We identified 770 DEGs (583 respectively, when ambiguous are removed) is seed coat between wild and domesticated peas. Due to the absence of complete pea genome and likely specificity of seed coat tissue, we could annotate 66% of MACE fragments. Moreover, between 36 and 41% produced ambiguous assignment (**Table S2**). For DEGs sequences assigned to GO terms, we observed differences within all three compounds: cellular components, molecular function, and biological process. Several GO groups were found differently expressed. In GO enrichment of DEGs between wild and cultivated the most interesting results belongs to Molecular function group (**Table S5**). The most DEGs were found in phenylpropanoid (17 genes in KEGG pathway) and flavonoid (11 genes) biosynthetic pathways (**Figures S4**, **S5**). These included O-hydroxycinnamoyltransferase (EC:2.3.1.133), dehydrogenase (EC:1.1.1.195), O-methyltransferase (EC:2.1.1.68), gentiobiase (EC:3.2.1.21), lactoperoxidase (EC:1.11.1.7), ligase (EC:6.2.1.12), reductase (EC:1.2.1.44), and 4-monooxygenase (EC:1.14.13.11) or synthase (EC:2.3.1.74), reductase (EC:1.3.1.77), O-hydroxycinnamoyltransferase (EC:2.3.1.133), isomerase (EC:5.5.1.6), 3′-monooxygenase (EC:1.14.13.21), 4-monooxygenase (EC:1.14.13.11) genes (**Table S5**), respectively. Enzyme 4-coumarate CoA ligase (MACE-S131) catalyzes conversion of 4-coumarate and other derivatives to corresponding esters serving to generate precursors for formation lignin, suberin, flavonoids. In general, main differences in gene expression was detected between enzymes that played important role in secondary metabolites biosynthesis. Different levels of expression were observed for the cellulose synthase enzyme (EC:2.4.1.12) and two enzymes of pectin metabolism pectate lyase (EC:4.2.2.2) and pectin methylesterase (EC:3.1.1.11), which may interfere together with enzymes from the phenylpropanoid and lignin biosynthesis to the structural composition of cell wall. Enzyme 3-hydroxyacyl-CoA (EC:1.1.1.35) dehydrogenase that belongs to fatty acid elongation pathways is also up-regulated in dormant (wild) genotypes. This enzyme participating in of fatty acid biosynthesis showed changes in expression between wild and cultivated genotypes (**Figure 6**). Out of 548 DEGs, 307 (56%) were assigned to GO-term groups, including 171 (56%) down-regulated and 136 (44%) up-regulated in the domesticated pea seed coat compared to wild samples. As shown in **Table S5**, the known DEGs were mainly classified into 40 functional categories and involved in 19 biological processes. The results showed that these DEGs mainly distributed in plasma membranes and nucleus after genes expression, and participated in the biological process

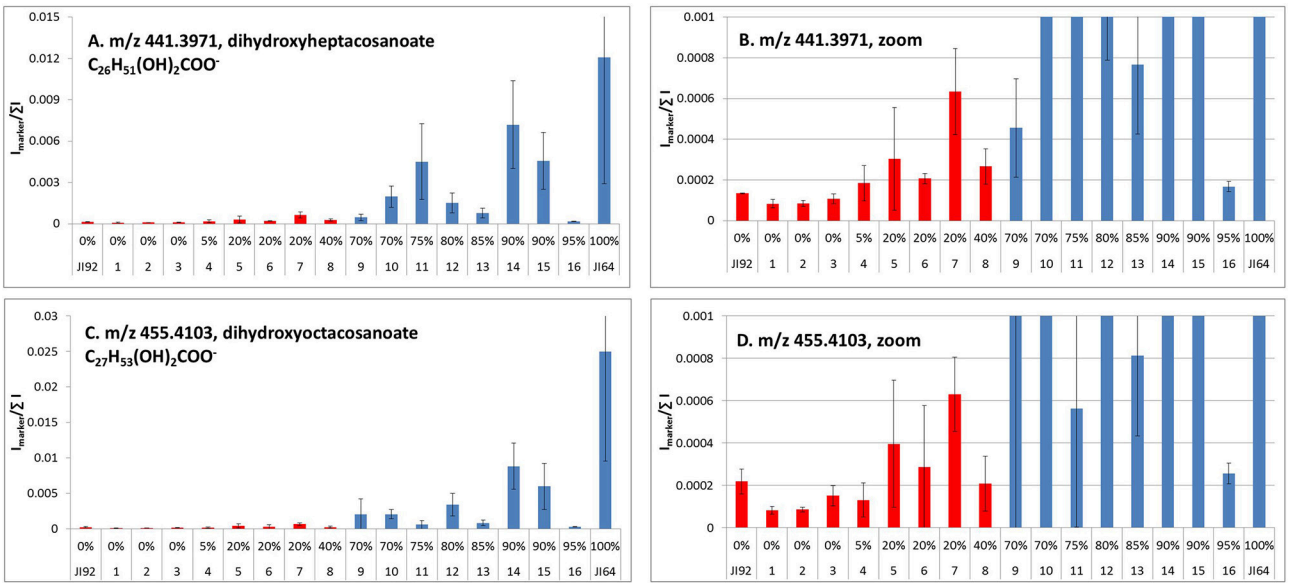

FIGURE 6 | LDI-MS analysis of dihydroxyheptacosanoate (A) and dihydroxyoctacosanoate **(C)** in seed coats of contrasting parents (JI64, JI92) and selected RILs (intensity of signals at particular m/z-values normalized to sum of all signals in mass spectrum; red, non-dormant; blue, dormant lines; error bars reflect the standard deviation of three replicated measurements, $\alpha = 0.05$); **(B,D)**, zoomed graphs.

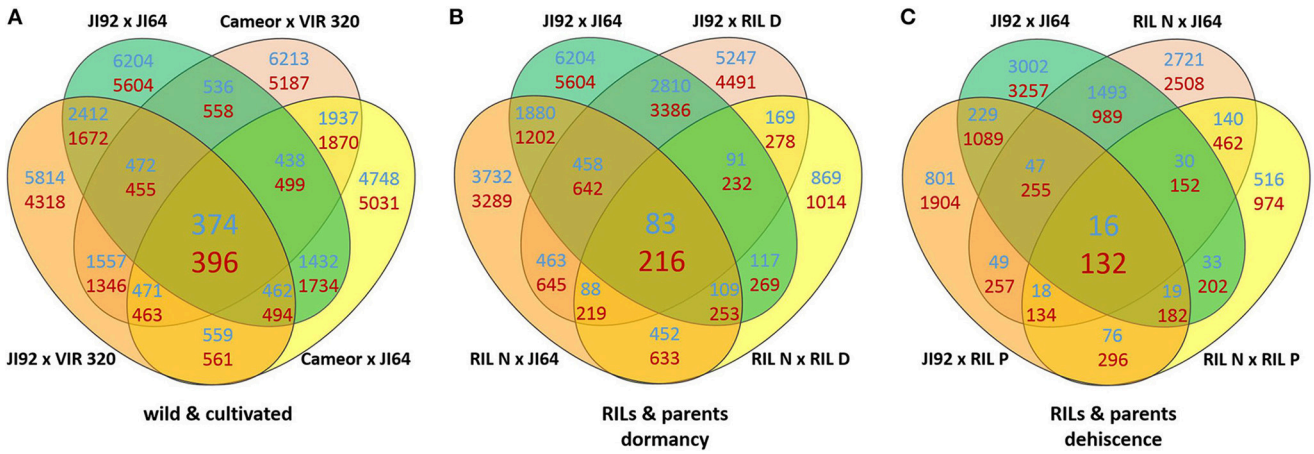

FIGURE 7 | Venn diagrams of differentially expressed genes (DEGs criteria $\log_2$ FC ≥ 2 and FDR ≥ 0.01) between seed coat (A) of the studied wild and cultivated genotypes, **(B)** and JI64, JI92 parents and resulting RILs and **(C)** between pod sutures of JI64, JI92 parents and resulting RILs. Blue counts are upregulated and red counts are downregulated in cultivated genotypes.

of biosynthetic process (60 genes, 12%), metabolism (183 genes, 36%), regulation of transcription (3 genes, 0.5%), transporting (17 genes, 3%), stress response (16 genes, 3%), cell division and differentiation (180 genes, 35%), localization (30 genes, 6%), establishment of localization (28 genes, 6%), lignin synthesis and so on. Through comparative analysis, the two most abundant sub-classes were biosynthesis processes and metabolic processes. The KEGG pathway analysis showed the presence of many genes PsCam051542, PsCam037704, PsCam043296, PsCam000856, PsCam038256, PsCam049689, PsCam016941, PsCam049689, PsCam050533 involved phenylpropanoid biosynthesis. Similarly, PsCam049689, PsCam038227, PsCam005153, and PsCam050665 were found to be associated with flavonoid biosynthesis (**Table S5**).

Pea Pod Dehiscence

The structure of pea pericarp follows the common arrangement in *Fabaceae*. The exocarp consists of thick walled epidermis, the relatively thick mesocarp is arranged in several layers of parenchyma and endocarp composed of lignified sclerenchyma on inside of which is thin-walled epidermis. Both exocarp and mesocarp are rich in pectins, as indicated with metachromatic staining of toluidine blue (**Figure 11**).

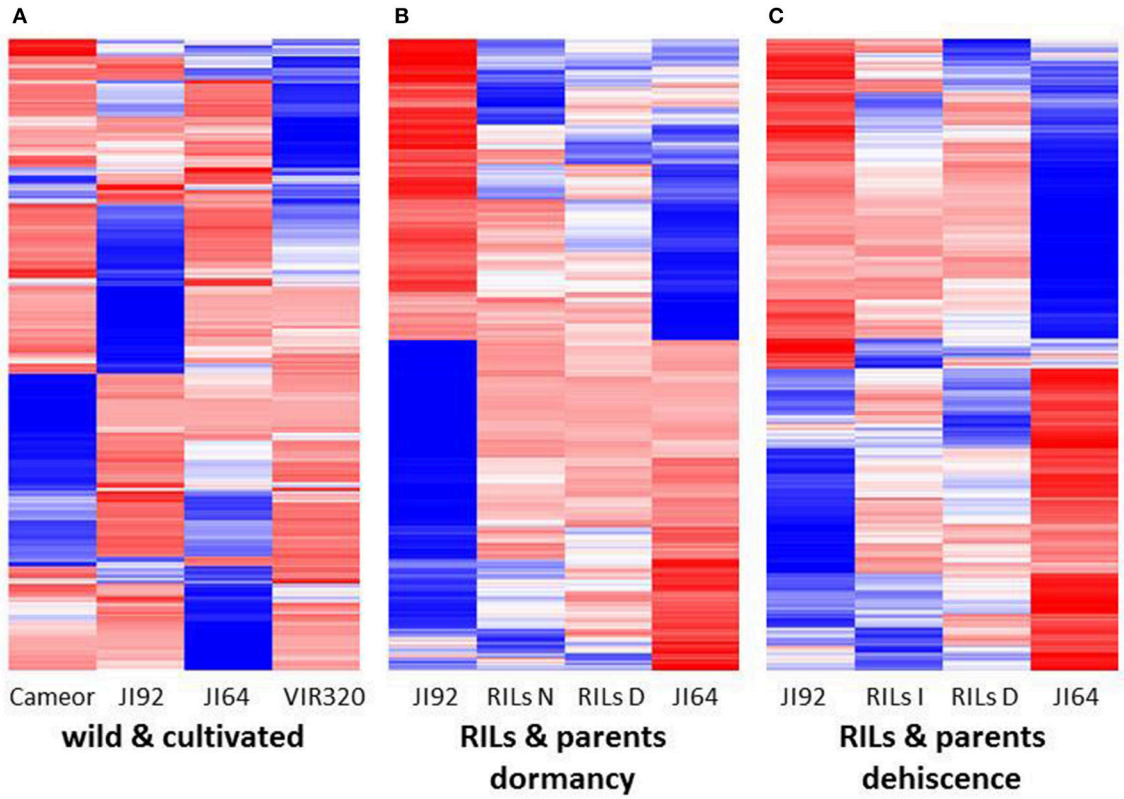

FIGURE 8 | The heat maps (A) of 1,000 genes with the highest variance among normalized expression values for cultivated and wild pea samples, **(B)** of 11,808 DEGs for dormancy, and **(C)** of 6,259 DEGs for dehiscence between RIL's parents samples in MACE experiments.

Differentially Expressed Genes between Dehiscent and Indehiscent Pods

In the case of pea pod dehiscence, we used MACE methodology to find differences in expression profiles between JI92 (domesticated, indehiscent pod) and JI64 (wild, dehiscent pod) and between two bulks of contrasting RILs. For each sample we used bulk of three developmental stages (2, 4 weeks and older) of dissected pod suture tissue (**Table S2**). Across all dehiscent and indehiscent libraries 148 DEGs were found (**Figure 7C**). Of these, 132 DEGs were down-regulated and 16 were up-regulated in indehiscent libraries. For gene expression analysis via qRT-PCR we selected 20 gene candidates with the most different expression in MACE analysis between contrasting lines (dehiscent and indehiscent pod). Nineteen of them were recognized by MACE as down expressed (with lower expression in domesticated indehiscent genotype) and one expressed gene candidate. In addition we tested also five other gene candidates (transcription factors) reported to be responsible for pod dehiscence in other plant species (bHLH Basic helix-loop-helix proteins *INDEHISCENT*, *SPATULA*, *SHATTERPROOF*, Basic Leucine Zipper Domain genes *bZIP* and *SHATTERING*; Ferrándiz et al., 2000; Girin et al., 2011; Dong et al., 2014). For these experiments we used RNA from pod suture tissue of parental line JI92 (domesticated, indehiscent pod) and JI64 (wild, dehiscent pod) as well as of eight contrasting RILs. As a result we detected over expression in indehiscent lines of two gene candidates (*SHATTERING* and *SHATTERPROOF*; **Figure 10C**) and the rest of tested genes (*INDEHISCENT, SPATULA, bZIP*) did not show differential expression. In the second experiment we tested gene expression of 20 gene candidates derived from MACE analysis. In this case, we tested parental lines JI64 and JI92 only. We used RNA from dorsal and ventral pod suture tissue of three developmental stages (10, 15, and 20 days after flowering). In this case we detected three genes (MACE-P004, MACE-P013, MACE-P015) corresponding to the down/over expression as in MACE results (**Figure 10A**). In *M. truncatula* genome homologs genes are: MACE-P004 transmembrane protein, putative (Medtr3g016200); MACE-P013 NADP-dependent malate dehydrogenase (Medtr1g090730), and MACE-P015 peptidoglycan-binding domain protein (Medtr2g079050). In one case (MACE-P009) qRT-PCR showed the opposite results than in MACE (**Figure 10A**). *Medicago* homolog of MACE-P009 gene is cathepsin B-like cysteine protease (Medtr7g111060). The rest of candidate genes for pod dehiscence generated by MACE were tested by qRT-PCR, namely: glycosyltransferase family 92 protein (MACE-P001, Medtr2g437660); serine carboxypeptidase-like protein (MACE-P002, Medtr3g434850); KDEL-tailed cysteine endopeptidase CEP1 (MACE-P003, Medtr3g075390); 60S ribosomal protein L3B (MACE-P005, Medtr1g098540); dormancy/auxin associated protein

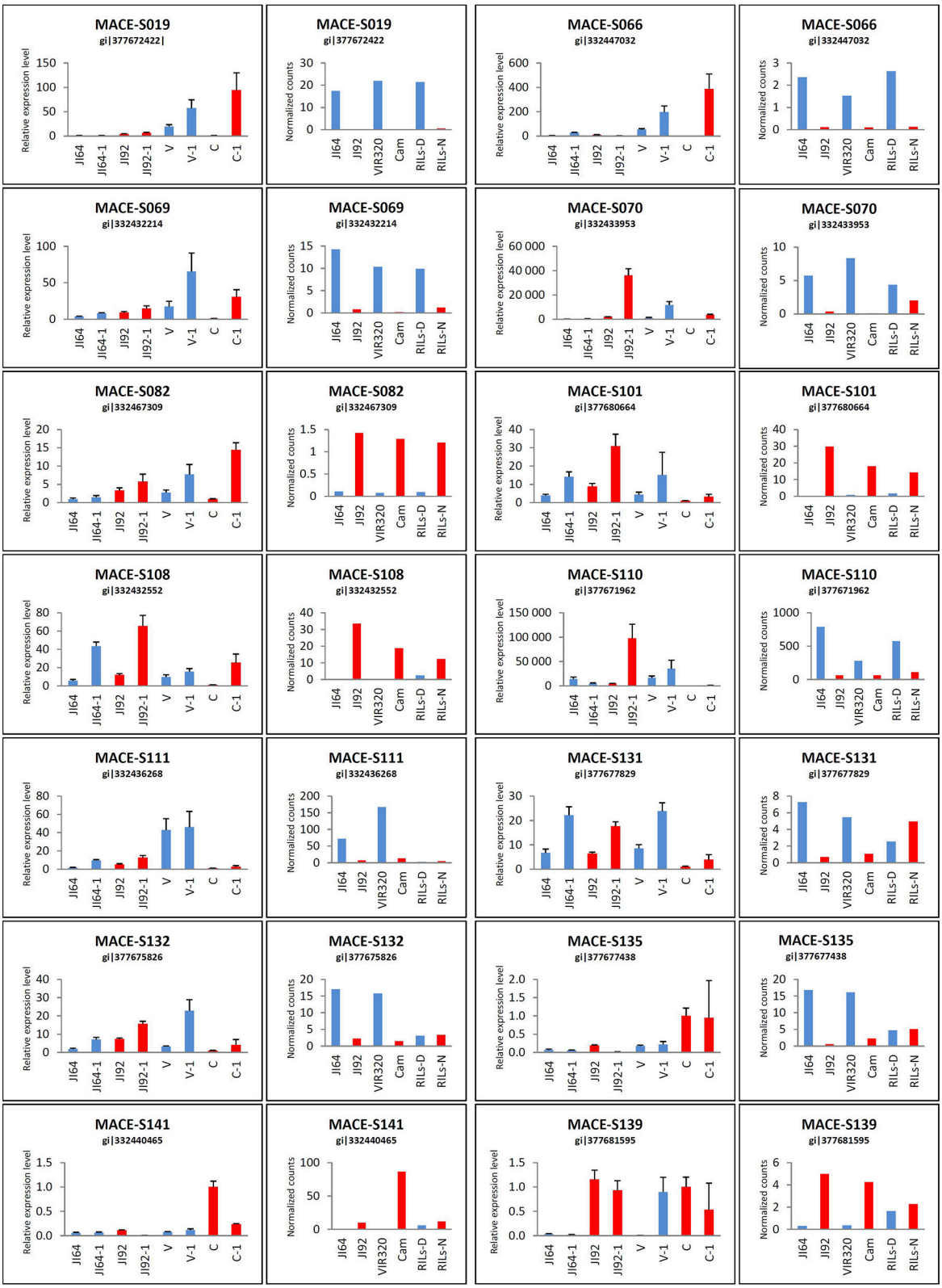

FIGURE 9 | qRT-PCR results of selected candidate genes in comparison with MACE analysis. qRT-PCR for four genotypes in two stages: JI64, JI92, V (VIR320), and C (Cameor)—younger stage; JI64-1, JI92-1, V-1, C-1—older stage and MACE for JI64, JI92, VIR320, Cam, RILs-D (dormant), and RILs-N (non-dormant).

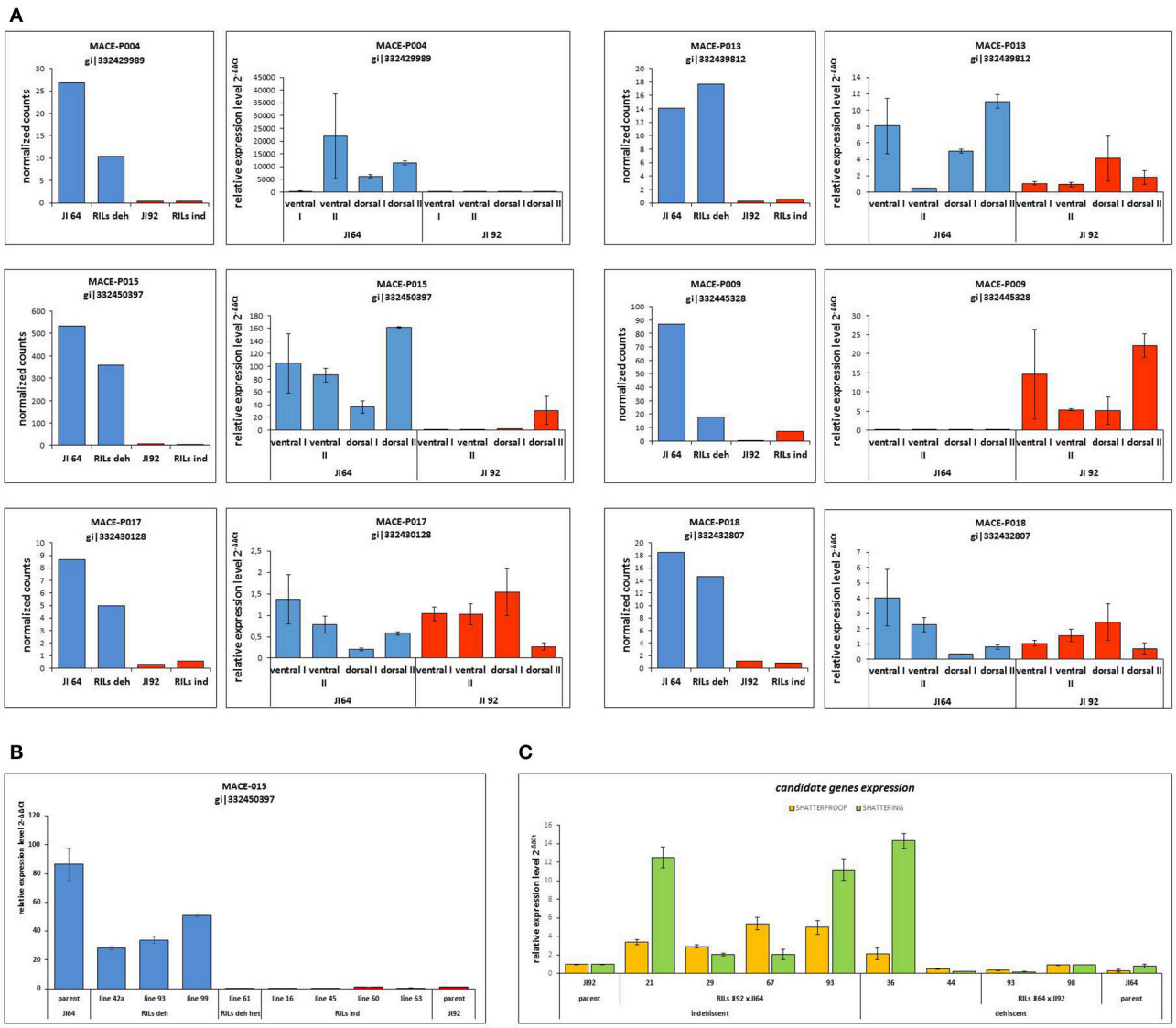

FIGURE 10 | qRT-PCR validation of selected DEGs detected to be associated with pod dehiscence. (A) MACE results in comparison with qRT-PCR of selected candidate genes. MACE-P004, MACE-P013, and MACE-P015 shows the same trend of over/down expression between MACE results of parental lines and RILs (JI 64—dehiscent pod, bulk of dehiscent RILs, JI 92—indehiscent pod, bulk of indehiscent RILs) and qRT-PCR of cDNA of parental lines (ventral or dorsal pod suture in two stages: I—younger stage, II—older stage). MACE-P009 showed the opposite results and MACE-P017 and MACE-P0018 without any trend of down or over expression in qRT-PCR. **(B)** qRT-PCR results of contrasting RILs (dehiscent/indehiscent pod) in comparison with parental lines of candidate gene MACE-P015. **(C)** qRT-PCR results of contrasting RILs (dehiscent/indehiscent pod) in comparison with parental lines of candidate genes SHATTERPROOF and SHATTERING.

(MACE-P006, Medtr7g112860); enoyl-CoA hydratase 2, peroxisomal protein (MACE-P007, Medtr3g115040); disease-resistance response protein (MACE-P008, Medtr2g035150); GASA/GAST/Snakin (MACE-P010, Medtr1g025220); dormancy/auxin associated protein (MACE-P011, Medtr7g112860); TCP family transcription factor (MACE-P012, Medtr2g090960); LL-diaminopimelate aminotransferase (MACE-P014, Medtr2g008430); zinc finger A20 and AN1 domain stress-associated protein (MACE-P016, Medtr2g098160); auxin-responsive AUX/IAA family protein (MACE-P017, Medtr1g080860); huntingtin-interacting K-like protein (MACE-P018, Medtr2g034010); transmembrane-like protein (MACE-P019, Medtr2g038550) and vacuolar processing enzyme (MACE-P020, Medtr4g101730). In the third experiment, we tested only candidates which showed differences in expression in both previous experiments using eight RILs with clearly determined phenotype (dehiscent or indehiscent pods). Four RILs were phenotypically dehiscent and four indehiscent. In this case, we used cDNA derived from the mixture of dorsal and ventral pod sutures tissue (**Figure 11**) for analysis. Finally, we tested four genes: MACE-P004 (homolog of transmembrane protein gene in *M. truncatula*, *P. sativum*

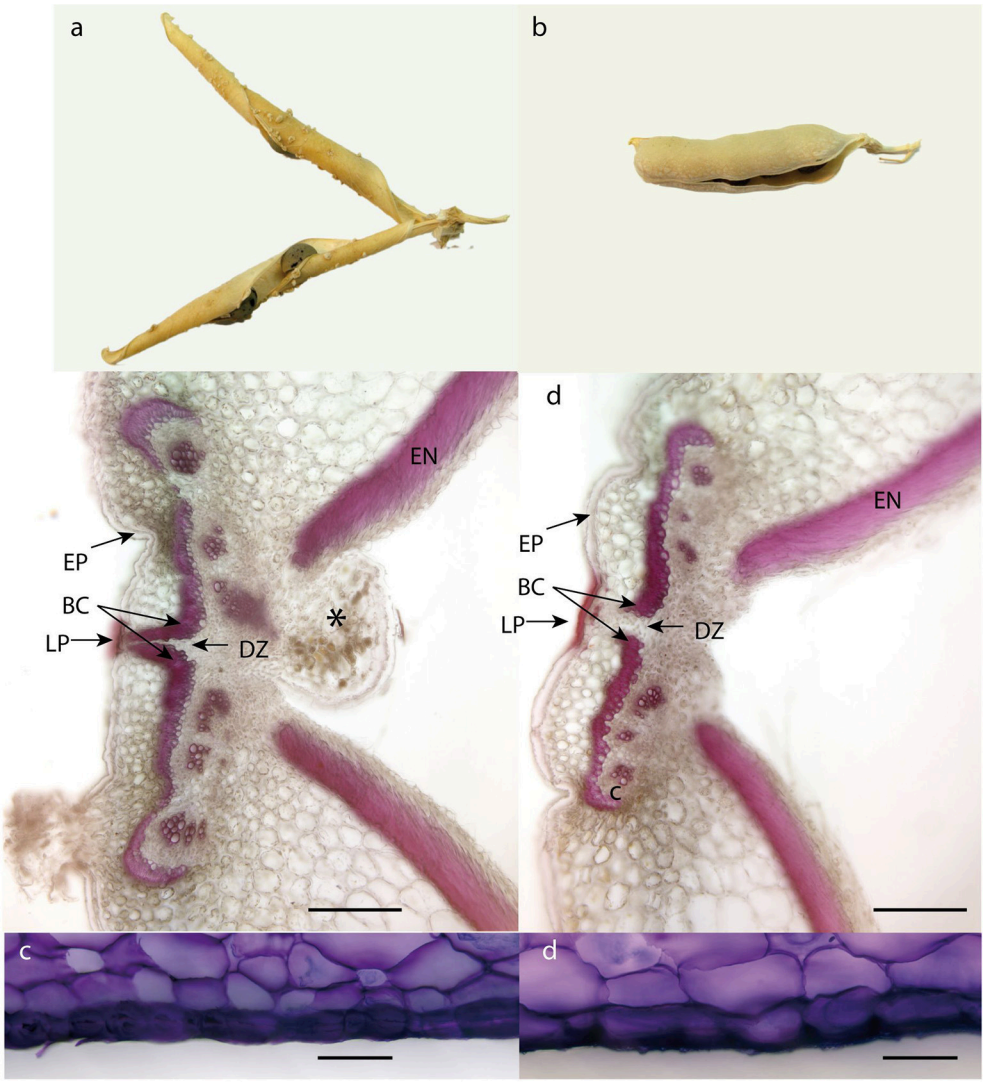

FIGURE 11 | Dorsal side of pea dehiscent and indehiscent pod. (a) Mature pod of dehiscent line (JI 64). **(b)** Mature pod of indehiscent line (JI 92). **(c,d)** Sections of dorsal side of dehiscent and indehiscent pea pod stained with fluoroglucinol: *, funikulus; BC, bundle cap; DZ, dehiscence zone; EN, endocarp, including inner sclerenchyma; EX, exocarp; EP, epidermis; DZ, dehiscence zone; LP, lignified epidermal plate.

LGIII: 24.5); MACE-P009 (homolog of cathepsin B-like cysteine protease in *M. truncatula*, *P. sativum* LGV: 20.3); MACE-P015 (homolog of peptidoglycan-binding domain protein gene in *M. truncatula*, *P. sativum* LGIII: 103.2); and Shatterproof (homolog of MADS-box transcription factor in *M. truncatula*, *P. sativum* LGIII: 89.3). As a result we didn't find any trend in down or over expression in contrasting RILs in relationship to dehiscence levels, with only one exception of MACE-P015 (homolog of peptidoglycan-binding domain protein gene in *M. truncatula*, *P. sativum* LGIII: 103.2) which showed trend of down expression in all tested indehiscent RILs (**Figure 10B**) with only one exception—RIL 61 (JI 64 × JI 92). Base on screening of PCR length polymorphism we recognized this line 61 (F_6) as heterozygous in MACE-P015 candidate gene because of presence of both parental alleles.

GO Annotation, KEGG

To annotate the DEGs in dehiscence-parents and RILs, the consensus MACE sequences were searched against the NCBI non-redundant protein database by blastx using *e*-value cut off of $1e^{-05}$. In biological process, most DEGs were found to be involved in metabolic processes (37 genes, 34%) which includes cellular metabolic process (28 genes, 26%) and primary metabolic process (28 genes, 26%), pigmentation (8 genes, 7%), regulation of biological process (8 genes, 7%), developmental processes such as anatomical structure development (3 genes, 3%; **Table S6**). In molecular function, the maximum DEGs were found to be involved in catalytic activity which includes mainly hydrolase activity (12 genes, 11%), transferase activity (8 genes, 7%) and oxidoreductase activity (7 genes, 6%). Next, the DEGs were mostly found to be involved in binding related activities such

as nucleic acid binding (10 genes, 9%) followed by nucleotide binding (9 genes, 8%) and nucleoside binding (5 genes, 5%). In the cellular component, the bulk of the DEGs belonged to cell part (41 genes, 38%) followed by membrane related proteins (22 genes, 20%), intracellular organelle (23 genes, 21%), and membrane-bound organelle (19 genes, 18%; **Table S6**). The KEGG pathway analysis showed that PsCam046431 is involved in phenylpropanoid biosynthesis, PsCam021037 in pentose phosphate, PsCam038465 in glycerophospholipid metabolism and PsCam042882 in carbon fixation pathways.

Genetic Mapping of Dehiscence Locus

We assume that dehiscent and indehiscent RILs bulks are contrasting for genomic regions which are responsible for dehiscence trait, because of selection for this trait and repeated selfing of RILs lines. On the other hand remaining genomic regions should be represented by polymorphic reads from RILs libraries due to mixing of RILs lines during bulking. When we compared polymorphism in reads from indehiscent RILs bulk and dehiscent RILs bulk three homozygous SNP rich genomic region associated with dehiscent trait were identified due to identification of homozygous SNP which indicated that this region was under selection during RILs lines development (**Figure S6**). Based on sequence homology first of them is located in the second half of *M. truncatula* chromosome 1. Second and third region are more clearer in contrast with first region. Second region is located at the beginning of chromosome 2 where homozygous SNP are concentrated around 2 megabase and third region is situated around 53 megabase at the end of chromosome 3. Others homozygous SNP are spread across all *Medicago* chromosomes and do not form distinct cluster.

DISCUSSION

Plant domestication process is interesting phenomenon of accelerated human directed evolution. To dissect genetic changes associated with this process, either wild to cultivated crosses and linkage mapping or newly genome wide association mapping are employed to infer on number of genes governing domestication traits. Although some of the genes underlying domestication traits were shown to be regulated at transcriptional level (Doebley et al., 1997; Konishi et al., 2006) limited studies were conducted to investigate transcriptomic changes between wild progenitors and cultivated crops, analyzing pod or seed tissues, such as wheat glumes (Zou et al., 2015). We used comprehensive transcriptomic, metabolomics, and anatomical analyses to compare domesticated and wild pea seed coats and pods in relation to the loss of seed dormancy and pod dehiscence.

Seed Coat Anatomical Structure and Histochemical Properties

Histological analysis of the seed coat in *M. truncatula* revealed changes in cell wall thickness in the outer integuments throughout seed development (Verdier et al., 2013a). In *Arabidopsis* and *Melilotus* (legume), seed permeability was modulated by mutations affecting extracellular lipid biosynthesis (in Verdier et al., 2013a). Similarly, in *M. truncatula*, cells of the outer integument showed abundant accumulation of polyphenolic compounds (**Figure 4**); which upon oxidation may impact seed permeability (Moïse et al., 2005). Current knowledge about physical dormancy mainly comes from studies on morphological structure, phenolic content, and cuticle composition in legume species (reviewed in Smýkal et al., 2014). Morphological observation indicated that seed hardness was associated with the structure of palisade and cuticular layer (Vu et al., 2014) and presence or absence of cracks (Meyer et al., 2007; Koizumi et al., 2008). Other authors have proposed that the compositions of carbohydrates, hydroxylated fatty acids, or phenol compounds in seed coats control the level of permeability (Mullin and Xu, 2000, 2001; Shao et al., 2007; Zhou et al., 2010). Mullin and Xu (2001) found that the seed coat of an impermeable genotype had a high concentration of hemicellulose, essentially composed of xylans, which would reduce the hydrophilicity of the seed coat. We have found considerable structural and functional differences in testa properties between wild and domesticated peas. Contrary to Ma et al. (2004), who found small cuticular cracks in soft but not hard seeds of soybean, the surface was similar among used lines with only small discontinuities over the whole surface (**Figure 3**). However, we cannot exclude that these small fissures resulted from the SEM sample preparation, similarly to the above mentioned work. In the non-dormant genotypes subjected to imbibition, large fissures appear preferentially in the hilar region and strophiole (not shown). However, those are the most likely consequence of embryo imbibition and its volume increase and thus we do not expect those as primary sites of water entrance. There is a lack of detailed description of the primary pathway of water entry in pea as well as in the whole *Fabaceae* family, although the topic is thoroughly discussed (e.g., Baskin et al., 2000; Meyer et al., 2007; Ranathunge et al., 2010; Smýkal et al., 2014). It is thus not clear whether the hilar or strophiolar region is the primary entrance of water in the non-dormant genotypes as suggested also by McDonald et al. (1988) or Korban et al. (1981) in soybean and common bean (Agbo et al., 1987) or if the minor fissures present in the cuticle and properties of the outer part of palisade macrosclereids make the difference as suggested by Ma et al. (2004). Interesting and generally neglected feature of macrosclereids is a presence of autofluorescent, phenolics containing lipidic material in the terminal caps of macrosclereids above the light line, which is not directly connected with the cuticle (**Figure S2**). There is no detailed information on the nature of this material or its possible functional significance. Obviously, detailed structure of outer part of the palisade macrosclereids deserves future attention. Dormant genotypes have thicker macrosclereids palisade layer, which might contribute to the water impermeability of coats of dormant pea genotypes as suggested by Miao et al. (2001). However, thickness alone does not necessarily account for water impermeability (de Souza and Marcos-Filho, 2001). Our results also suggest different thickness of palisade layer among the dormant genotypes with the most dormant JI64 having the thickest palisade layer (**Figure 2**). Metachromatic staining with toluidine blue revealed that the non-dormant non-pigmented genotype Cameor exhibits high level of polyanionic pectins

with exposed free carboxyl groups (**Figure S3**). On the other hand, the non-dormant, but well-pigmented JI92 showed lower abundance of pectins related anions, similarly to pigmented dormant genotypes. There is clear connection between toluidine blue stainability and seed coat pigmentation—anthocyanidin presence. Taken together results from toluidine and Alciane blue with vaniline and DMACA suggest that tannins in the seed coats of pigmented peas are probably bound to other compounds of the cell walls, changing the staining properties of cell walls. Nature of these linkages is unknown, but covalently bound tannins might be indicated as alkaline hydrolysis releases proanthocyanidins from the cell walls (Krygier et al., 1982). Such expectation might be further supported by presence quercetin-3-rhamnoside fragments from LC/MS and MALDI-MS study. The detected increase in metachromatic staining of testa cell walls after weak acid treatment might be indicative of a crosslinking of pectins with other compounds in dormant as well as JI92 genotype, which is released in acid environment. Interestingly, it was reported that condensed tannins might be released from linkages in acid environment (Porter, 1989). We can speculate whether the intensity of pectin—tannin crosslinking is associated with physical dormancy of pea. There are scarce references indicating possible role of tannins in cell wall polymer network and its properties (Pizzi and Cameron, 1986). There is some supposition for callose deposition in the light line area (e.g., de Souza et al., 2012). However, strong staining with aniline blue fluorochrome staining in the upper part of macrosclereids including the light line (**Figure S2**) cannot be attributed to callose. Such assumption is consistent with the specific callose antibody localization which led us to the conclusion that the signal for aniline blue is probably signal for one or more other compounds structurally similar to callose. The interaction of aniline blue fluorochrome with other 1,3- or 1,4-β-D-glucans was described by Evans et al. and this interaction depends on the degree of polymerization, and nature of substitution of the 1,3-β-D-glucan chain as well as on the concentration of phosphate in the staining solution (Evans et al., 1984). Deeper anatomical and histochemical analysis of seed coat and more reliable detection of primary entrance point of water during early rehydration phase is needed.

Differentially Expressed Genes during Seed Coat Development and Seed Dormancy

Seed development has been thoroughly studied in number of crops including legumes, especially with focus on embryo development (Bewley et al., 2013). In our study, we have made comparative transcriptomics analysis in order to dissect candidate genes/pathways associated with domestication imposed changes on seed coat properties. Temporal transcriptional changes during seed and pod development were studied in pigeonpea (Pazhamala et al., 2016), soybean (Aghamirzaie et al., 2015; Redekar et al., 2015), *Medicago* (Gallardo et al., 2007; Benedito et al., 2008; Verdier et al., 2013a; Righetti et al., 2015), peanut (Zhu et al., 2014; Wan et al., 2016), and pea (Liu et al., 2015) seeds but no study was made on comparison of wild progenitor and cultivated crop. Moreover, these studies analyzed either entire seed/pod or developing embryos, while in our work we have used excised seed coat or dissected pod suture. During the RNA isolation from the seed coat tissue, we have experienced great difficulties when working with wild pea seed samples. As reported earlier for pigmented soybean seeds (Wang and Vodkin, 1994) proanthocyanidins binds to RNA and prevent its extraction. We failed when using standard phenol/chloroform (McCarty, 1986) or guanidium thiocyanate (Chomczynski and Sacchi, 1987) methods, as well as common plant tissue RNA isolation kits.

There is problem of ambiguously mapped reads, which are the major source of error in RNA-Seq quantification (Robert and Watson, 2015). Short-read alignment is a complex problem due to the common occurrence of gene families. In contrast to RNA-seq, MACE methodology is derived from 3′ UTR end of transcript and each is represented by single molecule. The choice of quantification tool also has large effect, as these also differ in the way they handle aligned data and multi-mapped/ambiguous reads (Robert and Watson, 2015). In order to exclude or minimalize that identify DEGs are solely due to the genetic differences between contrasting parental genetic stocks we have used phenotypically classified bulks derived from RILs (**Table S1**, **Figure S1**). The concept of Bulk segregant analysis (BSA) was established as a method to detect markers in a specific genomic region by comparing two pooled DNA samples of individuals from a segregating population (Michelmore et al., 1991). Coupling BSA with the high throughput RNA sequencing has been shown to be an efficient tool for gene mapping and identification of differentially expressed genes (Chayut et al., 2015; Bojahr et al., 2016). One possible bottleneck of our analysis was bulking of several developmental stages into single MACE sample, where temporal and spatial expression might be hidden, resulting in differences between MACE and qRT-PCR data. Dynamic nature of gene expression both in spatial and temporal levels is clearly seen at qRT-PCR analysis of selected DEGs (**Figures 9**, **10**). The bulking of various developmental stages, moreover from different genotypes (in case of RILs) is the source of imprecision which can result in masking of DEGs. The key to the successful use of BSA is precision of phenotypic assignment. Although some imprecision in phenotypic classification and comparable low number of RILs used for bulking (**Table S1**), transcriptomics analysis has provided valid results. As shown on heat-map (**Figure 8**), RIL bulks are indeed genetic mixture of parental genotypes. MACE method (Kahl et al., 2012) detects allele-specific SNPs and indels associated with the defined genotypes that can be instantly used in genetic mapping (Bojahr et al., 2016). As result, there was significant clustering of homozygous SNPs associated with seed dormancy (e.g., respective parental alleles) on *Mt* chromosomes 3 and 4, or chickpea chromosomes 5 and 7, respectively (not shown). These correspond to pea linkage groups (LG) III and IV. Using identical RIL mapping population and DARTseq markers, we mapped seed coat thickness to LG I, III, IV, and VI (unpublished) and percentage of seed germination to LGII. These indicate that there is likely more than single major gene involved in seed dormancy, acting at different stages (testa thickness, permeability).

Despite that detected DEGs between dormant and non-dormant pea seeds belongs to various GO and KEGG pathways, the largest number of annotated ones was found within phenylpropanoid and flavonoid pathways (**Figure S4**). These

are involved in various activities such as UV filtration, fixing atmospheric nitrogen, and protection of cell walls (Zhao et al., 2013). Analysis of soybean mutant defective in seed coat led to identification of differentially expressed proline-rich and other cell wall protein transcripts (Kour et al., 2014). Moreover, this single gene mutation has resulted in differential expression of 1,300 genes, pointing out that complex series of events, many manifested at the transcript level, lead to changes in physiology, and ultimately structure of the cell wall. Similarly, we speculate that gene/-s causative of pea seed dormancy results in complex transcriptional and metabolomics changes. Recently, KNOTTED-like homeobox (KNOXII) gene, KNOX4, was found responsible for the loss of physical dormancy in the mutant *Medicago* seeds (Chai et al., 2016) resulting in differences in lipid monomer composition. These findings are in agreement with our data obtained by laser desorption-ionization mass spectrometric comparative analysis between dormant and non-dormant pea genotypes. Especially long chain hydroxylated fatty acids such as mono and dihydroxylated hexacosanoate, heptacosanoate, and octacosanoate were found in higher concentration in dormant peas compared to non-dormant ones (as shown in **Figure 8** for dihydroxyheptacosanoate and dihydroxyoctacosanoate), implying that the presence of a greater proportion of hydroxylated fatty acids may provide a greater interconnectivity of cutin hydrophobic components improving its stability and impermeability for water as discussed also by Shao et al. (2007). As downstream targets of KNOX4 gene, several key genes related to cuticle biosynthesis were identified, such as the cytochrome P450-dependent fatty acid omega-hydroxylase and fatty acid elongase 3-ketoacyl-CoA synthase (Chai et al., 2016). We have not found homologs genes when searched within our DEGs set. It can be hypothesized that different genes have been altered in independently domesticated crops, although possibly acting on identical pathways. There are limited studies combining of transcriptomic and metabolomics analysis (Enfissi et al., 2010) or a combination of both techniques including proteomics (Barros et al., 2010; Collakova et al., 2013). Such integrative approach enables not just to identify transcript and metabolite changes associated with given process, but also to focus on biochemical pathways relevant to studied trait and possibly also delimit candidate genes. As shown in *Medicago* (Verdier et al., 2013a) and soybean (Ranathunge et al., 2010) the gene expression in seed coat is complex and dynamic. Since we could not currently annotate 10% of detected transcripts, it can be expected that with available pea genome this could be further improved. They might include putative target candidate/-s for seed coat permeability.

Dormant Pea Seed Coat Accumulates More Proanthocyanidins

In legume seeds, there are three parts: the seed coat, the cotyledon, and the embryonic axis which, on average, represent 10, 89, and 1%, respectively, of the seed content. Seed coat pigmentation was shown to correlate with imbibition ability in several legumes, including common bean (Caldas and Blair, 2009), chickpea (Legesse and Powell, 1996), yardlong bean (Kongjaimun et al., 2012), faba bean (Ramsay, 1997), and pea (Marbach and Mayer, 1974; Werker et al., 1979). The presence of proanthocynidins (PAs) in seed coats can be assessed by the appearance of brownish coloration, which is the result of PA oxidation by polyphenol oxidase (Marles et al., 2008). In soybean (*Glycine max*), the recessive *i* allele results in high anthocyanin accumulation in the seed coat, resulting in dark brown or even black color (Tuteja et al., 2004; Yang et al., 2010). In contrast, the dominant *I* allele, which silences chalcone synthase (CHS) expression and hence blocks both anthocyanin and PA biosynthesis, results in a completely colorless seed coat. However, there is not simple relationship between the testa pigmentation imposing dormancy, as numerous cultivated pea varieties have colored testa yet do not display seed dormancy as illustrated by this study used JI92 landrace. Mendel's *A* gene beside flower color has pleiotropic effect including seed coat pigmentation (Hellens et al., 2010), yet these traits can be decoupled by recombination (Smýkal, unpublished). Second, *B* gene of pea encodes a defective flavonoid 3′, 5′-hydroxylase, and confers pink flower color, by control of hydroxylation of flavonoid precursors (Moreau et al., 2012). Neither this mutation results in alteration of seed dormancy. Comparably more is known on *Arabidopsis* and *Medicago* seed development owing to available mutants. Many of these mutations indicate the important role of proanthocyanidins and flavonoid pigments in testa development (Graeber et al., 2012) including effect on seed dormancy. The proanthocyanidins (PAs) received particular attention due to their abundance in seed coats (Dixon et al., 2005; Zhao et al., 2010) including pea (Ferraro et al., 2014). PAs are also known as the chemical basis for tannins, which are considered to be important part of physical dormancy in some species (Kantar et al., 1996; Ramsay, 1997). Flavan-3-ol-derived PA oligomers and anthocyanins are derived from the same precursors, proanthocyanidins (Lepiniec et al., 2006) and chemical diversity is introduced early in the pathway by cytochrome P450 enzymes (reviewed in Li et al., 2016a). Anthocyanidin synthase, anthocyanidin reductase, and leucoanthocyanidin reductase were studied at transcriptional level by Ferraro et al. (2014) in cultivated pea varieties and showed to be developmentally regulated. In our comparative transcriptome profiling we have not found any of these genes to be among DEGs, suggesting that differences in metabolites (quercetin, gallocatechin) found by chemical analysis are not at these steps of PA biosynthesis. Anthocyanidins are either immediately modified by glycosylation to give anthocyanins by anthocyanidin 3-O-glycosyltransferases (UGTs) or reduced to generate flavan-3-ols (such as epicatechin) by anthocyanidin reductase for PA biosynthesis (Xie et al., 2003, 2004). There are several described *Medicago* mutants defective in respective genes, resulting in reduced testa pigmentation (Li et al., 2016b), although the relationship to seed dormancy was not specifically investigated. Indeed, we have detected several differentially expressed UDP-glycosylases, two of them studied by qRT-PCR (**Figure 9**). The glycosyltransferase superfamily consists of 98 subfamilies and only few have been characterized so far. Only a few members of the UGT72 family been shown to have activity toward flavonoids; such as the seed coat-specific UGT72L1 from *Medicago* (Zhao et al., 2010) and several seed specific

UGTs in *L. japonicus* (Yin et al., 2017). The UGT72L1 catalyzes (Zhao et al., 2010) formation of epicatechin 3′-O-glucoside (E3′OG), the preferred substrate for MATE transporters. MATE1 mutant display altered seed coat structure and PAs accumulation (Zhao and Dixon, 2011) and also has significantly lower seed dormancy levels (Smýkal, unpublished). The mechanism of PA polymerization is still unclear, but may involve the laccase-like polyphenol oxidase (Zhao et al., 2010). Notably, *Medicago myb5* and *myb14* mutants exhibit darker seed coat color than wild-type plants, with *myb5* also showing deficiency in mucilage biosynthesis, and accumulating only of the PA content of wild-type plants. When *myb5* seeds are exposed to water, they germinate readily without dormancy typical of wild type *Medicago* seeds (R. Dixon, personal communication and P. Smýkal, unpublished). All these observations suggest that PA oligomers play indeed a role in seed coat mediated dormancy.

Our LC/ESI-MS experiments confirm the presence of significantly higher contents of dimer and trimer of gallocatechin (i.e., soluble tannins of prodelphinidin type) in dormant compared to non-dormant pea genotypes. Catechin dimer and trimer was also found in the pea seed coat extracts but their differences between dormant and non-dormant peas are much less significant than their gallocatechin counterparts. This fact points out the significance of hydroxylation of B-ring of PAs in relation to dormancy. The insoluble PAs are the result of oxidative cross-linking with other cell components. Variation in PA content in the pea seeds has been reported (Troszyńska and Ciska, 2002) but not in comparison of wild vs. cultivated peas. PAs play also important roles in defense to pathogens, and because of the health benefits are of industry and medicine interest. PA biosynthesis and its regulation have been dissected in *Arabidopsis* using *transparent testa* (*tt*) mutants, which regulate production, transport or storage of PAs (Lepiniec et al., 2006), and 20 genes affecting flavonoid metabolism were characterized at the molecular level (reviewed in Bradford and Nonogaki, 2009). Many of these flavonoid biosynthesis pathway genes have been found to affect dormancy of *Arabidopsis* seeds, indicating the role of pigments in this process (Debeaujon et al., 2000). Similarly *Medicago* also synthesizes PAs in the seed coat, which consists essentially of epicatechin units (Lepiniec et al., 2006; Zhao et al., 2010). Polymerization of soluble phenolics to insoluble polymers is promoted by peroxidases (Gillikin and Graham, 1991) and catecholoxidases (Marbach and Mayer, 1974; Werker et al., 1979), which are abundant in legume seed coats. Positive correlation in content of phenolics, the requirement of oxidation and the activity of catechol oxidase in relation to seed dormancy (germination) in wild vs. domesticated pea seeds have been shown by Marbach and Mayer (1974) and Werker et al. (1979). Recently, epicatechin, cyanidin 3-O-glucoside, and delphinidin 3-O-glucoside were isolated in wild compared to cultivated soybean seed coats (Zhou et al., 2010) with epicatechin being in significant positive correlation with hardseededness.

Beside proanthocyanidin also flavonols, first of all quercetin derivatives, are frequently found in legumes including pea (Dueñas et al., 2004). We found significantly higher content of quercetin rhamnoside in dormant JI64 genotype compared to non-dormant JI92. Similarly, its hydroxylated analog, i.e., myricetin-3-rhamnoside appeared to be a marker of dormancy. This compound was found in many legumes including chickpea, horse gram (Sreerama et al., 2010) and pea (Dueñas et al., 2004). As described in review of Agati et al. (2012) the antioxidant properties of flavonoids represent a robust biochemical trait of organisms exposed to oxidative stress of different origin during plant-environment interactions (regulation of the action of reaction oxygen species, ROS). The effect of ROS on the plant developmental processes including seed germination was described by Singh et al. (2016) including increase of free radical scavenging during pea seed germination (Lopez-Amoros et al., 2006). Presence of phenolic compounds in seed coat might help to protect against fungal diseases during germination as shown in lentil (Matus and Slinkard, 1993).

Pod Dehiscence

Pod maturation might terminate with pod shattering, which is an important trait for seed dispersal of wild species but generally unwanted trait in crops (Fuller and Allaby, 2009). Central to the ballistic seed dispersal in *Pisum* is the dehiscent pod (single carpel fused along its edges) where the central pod suture undergoes an explosive rupturing along a dehiscence zone (Ambrose and Ellis, 2008). During pod shattering, the two halves of the pod detach due to a combination of the diminished cell walls adhesion in the dehiscence zone, and the tensions established by the specific mechanical properties of drying cells of endo and exocarp of the pod shell. These two principal aspects are shared among families producing dry dehiscent fruit, such as *Fabaceae* and *Brassicaceae* (Grant, 1996; Dong and Wang, 2015). The spring-like tension within the pod shell is generated during differential drying-induced shrinkage of endocarp and the outer part of the shell—exocarp (Armon et al., 2011). Properties of both the obliquely arranged rigid and lignified inner sclerenchyma as well as the pectin rich exocarp cell with longitudinal orientation are crucial factors of springing in Fabaceae pods. Regulators of their development affecting geometrical arrangement of the layers and their histological properties (cell wall thickness and composition, lignification, hydration) will be key factors generating required tension. Composition and characteristics of pod cell shell cell walls correlate with shattering of yardlong bean and wild cowpea (Suanum et al., 2016) and thickness of the shell and extend of sclerenchymatous dorsal bundle caps was connected with shattering in soy (Tiwari and Bhatia, 1995). The major QTL controlling pod dehiscence in soybean is *qPDH1* (QTL for Pod Dehiscence 1). *qPDH1* had been recently cloned and shown to encode a dirigent-like protein expressed in the sclerenchyma of differentiating endocarp and modulating the mechanical properties of the pod shell. Lignin biosynthesis is the most likely process affected by *qPHD1* (Suzuki et al., 2010; Funatsuki et al., 2014), which might be connected with modulation of torsion within drying pod walls (Funatsuki et al., 2014). However, the precise biochemical activity of *qPHD1* is still unclear. Similarly the sclerenchyma differentiation and lignification of the endocarp and valve margin cells of Arabidopsis are central to silique dehiscence with *NAC SECONDARY WALL THICKENING PROMOTING FACTOR 1*

(NST1) and *SECONDARY WALL ASSOCIATED NAC DOMAIN PROTEIN 1 (SDN1)* being identified as master regulators of their differentiation (Zhong et al., 2010). Lignification of endocarp and valve margin cells is lost together with dehiscence in the *nst1snd1* double mutant (Mitsuda and Ohme-Takagi, 2008). The other decisive point of pod dehiscence is mechanical stability/instability of dehiscence (suture) zone which might trigger the explosive release of pod shell tension (Grant, 1996). Decreased cell to cell adhesion, might be carried out by action of endo-1,4-glucanases and endopolygalacturonases disintegrating the middle lamella in the separation layer (Christiansen et al., 2002). Degradation of pectin in the middle lamella of abscission zone is common theme in fruit shattering (Dong and Wang, 2015). Contrary, mechanism of pod shattering resistance due to reinforcement of the suture has been described in domesticated soybeans *NST1/2* homologous transcription factor *SHATTERING1-5* (*SHAT1-5*) from NAC family has been unveiled, inducing excessive secondary cell wall deposition and lignification in the outer part of the suture (fiber cap cells; Dong et al., 2014). Up-regulated expression of *SHAT1-5* in domesticated soy "locks" the dehiscence zone interconnecting the vascular bundle caps of sclerenchymatous fibers in the suture vicinity preventing shattering.

The fundamental elements of fruit shattering regulatory network is being uncovered recently in *Arabidopsis* and homologous genes were identified also in other species and crops (*Zea mays*, *Triticum aestivum*, *Oryza sativa*, *Glycine max*, *Sorghum bicolor*, *Sorghum propinquum*, or *Solanum lycopersicum*; for review see Dong and Wang, 2015; Ballester and Ferrandiz, 2016). MADS box genes of *Arabidopsis SHATTERPROOF1 (SHP1)* and *SHATTERPROOF2 (SHP2)* participate in the dehiscence zone specification (Liljegren et al., 2000). *INDEHISCENT (IND)* b-HLH transcription factor act down-stream to SHP1/2 as regulator sclerenchyma differentiation in endocarp and valve margin. The *shp1/2* double mutant as well as *ind* produces indehiscent siliques devoid of proper cell specification and differentiation in the dehiscence zone (Liljegren et al., 2004; Dong and Wang, 2015). SHP1 and SHP2 are required for the proper specification of the different cell types within the valve margin and the DZ and both genes probably represent the top of the hierarchy regulating DZ formation (Liljegren et al., 2000). *SHATTERPROOF* genes along with *INDEHISCENT* (*IDEH*) are the main regulators of establishment of lignified layer, which causes pod dehiscence. Another MADS box gene involved in dehiscence zone formation is *FRUITFULL* (*FUL*), which expression appears at the inception of the carpel primordia, and soon after becomes restricted to the cells that will give rise to the valves, a pattern that is complementary to that of the SHP genes (Liljegren et al., 2004; Dong and Wang, 2015). Significant up-regulation of *SHATTERING1–5* (*SHAT1–5*) in the fiber cap cells (FCC) of cultivated soybean was shown to be responsible for the excessive cell wall deposition in the FCC, which in turn prevents the pod from committing dehiscence after maturation (Dong et al., 2014). Homologous genes defining dehiscence zone identity and its differentiation *INDEHISCENT*, *SPATULA*, *SHATTERPROOF*, *bZIP*, and *SHATTERING* (Ferrándiz et al., 2000; Girin et al., 2011; Dong et al., 2014) were identified in *Pisum* genome and their abundance was tested in RNA isolated from pod suture tissue of wild and domesticated pea as well as of contrast RILs incurred from crossing of wild and domesticate parent with dehiscent or indehiscent pods. In case of *SHATTERPROOF* and *SHATTERING* homologous genes, we found differences in expression between parental lines of pea but we didn't find the similar results in case of contrast RILs. The other homologous genes (*INDEHISCENT*, *SPATULA*, and *bZIP*) did now exhibit any significant difference in expression between dehiscent and non-dehiscent phenotypes.

In the legumes, the pod shattering trait is controlled by one or two dominant genes or QTL. In pea and lentil, genes controlling pod shattering map to a syntenic region, suggesting that the same genes may have been modified during the domestication of the two cool-season legumes (Weeden et al., 2002; Weeden, 2007). Single locus control of pod dehiscence was found in lentil (Ladizinsky, 1998), while two loci in mungbean (Isemura et al., 2012), yardlong bean (Kongjaimun et al., 2012), one controlling the number of twists along the length of the shattered pod, and second the percentage of shattered pods, similarly to two loci found in pea (Weeden et al., 2002; Weeden, 2007), and common bean (Koinange et al., 1996). Bordat et al. (2011) localized *Dpo* locus responsible for loss of pea pod dehiscence on LGIII. We obtained the similar result by the genome-wide DArTseq analysis. Based on comparison of our candidate gene position in *M. truncatula* genome and SNPs map (Tayeh et al., 2015) we localized our candidate genes in pea genome. In total of our 25 candidate genes 7 are localized on LGIII, 6 on LGVI, 4 on LGII, 3 on LGV, 2 on LGVII, 2 on LGIV, and 1 on LGI (not shown). MACE-P015, the main candidate gene possibly responsible for pod dehiscence localized on LGIII, is a homolog of peptidoglycan-binding domain protein (PGDB) of *M. truncatula* (Medtr2g079050). These proteins may have a general peptidoglycan binding function and this motif is found at the N or C terminus of a variety of enzymes involved in bacterial cell wall degradation. Many of the proteins having this domain are so far uncharacterized. Matrix metalloproteinases (MMP), which catalyze extracellular matrix degradation, have N-terminal domains that resemble PGBD (Seiki, 1999). On the other hand our candidate MACE-P015 has also 80% match with *Cicer arietinum* proline-rich extensin-like protein EPR1 (XM_004488673). Extensins are plant specific structural cell-wall proteins (Lamport et al., 2011); they can account for up to 20% of the dry weight of the cell wall and can significantly modulate mechanical cell wall properties through linkages to other cell wall component, which can play a role in pod dehiscence.

New Insights into Pea Seed and Pod Development in Relation to Domestication

Study of biochemical and molecular mechanisms underlying plant domestication process is important area of research in plant biology. In the current study we used a comparative anatomy, metabolomics, and transcriptome profiling of pods and seed coats in wild and domesticated pea in order to identify genes associated with loss of seed dormancy as well as pod dehiscence.

We have identified genes showing differential expression in respective parents as well as phenotypically contrasting RILs. Among others, there were number of genes belonging to phenylpropanoid pathway, which was also identified by metabolomics analysis of seed coat. Our results support the role of proanthocyanidins and their derivatives in physical seed coat mediated dormancy. One of the identified differentially expressed gene involved in pod dehiscence showed significant down-expression in dorsal and ventral pod suture of indehiscent genotypes. Moreover, this homolog of peptidoglycan-binding domain or proline-rich extensin-like protein mapped correctly to predicted *Dpo1* locus on PsLGIII. This integrated analysis of the seed coat in wild and cultivated pea raised new questions associated with domestication and seed dormancy. Having underlying gene(s) in hands for various independently domesticated legume crops it would help our understanding of genetic and molecular processes involved in seeds dormancy. Moreover, extended knowledge on control seed dispersal and seed dormancy is necessary for diverse applications—biodiversity conservation as well as breeding.

AUTHOR CONTRIBUTIONS

Conceived and designed experiments: PS, PB, AS, and PH. Performed experiments: IH, PS, AJ, PH, LP, MC, and MV. Analyzed the data: IH, AJ, PB, OT, and KA. Wrote the paper: IH, AJ, OT, PH, and PS. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00542/full#supplementary-material

Table S1 | Germination characteristics of parental and RIL lines.

Table S2 | Summary statistics for MACE sequencing data and annotation to the Pisum mRNA sequences from NCBI and to the contigs based on the *de novo* assembly of sequences.

Table S3 | List of primers used for qRT-PCR.

Table S4 | Complete set of DEGs detected in seed dormancy and pod dehiscence experiments, between wild and cultivated parental genotypes as well as RILs, with indicated homology to annotated pea transcriptome (Tayeh et al., 2015) and MACE expression values.

Table S5 | GO annotations of seed dormancy genes.

Table S6 | GO annotations of pod dehiscence genes.

Figure S1 | Germination indexes of all 126 RIL lines tested dat 25C over the period of 7 days. Ordered by cummulative germination percentages **(A)** with shown Coefficient of velocity **(B)**, Timson indexes **(C)**, and Mean germination time **(D)** arranged accordingly.

Figure S2 | Seed coat transverse sections from extrahilar region: Cameor **(a)**, JI92 **(b)**, JI64 **(c)**, and VIR320 **(d)**. UV excited autofluorescence (**a–d):** white arrow, cuticle; asterisk, light line. Sudan Red 7B staining of terminal parts of macrosclereids **(e–h)**: white arrow, cuticle; black arrow, lipidic material different from the cuticle). Aniline blue fluorochrome staining under UV excitation **(i–l)**: asterisk, light line; white arrow, the edge between pigmented and non-pigmented interface of JI92, inlay in **(i)** callose immunodetection (blue excitation); scale bars = 25 μm)

Figure S3 | Seed coat transverse sections from extrahilar region: Cameor **(a)**, JI92 **(b)**, JI64 **(c)**, and VIR320 **(d)**. Metachromatic toluidine blue staining is indicative of high density of polyanionic surface **(a–d)**: asterisk, light line. Toluidine blue staining after mild acid treatment **(e–h):** black arrow = the edge between pigmented and non-pigmented interface of JI92; scale bar = 50 μm.

Figure S4 | KEGG phenylpropanoid (A) and flavonoid **(B)** pathways of DEGs between dormant and nondormant seeds.

Figure S5 | KEGG phenylpropanoid pathway of DEGs between dehiscent and indehiscent pods.

Figure S6 | Strictly homozygous SNP between dehiscence and indehiscence RIL bulks mapped to the eight *Medicago truncatula* chromosomes.

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Appendix II (Paper II)

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Towards Better Understanding of Pea Seed Dormancy Using Laser Desorption/Ionization Mass Spectrometry

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Abstract: Seed coats of six pea genotypes contrasting in dormancy were studied by laser desorption/ionization mass spectrometry (LDI-MS). Multivariate statistical analysis discriminated dormant and non-dormant seeds in mature dry state. Separation between dormant and non-dormant types was observed despite important markers of particular dormant genotypes differ from each other. Normalized signals of long-chain hydroxylated fatty acids (HLFA) in dormant JI64 genotype seed coats were significantly higher than in other genotypes. These compounds seem to be important markers likely influencing JI64 seed imbibition and germination. HLFA importance was supported by study of recombinant inbred lines (JI64xJI92) contrasting in dormancy but similar in other seed properties. Furthemore HLFA distribution in seed coat was studied by mass spectrometry imaging. HLFA contents in strophiole and hilum are significantly lower compared to other parts indicating their role in water uptake. Results from LDI-MS experiments are useful in understanding (physical) dormancy (first phases of germination) mechanism and properties related to food processing technologies (e.g., seed treatment by cooking).

Keywords: pea; fatty acid; seed coat; seed dormancy; seed hardness; laser desorption-ionization mass spectrometry; imaging mass spectrometry; multivariate statistics

1. Introduction

Seed germination is a key step of plant life predetermining spread of particular plant species on the Earth [1]. In a population of wild seeds only a certain fraction of individuals starts to germinate in favorable conditions. Their inactive counterparts can survive adverse periods and thus ensure continuation of given plant species for long time. The extent of seed inactivity is expressed by dormancy [2]. Dormancy is a regulatory and adaptive trait in virtually all seed-plant species. There are several types of seed dormancy, with one of them being physical dormancy, executed by water impermeable seed coat [3]. This type is prevalent in legumes [4]. Its detailed description and classification, importance for agriculture and food industry and connection with other seed properties (i.e., seed hardness, pre-harvest sprouting, etc.) is given in two our recent papers [4,5] and in citations listed therein.

Activation of a seed starts with water imbibition and penetration of gases through the seed coat (their restriction is described as physical dormancy) [3]. These processes are connected with

structure and chemical composition of the most outer cell layers of seed coats [4]. A number of analytical techniques was used for chemical analysis of seed composition (including characterization of separated seed coat) starting with a selective staining [4], over standard instrumental techniques, i.e., gas chromatography with flame ionization detection (GC-FID) [6], high performance liquid chromatography (HPLC) with ultraviolet/visible (UV/Vis) spectrophotometric detection [7,8] up to hyphenated techniques, first of all gas chromatography-mass spectrometry (GC/MS) and liquid chromatography mass spectrometry (LC/MS) [9–11]. All these techniques utilize an interaction of plant material with a solvent (soaking or liquid extraction), often in combination with further chemical treatment (i.e., hydrolysis and derivatization). GC/MS was used for analysis of saponified and methylated seed coats [12]. These techniques dispose with high selectivity towards extractable analytes.

Number of techniques allow direct analysis of seeds in dry state or after a very short contact with a solvent (i.e., spray covering of surface with a matrix prior to a matrix assisted laser desorption/ionization mass spectrometric analysis (MALDI-MS), etc.) preventing (or significantly restricting) the initiation of the imbibition processes during sample preparation or (reversely) to study the changes after defined wetting of seed coat. Especially, spectrometric techniques such as MALDI-MS, desorption electrospray mass spectrometry (DESI-MS), direct analysis in real time mass spectrometry (DART-MS), nuclear magnetic resonance spectroscopy (NMR), Fourier-transform infrared spectroscopy (FTIR) and laser ablation–inductively coupled plasma–mass spectrometry (LA-ICP-MS) have been already used for analysis of seeds as recently reviewed [13–20]. Notably none of those techniques was used for direct measurement of separated seed coat tissue from embryo and endosperm.

Majority of later methods possesses spatial information (imaging methods). Perhaps the most widespread and powerful technique for the analysis of seed surface layers with respect to organic molecules is laser desorption/ionization imaging mass spectrometry. It provides high versatility often with acceptable selectivity and sensitivity. Mass spectrometry is very popular technique in proteomic and metabolic profiling of plant tissues. MALDI-MS technique combined with gel electrophoresis was used in study of proteomic composition of *Medicago truncatula* [21], *Lotus japonicas* and other legume seeds [22]. MALDI-mass spectrometry imaging (MALDI-MSI) protocols for the detection of small molecules in cryodissected immature barley grains are described in the work of Peukert et al. [23]. Bhandari et al. published a comprehensive high resolution MS imaging analysis of cryosections of two types of seeds (oil-seed rape and wheat) and other plant tissues (wheat rachis, stem base, rice root) concerning with germination and seed maturation [24]. Besides, Gorzolka et al. have shown the possibility of MALDI-MS imaging for spatial-temporal metabolite profiling during the germination of barley seeds [25].

To the best of our knowledge this is the first report of the utilization of laser desorption/ionization mass spectrometry (LDI-MS) for the analysis of seed coat composition in the relation to the level of seed dormancy. The main aim of this work was to study the potential of laser desorption/ionization mass spectrometry for the surface analysis of pea seed coat in mature dry state with respect to physical dormancy. Multivariate statistics on raw MS data was used for classification of pea genotypes and lines and obtained outputs correlated with level of dormancy. Dormant and non-dormant genotypes (possessing different propensity to imbibition and germination) were profiled and content of hydroxylated long-chain fatty acids (HLFA) was identified as the important discriminating factor. The obtained information is significant also for agricultural and food industry.

2. Results and Discussion

2.1. LDI-MS Measurement and Utilization of PCA and OPLS-DA for Data Analysis

LDI-MS spectra of outer surface of six different genotypes (JI92, Cameor, Terno, JI64, VIR320 and L100) were measured in positive and negative ion mode. These genotypes represent both wild and domesticated pea types commonly used in genetic and biological studies [5]. They contain both pigmented and non-pigmented seed coats. Strong signals at m/z 98.9769, 112.9229, 154.9325, 196.9439,

206.9943, 291.1685, 317.11198, 369.1229, 377.1309 and 485.3076 were observed in positive mode and those at m/z 121.0060, 132.0010, 143.9992, 156.0039, 169.0079, 193.0115, 204.0037, 214.0169, 217.0682, 268.0770, 282.1081 306.1075, 319.1063 and 323.1524 in negative ion mode. Differences in those major signals, however, do not allow a resolution of wild/dormant from cultivated/non-dormant genotypes. Although certain differences among some genotypes are visible (i.e., Terno in positive and Cameor in negative ion modes), the direct interpretation of MS spectra do not allow to find characteristic signals for cultivation/dormancy (raw LDI-MS and MALDI-MS spectra are given in supplemental material, Figure S1a–d). Since comprehensive evaluation of differences by direct raw MS spectra comparison was not possible, multivariate analysis was applied. Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) are generally the most proven methods to differentiate between classes in highly complex data sets. We started with application of classical PCA to visualize the chemical differences among samples by unsupervised (independent) way. Utilization of PCA as starting point during multidimensional data treatment was recommended for instance by Worley and Powers for metabolomics representing similar exploratory area [26]. Figure 1 shows the 3D Score plots obtained by PCA of raw LDI-MS and MALDI-MS data in positive and negative ion modes.

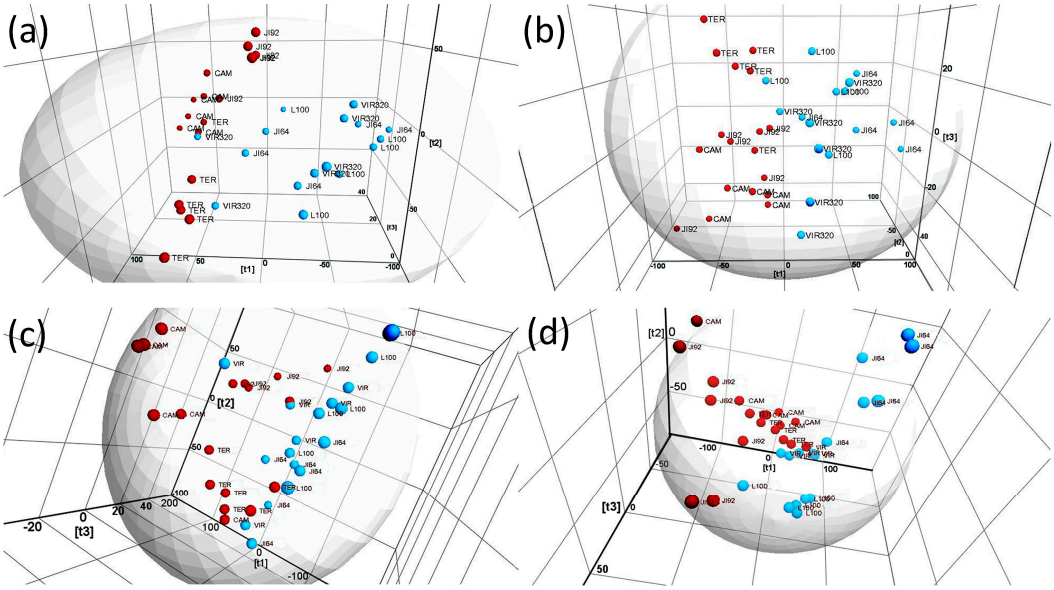

Figure 1. 3D Score plots obtained by Principal Component Analysis of MALDI-MS and LDI-MS data. Upper plots—measurement in negative ionization mode, (**a**) with matrix and (**b**) without matrix; bottom plots—measurement in positive ionization mode, (**c**) with matrix and (**d**) without matrix; dormant species are marked with blue and non-dormant ones with red bullets; Pareto scaling and marker intensity threshold 1000 was used for data matrix processing.

Despite a relatively large variation of replicated measurements, separation of dormant and non-dormant genotypes is evident in each ionization mode regardless whether matrix is used or not. Roughly, non-dormant samples are located on left and dormant ones on the right side in each 3D Score plot, respectively. These results suggest that laser desorption ionization mass spectrometry can be used for classification (resolution) of pea genotypes with regard to surface composition related to domestication/dormancy by direct analysis of dry maturated seeds as alternative method to classical study of germination. It should be emphasized that the resolution can be achieved without selection and knowledge of the origin of markers (signals significantly differing in content between dormant and non-dormant samples). Nevertheless, identification of one important group of markers was successful as described in next Chapters. In negative ion mode (Figure 1a,b), the first three dimensions of the Score plots explain 78.53% and 76.46% of data variability in matrix assisted and matrix free experiments,

respectively. In positive ion mode similar level of explained variability was achieved (80.28% and 75.58%, respectively). Complete datasets related to those Score Plots are given in supplementary material, Table S1a–d. Surface morphology differs significantly among genotypes (e.g., presence of "gritty" surface on seed coats of some genotypes, Figure S2). Such differences at surface can alter the mechanism of water penetration through the seed coat (the mechanism of seed imbibition and consequently dormancy)—from a biological point of view—as well as ionization efficiency during LDI-MS measurement—considering the used analytical methodology. To conclude, the resolution of dormant and non-dormant species was unambiguously achieved but surface morphology affects significantly the distribution of particular data-points.

As already mentioned, the resolution of dormant and non-dormant genotypes is achieved regardless the presence of matrix. Application of matrix using standard automatized solution spraying technique causes torsion of samples attached to MALDI plate. We observed that this process is more pronounced in non-dormant genotypes. Such torsion is connected with a tension among different seed coat cell layers during their contact with water and its extent is possibly due to thiner seed coat of non-dormant compared to dormant species [5]. When occurring on an intact seed in nature, this phenomenon could contribute to seed coat rupture, opening the seed for water and (consequently) accelerating germination [5]—hence the stronger connection of the torsion on MALDI plate with non-dormant (faster germinating) seed coats. Although the measurement of deformed seed coat pieces was possible and similar degree of distinction of samples according to dormancy level was achieved in matrix assisted and matrix free experiments, the changes of the surface cell layers by their swelling with the applied solvent during and after spraying represent a phenomenon negatively affecting both the sample preparation as well as signals in MS spectra. The matrix-free method should be therefore further preferred from methodological point of view. The effect of matrix presence on the significance of identified signals in terms of OPLS-DA (the position of corresponding markers in particular S-plots) will be discussed later.

The differences in chemical composition of individual dormant genotypes were studied against non-dormant genotypes (all three non-dormant genotypes as reference set) by OPLS-DA. Obtained signals almost completely differ when compared particular dormant genotypes with two exceptions (given by italics, Table S2) that could contribute to differences in the process of water imbibition. Signals of particular hydroxylated long-chain fatty acids appeared among the most important markers of JI64 genotype as discussed later in more details.

As already indicated, the distinctness in seed characters (i.e., size, pigmentation, surface properties, etc.) influences the LDI-MS experiment. We therefore further focused on JI64 (D) and JI92 (N) pair that is less contrasting in term of seed coat appearance but strongly different in seed coat permeability and dormancy (for details see Section 3.2. and [5]). Note that those two genotypes are very well separated by unsupervised PCA (see all 3D Score Plots, Figure 1). Data obtained by their measurement were also analyzed by OPLS-DA. Figure 2 shows the S-plots reflecting the differences in signals between both genotypes in negative ion mode (all markers and their coordinates in S-plots are given for both ionization modes in supplemental material, Table S3a–d).

The area of significant signals (further referred as dormancy markers, DM) in each S-plot corresponds with low risk region [27] that is given in this study as a box with following coordinates: CoeffCS(2) = 10–100% and p(corr)(1) = 75–100% from the highest value at CoeffCS(2)-axis (x-axis) and p(corr)(1)-axis (y-axis), respectively, in "dormant" part (the first quadrant) of the S-plot. Signals at m/z 255.2331, 281.2393, 283.2613, 411.3850, 425.3990, 437.3946, 441.3950 and 455.4065 fell into this region and are thus classified as significant DM in matrix free LDI-MS experiments (Figure 2a). Analogous signals were observed in MALDI-MS spectra as well but some of them out of low risk region of related S-plot (Figure 2b).

Those signals correspond well with theoretical m/z values of deprotonated molecules of common fatty acids (palmitic, oleic and stearic acid) and, perhaps more importantly, with hydroxylated long chain fatty acids (HLFA). Table 1 shows details of these markers. HLFA have been already considered as hydrophobic components increasing impermeability of cutin layers for water [5,6].

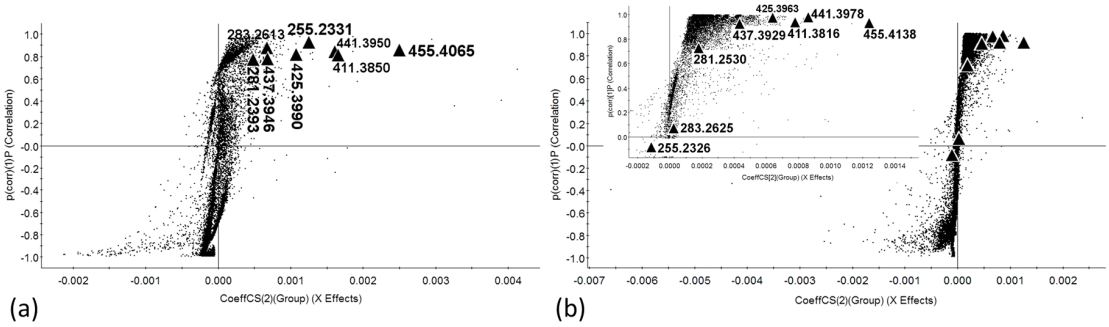

Figure 2. S-plots obtained by OPLS-DA of JI64 a JI92 genotypes. (**a**) Matrix free experiments; (**b**) matrix assisted experiments (inset in (**b**) shows zoomed dormant part of the appropriate S-plot); enlarged triangles indicate identified markers of physical dormancy.

Table 1. List of fatty acids found among dormant markers by LDI-MS, MALDI-MS and OPLS-DA analysis. (* deviation of measured mass (m/z) from theoretical value calculated from elemental composition).

Elemental Composition	Matrix Free Experiments, Figure 3a				Matrix Assisted Experiments, Figure 3b			
	[M–H]$^-$	dtm (ppm) *	Coordinates in S-Plot		[M–H]$^-$	dtm (ppm) *	Coordinates in S-Plot	
			CoeffCS(2)	p(corr)(1)			CoeffCS(2)	p(corr)(1)
$C_{16}H_{31}O_2^-$	255.2331	0.78	0.00125	0.90637	255.2326	−1.18	−0.00012	−0.09994
$C_{18}H_{33}O_2^-$	281.2393	−33.07	0.00049	0.74660	281.2530	15.64	0.00017	0.70174
$C_{18}H_{35}O_2^-$	283.2613	−10.24	0.00066	0.85055	283.2625	−6.00	0.00002	0.04306
$C_{26}H_{51}O_3^-$	411.3850	2.67	0.00162	0.80646	411.3816	−5.59	0.00079	0.92125
$C_{27}H_{53}O_3^-$	425.3990	−1.18	0.00107	0.81814	425.3963	−7.52	0.00065	0.96154
$C_{28}H_{53}O_3^-$	437.3946	−11.20	0.00067	0.77322	437.3929	−15.09	0.00042	0.91775
$C_{27}H_{53}O_4^-$	441.3950	1.36	0.00160	0.83272	441.3978	7.70	0.00087	0.96626
$C_{28}H_{55}O_4^-$	455.4065	−7.69	0.00249	0.84469	455.4138	8.34	0.00126	0.91329

There are several former reports confirming that LDI-MS is generally suitable technique for analysis of fatty acids. Pirkl et al. dealt with the direct profiling of fatty acids in insect tissues by LDI-MS [28], for instance. Budimir et al. published laser desorption/ionization on porous silicon mass spectrometric analysis of nonadecanoic acid and heneicosanoic acid [29]. Besides, Shroff et al. have shown that MALDI-MS can be used for very sensitive analysis of various acids including fatty acids in negative ion mode [30]. Since the defined standards of HLFA are not commercially available, our LDI-MS method was verified using a mixture of common palmitic and oleic acid. Both acids provided the signal of appropriate anions when measured separately and also when applied at the pea seed coat surface, i.e., signals at m/z 255.2312 and 281.2465 were achieved in both experiments (deviation from theoretical mass, dtm, was 6.7 and 7.5 ppm, respectively, corresponding MS spectra are given in supplementary material, Figure S3a,b). Those results confirm the capability of our method to analyze fatty acids present on the surface of seed coats. Tandem MS/MS experiments confirmed the identity of HLFA markers on seed coat surface (see Figure S4 showing LDI-MS/MS spectrum of dihydroxyoctacosanoate measured with Trap CE 30 eV yielding the highest signal of characteristic fragments). Loss of water from parent ion was observed providing the fragment at m/z 437.3940 ($C_{28}H_{53}O_3^-$). This loss suggests the presence of a hydroxyl group in the molecule. The signal of fragment arising by two consequent losses of water (confirming the presence of two hydroxyls in parent ion) was also observed but with low intensity (fragment at m/z 419.3841, $C_{28}H_{51}O_2^-$). Loss of CO_2 was not observed. Structure of dihydroxyoctacosanote fragments at m/z 183.1372, 253.2503 and 267.2664 was considered as well. The fragment at m/z 183.1372 corresponds with mass of undecenoate that can be formed by a cleavage of eleven-carbon chain from carboxyl end of dihydroxyoctacosanoate and elimination of water ($C_{11}H_{19}O_2^-$). The latter two fragments could arise by a cleavage of C17 and C18 chain and elimination of water ($C_{17}H_{33}O^-$ and $C_{18}H_{35}O^-$, respectively). A higher deviation

of measured m/z values from theoretical ones is due to weak signal of rising fragments in MS/MS spectra. The fragmentation pattern suggests the C10 and/or C12 hydroxylation, however, the detailed elucidation of the position of hydroxyl is the objective of future research. Analogous fragmentation pattern was described by Nilsson et al. [31]. Besides, the formation of fragments that do not bear a carboxy group was described also by Kerwin et al. [32]. The presence of the above discussed fragments further confirms the identity of the found HLFA. The other HLFA did not provide utilizable signals of fragments in MS/MS spectra due to lower intensity of corresponding parent ions. Loss of water (but with very low intensity) was observed also in MS/MS spectra of monohydroxylated HLFA (i.e., hydroxyhexacosanoate, parent ion at m/z 411.3865 and hydroxyheptacosanoate, parent ion at m/z 425.3927).

The identified HLFA provided significantly higher signal in spectra of dormant JI64 genotype compared to non-dormant JI92 confirming the results of untargeted multivariate statistics. Figure 3 shows the MS spectra of external and internal surface of seed coat of both in dormancy contrasting genotypes.

Signals of HLFA are visible in the spectrum of external surface of JI64 (Figure 3a). They appear very close to another (stronger) signals with the same nominal mass (arising probably by a thermal destruction of polysaccharides and lignin). These HLFA signals are missing in the other LDI-MS spectra measured on external surface of JI92 genotype as well as on internal seed coat surface and its cross sections of both genotypes (Figure 3b–f). The resolution of the HLFA signals can be improved using ion mobility separation. The signal of dihydroxyoctacosanoate dominates in the spectrum obtained by averaging over its ion mobility peak and baseline correction being well separated from the interfering signal at m/z 455.1493 (Figure 3g shows mobilograms reconstructed for the interfering signal, upper trace and dihydroxyoctacosanoate, bottom trace; Figure 3h shows the spectrum averaged over the whole mobility range, upper spectrum and over the mobility peak with apex at 125 bins, bottom spectrum). Similar ion mobility separation can be achieved for the other HLFA.

The yield of signal of the identified HLFA with respect to sum of all signals present in spectra (normalized signal) was further optimized. The effect of ion source parameters on the normalized response of analytes and on the total ion current in MS spectra was studied in JI64 genotype. The effect of hexapole RF amplitude, sample plate voltage, laser energy and laser step rate on the normalized signal of dihydroxyoctacosanoate as the HLFA with the highest mass and response was studied (supplementary material, Figure S5). The effect of hexapole RF amplitude is quite significant. This parameter is important for focusing and transmission of ions in a lower vacuum region and it is m/z dependent in a broad range. The optimal value of this parameter is 300 V. Besides, the decreasing of sample plate voltage from the default 25 to 10 V caused utilizable improvement of signal. The optimal value of laser energy was set at 300 (dimensionless parameter, the value affects position of neutral density filter that attenuates/amplifies the laser beam to provide energy setting functionality). Laser step rate (velocity of the laser movement) influences the signal of dihydroxyoctacosanoate significantly as well (optimal value is 30). Slower motion of laser burns seed coats and HLFA signals are suppressed probably due to pyrolysis products that rise in more amounts. The effect of this parameter is connected with applied the energy of laser beam. However, for MSI mode (discussed in detail in the next chapter) operating with more focused laser beam to achieve better spatial resolution (beam size 60 μm, pixel size 50 × 50 μm) the optimal laser energy was lower (250–300). Higher value than 300 rapidly burnt analyzed seed coats.

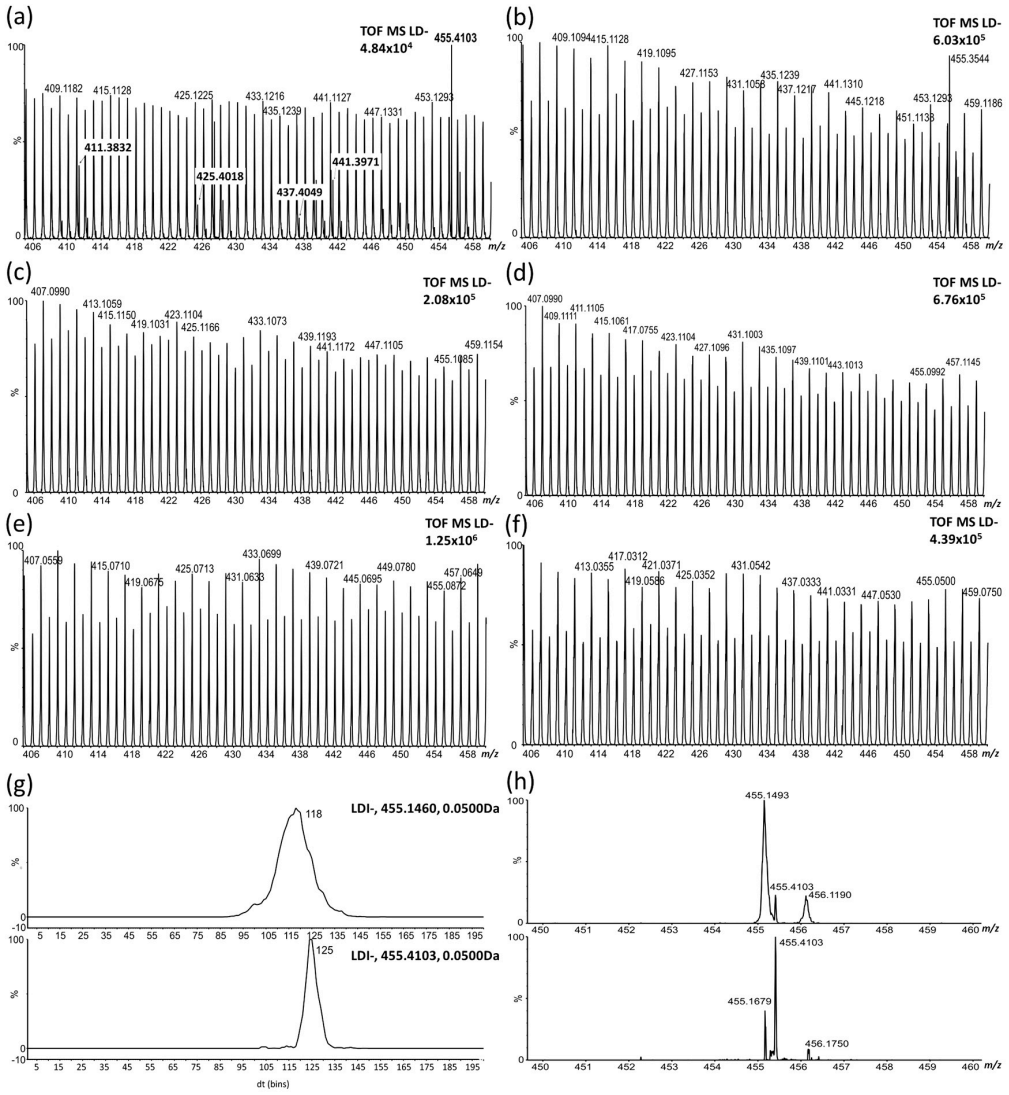

Figure 3. LDI-MS spectra in negative ionization mode. (**a**) external seed coat surface of JI64 genotype; (**b**) external seed coat surface of JI92; (**c**) internal surface of JI 64; (**d**) internal surface of JI92; (**e**) seed coat cross section of JI64 (MSI); (**f**) seed coat cross section of JI92 (MSI); (**g**) upper mobilogram (record of ion mobility scan) reconstructed at m/z 455.1460 and bottom mobilogram at m/z 455.4103; (**h**) upper MS/MS spectrum averaged over the whole mobility range and bottom MS/MS spectrum averaged over the second mobility peak (with maximum at 125 bins).

2.2. Study of the Distribution of Hydroxylated Long-Chain Fatty Acids on the Pea Seed Coat Surface

As already mentioned, water penetration through the seed coat is to certain extent driven by the composition of the outermost seed coat layer formed by hydrophobic compounds like cutin [4] and most likely also subcuticular lipids present in the macrosclereids. Low content of HLFA in some parts of seed coat outer surface (inhomogeneity of HLFA distribution over the outer surface) would be a primary place for water intrusion even when other parts of the surface are very homogeneous and rich in fatty acids. Imaging mass spectrometry was used to study the distribution of the HLFA previously detected as the dormancy markers as discussed in the previous chapter. Figure 4b compares the HLFA distribution (signals of particular m/z values) on the surface of dormant JI64 and non-dormant JI92 genotypes (the photo in Figure 4a shows the imaged seed coats). Homogenenous distribution of HLFA over the major parts of seed coats surface of both genotypes was observed and significantly higher signal of HLFA can be seen at the surface of JI64. However, Figure 4d demonstrates that hilum and strophiole

(small brown part occurring near hilum, see Figure 4c) contain a significantly lower content of HLFA (dihydroxyoctacosanoate, m/z 455.4103, in this case, see mass images at Figure 4d). The distribution of the other HLFA exhibits similar pattern as given in supplementary material (Figure S6).

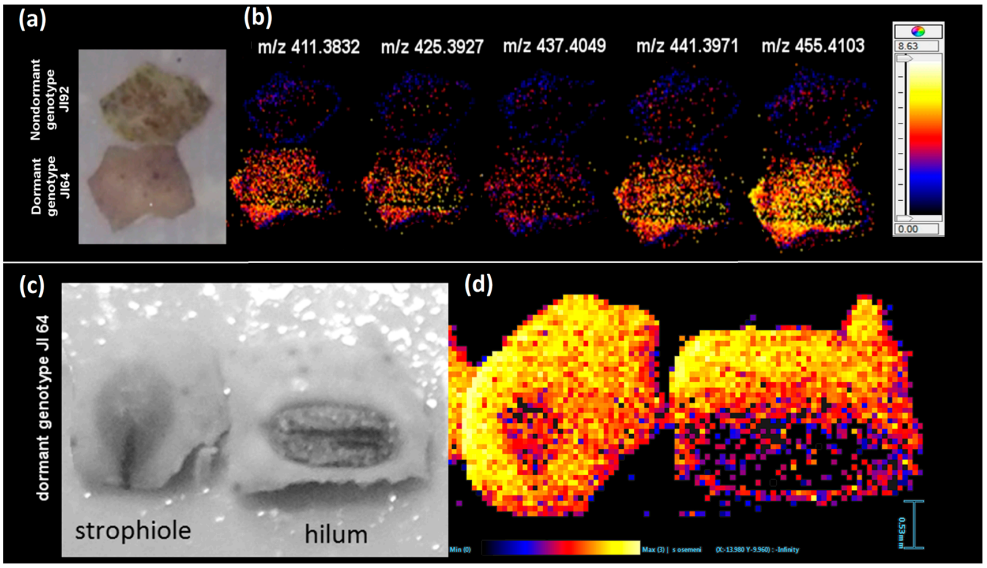

Figure 4. LDI-MSI analysis of external seed coat surfaces. (**a**) Optical image of analyzed seed coats used for mass imaging; (**b**) comparison of distribution of signals (m/z values) of hydroxylated fatty acids on seed coat of dormant JI64 (bottom mass images) and non-dormant JI92 (upper mass images); (**c**) optical image of parts of JI64 seed coats containing strophiole and hilum used for mass imaging; (**d**) distribution of dihydroxyoctacosanoate (m/z 455.4103) over the external surface containing strophiola and hilum; matrix free MSI measurement. Colors in the mass images, i.e., (**b**,**d**), correspond with intensity of particular HLFA markers (their amounts on seed coat surface) in MS spectra (yellow reflects the highest signal and black the lowest one as displayed by inserted color scales).

These differences should be related to anatomical structure–presence of chemically different surface layers (e.g., counterpalisades in hilum area) [4]. Our data point to a possible relationship of strophiole and hilum to the water uptake. Those two anatomical parts were already suggested to play a role during imbibition by Karaki et al. [33]. We should remind that no signal of those hydroxylated fatty acids was observed on internal surface (see Figure 3c,d) of dormant/nondormant testa at the same experimental conditions confirming that the changes in HLFA distribution in seed coat are related only to its outermost layers. Consequently, LDI-MSI was used for analysis of HLFA in seed coat cross-sections. These experiments showed that HLFA signals are not present inside of seed coat tissue (Figure 3e,f). The extracellular lipids rich in hydroxylated fatty acids located in the outermost layers of dormant seed coats are probably connected with blocking effect of seed coat to water transport into the endosperm. Hydrophobic effect might be enhanced with structure crosslinking of hydroxyl groups parted on the carbon chain with carboxyl functional group. The distribution of common non-hydroxylated fatty acids over the all seed coat surface is homogenous and their signals are higher in dormant JI64 compared to nondormant JI92. It should be emphasized that the signal of non-hydroxylated fatty acids in strophiole and hilum is not as different from the rest of surface as it is observed in the case of HLFA (supplementary material, Figure S6). This fact could point out the higher significance of HLFA surface distribution with respect to dormancy compared to the distribution of non-hydroxylated FA.

2.3. LDI-MS Analysis of HLFA in Recombinant Inbred Lines (RILs)

As previously discussed, our analyses clearly confirmed the significantly higher content of HLFA in seed coat surface of dormant (wild) JI64 genotype compared to non-dormant (domesticated) ones.

However, the composition of seed coat (including the fatty acid profile) can be predetermined not only by reproduction regulatory mechanism (physical dormancy) but also by other adaptation traits, i.e., protection from predators, fungi, physicochemical properties of environment. Variance in chemical composition among particular genotypes is expressive as reflected also by visible differences in seed coat morphology. As indicated above, there are certain genotypes relatively similar in morphology but differing strongly in dormancy levels, i.e., JI64 and JI92. Further elimination of differences in another properties and preservation of differences in dormancy at the same time can be achieved genetically by cross of those two genotypes and establishment of recombinant lines (RILs) [5]. The potential of the "RILs approach" for mapping have been demonstrated by Bagheri et al. which used recombinant inbred line (RIL) population for study of *Brassica rapa* seed metabolites. [34].

The seeds from the sufficiently genetically homozygous sixth generation of phenotypically evaluated RILs were analyzed by LDI/MS and LDI/MSI methods. Figure 5a shows signal intensities of HLFA on the seed coat surface of chosen RIL pea genotypes placed in order of increasing dormancy percentage.

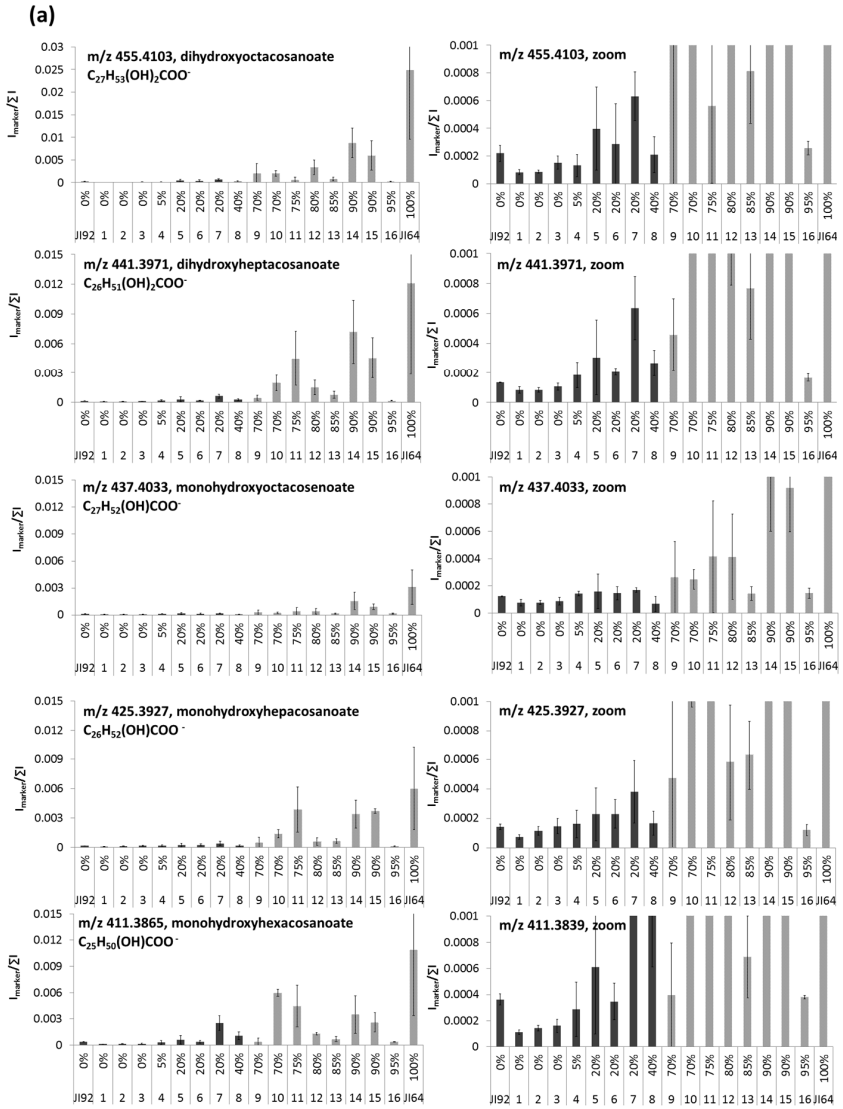

Figure 5. *Cont.*

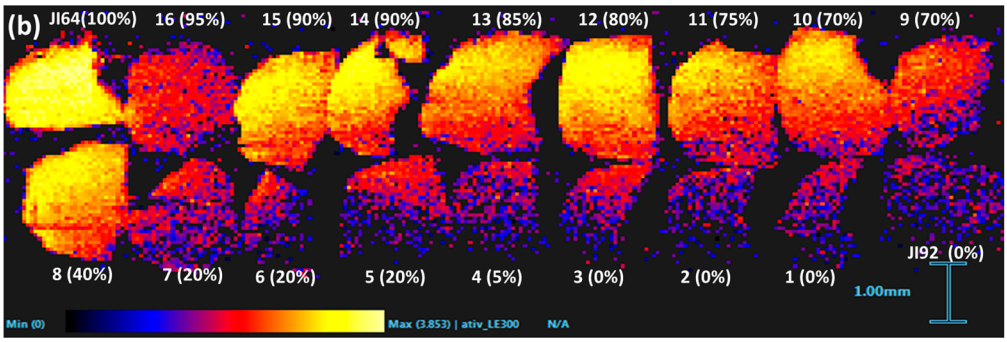

Figure 5. Relationship of HLFA signals to the dormancy level of JI64, JI92 and respective recombinant inbred lines. (**a**) signals of individual hydroxylated long chain fatty acids in parent genotypes and RILs obtained by LDI-MS measurement, black bars denote non-dormant and gray ones dormant lines; signal is expressed as an average of normalized intensities of four repeated measurements; (**b**) distribution of dihydroxyoctacosanoate on the RILs' surface; numbers at x-axis denote particular RIL lines (1–16), numbers given above the codes of lines in graphs or in parenthesis in mass images, respectively, reflect the dormancy level in percents.

Generally, the higher dormancy level the higher signal of HLFA is obtained. There are only several exemptions from that rule that deserve a further biological study. Those differences are easily visible from simultaneous mass image (Figure 5b and Figure S7d–h). The distribution of fatty acids over the seed coats of RILs is mostly homogeneous except of strophiole and hilum parts of dormant RILs. Those findings strongly support the significance of HLFA for regulation of water uptake by seed and early stages of germination. Our results are in agreement to the recent work of Chai et al. that studied the differences between *Medicago truncatula* wild (D) and mutant (N) seeds and revealed significant reductions in content of long chain acids (especially 18:2 18–OH at individual monomer level) in non-dormant mutants [35].

Note that also palmitic, stearic and oleic acids exhibit some differences among dormant and non-dormant RILs (Figures S7a–c and S8) although, due to missing hydroxyl groups in their chain, their role in extracellular lipid composition is probably somewhat different (they provide only terminal hydrophobic instead of connecting chains of HLFA).

3. Materials and Methods

3.1. Chemicals

Methanol (gradient grade), palmitic acid (p.a.), oleic acid (p.a.), 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP, p.a.), 4-aminoquinoline (AQ, p.a.), acetone (for HPLC), acetonitrile (gradient grade) and red phosphorus (p.a., standard for TOF calibration) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Ultrapure water from Milli-Q apparatus (Merck, Kenilworth, NJ, USA) was used for preparation of all solutions.

3.2. Plant Material

Seeds of *Pisum species*, namely wild dormant (D) pea *Pisum sativum* subsp. *elatius* (JI64, VIR320, L100) and cultivated non-dormant (N) *Pisum sativum* subsp. *sativum* (JI92, Cameor, Terno) [5] were used. *P. elatius* JI64 originates from Turkey and JI92 is cultivated Afghan landrace (e.g., primitive local type) *P. sativum* (both from John Innes Pisum Collection, Norwich, UK). L100 genotype originates from Israel. Wild *P. elatius* VIR320 is from Vavilov Institute Research of Plant Industry (St. Petersburg, Russia) and was used previously for incompatibility study [36]. Cultivated *P. sativum* cv. Cameor (used for pea genome sequencing) originates from INRA France while Czech cv. Terno is commonly used in cultivation and represents modern variety. Cross of cv. Terno with L100 is being used for

establishment of introgression lines [37] while F_6 (sixth generation) recombinant inbred lines (RILs) derived from JI64 and JI92 cross [38] were used for genetic mapping and transcriptomic analysis [5]. JI64 and JI92 pair is less contrasting in term of visual seed coat appearance [4,5] except of differences in seed coat permeability and dormancy. Plants were cultivated in controlled glasshouse conditions during February–April (2016, 2017) and mature seeds were manually harvested.

Mature pea seeds (*Pisum* sp.) were air dried and stored at laboratory temperature in a dark and dry place until the analysis. The dormancy of particular genotypes and lines was measured by standard method [5]. Briefly, twenty five seeds per line were incubated in Petri dishes (9 cm diameter) over two layers of medium speed qualitative filter papers (Whatman, Maidstone, UK, grade 1) wetted with 3 mL of tap water and incubated in a 25 °C incubator in darkness. Imbibition was scored at 24 h intervals based on changes in seed swelling and germination was determined based on the radicle breaking through seed coat. Non germinated seeds were mechanically scarified after 60 days to determine the percentage of viable seeds.

3.3. Sample Preparation for Surface Analysis

Seed coats were mechanically separated from embryos and broken into small pieces. These small pieces (approx. 2 mm in diameter) were fixed on stainless steel MALDI plate with outer surface facing up using a double sided tape (Ulith, Prague, Czech Republic). Six repetitions of each genotype/line were measured. The samples with internal surface oriented up were analyzed for comparison as well. Samples were analyzed directly in dry, intact state (LDI-MS) or after spraying with matrix (MALDI-MS; THAP matrix: 1 mg/mL, dissolved in acetonitrile:water, *v:v*, 1:1 for positive ion mode; AQ matrix: 1 mg/mL, dissolved in acetone for negative ion mode). The matrix solution was sprayed using standard SunCollect sprayer (SunChrom GmbH, Friedrichsdorf, Germany) on the seed coat samples in successive six layers.

For study of distribution of analytes on cross-section (vertical distribution), samples of seed coats were dissected from dry seed and saturated with 2% sucrose solution under vacuum. Equal volume of cryo-gel (Cryomatrix Shandon, Thermo Fisher Scientific, Waltham, MA, USA) was added to the sample and shaken overnight to improve subsequent sectioning. Saturated samples were mounted into cryo-gel on the alum chuck, frozen down to −25 °C and cut into 20 μm transversal sections [39]. The prepared slices were put on the normal glass slide. The prepared glass slides were stored at laboratory temperature at dark and dry place until analysis.

3.4. Instrumentation

Measurements were done using a high resolution tandem mass spectrometer equipped with a vacuum MALDI ion source, a hybrid QqTOF mass analyzer and an ion mobility cell (Synapt G2-S, Waters, Milford, MA, USA). The MALDI source was equipped with a 350 nm 1 kHz Nd:YAG solid state laser. Mass spectra were collected in positive and negative ionization modes in mass range 50–1200 Da. The effect of laser energy was studied in the range 300–450 and laser step rate in the range 1–30, Hexapole RF amplitude in the range 250–450 V and sample plate voltage in the range 0–25 V. MALDI Extraction voltage was set at 10 V, Hexapole Bias at 10 V and Aperture at 5 V. (MA) LDI-MS analyses were performed for 6 min (300 scans were collected). Acetone solution of red phosphorus was used for TOF calibration (supernatant of suspension prepared at concentration 1 mg/mL). The control of the mass spectrometer and data collection were performed using MassLynx 4.1 software (Waters, Milford, MA, USA). Trap collision energy (TrapCE) was set to 4 eV and transfer collision energy to 2 eV for MS scan. For MS/MS experiments the effect of collision energy in both collision cells on the yield of fragments was tested in the range 10–50 eV (the optimal values for particular experiments are given in Results and Discussion). The exact mass measurement involved external calibration and automatic lock mass measurement (correction) using red phosphorus (see above). Spectra for lock mass correction were collected as a part of each analysis prior to seed sample measurement. The identification of

selected markers (signals differing significantly between dormant and non-dormant samples) was based on exact mass measurement and consequent target MS/MS experiments.

Laser Desorption/Ionization Imaging Mass Spectrometry (LDI-MSI)

LDI-MSI technique was used for analysis of surfaces as well as cross sections of seed coats. HDImaging software (version 1.4, Waters) was used for the setup of experiment and the data collection. The LDI-MSI measurement of seed coat surface was taken in negative ionization mode with 60 μm laser beam size. Four laser energies (200, 250, 300, 350) were tested in order to find the highest yield of signals of studied markers (details are given in Results and Discussion). Laser repetition rate was set at 1000 Hz and scan time at 1 s. The Hexapole RF Amplitude was the same as given in Results and Discussion. Filtration of low abundant signals was applied during measurement to decrease the number of datapoints (the number of the most intense peaks included in raw datafile was set to 300,000 and the signal threshold was set to 10 counts). No matrix was used for MSI experiments.

3.5. Data Processing

The raw MS data were firstly processed by MarkerLynx XS software that is an extension of MassLynx platform (Waters). This software provided extraction, normalization and alignment of data (creation of data matrix). Parameters of optimized MarkerLynx XS method were set as follows: marker intensity threshold 1000 (optimal value for resolution of dormant and non-dormant species in PCA plot), peak separation 0.05 Da, mass range 50–1200 Da. "Combined Scan Range" was used as the type of analysis. The obtained data matrices were studied by multivariate statistics. Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were applied using EZinfo software (version 2.0, Umetrics, Malmö, Sweden) with Univariate or Pareto scaling. Significance of obtained markers was evaluated according to their position in appropriate S-plots.

4. Conclusions

LDI-MS in combination with PCA and OPLS-DA proved to be a useful tool for the classification (distinction) of dormant and non-dormant genotypes of pea by the direct analysis of seed coat outer surface. Minimum sample treatment is necessary, even the application of matrix can be omitted and seeds can be analyzed in dry (inactive) state. Particular dormant genotypes exhibited strong differences in signals of dormancy markers suggesting differences in the process of their water imbibition. Detailed study of morphologically similar but in dormancy levels strongly different pair of genotypes, i.e., JI64 and JI92 and derived RILs, revealed significant differences in content of hydroxylated long chain fatty acids (ranging from C26 to C28). Significantly higher signals of HLFA were found in dormant JI64 and dormant RIL lines.

The effect of experimental conditions on the signal of HLFA was studied in detail. Application of matrix caused mechanical changes of samples and matrix free analysis provided better results. Hexapole RF amplitude, sample plate voltage, laser energy and laser step rate appeared to be significant LDI parameters for HLFA signal and their values were optimized. Ion mobility experiments allow effective filtration of HLFA signals from ballast ones. Laser desorption/ionization mass imaging experiments reveal homogeneous distribution of HLFA on the outer seed coat surface with the exemption of strophiole and hilum in JI64 seed coat that show significantly lower content of HLFA. Analysis of JI64–JI92 recombinant inbred lines strongly supports the relation of physical dormancy with the distribution of HLFA. The obtained information contributes to a deeper insight into the mechanism of water absorption by seed that is important also for food and agricultural research.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/18/10/2196/s1.

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Appendix III (Paper III)

HRADILOVÁ I., DUCHOSLAV M., BRUS J., PECHANEC V., HÝBL M., SMRŽOVÁ L., ŠTEFELOVÁ N., VÁCLAVEK T., BARIOTAKIS M., MACHALOVÁ J., HRON K., PIRINTHOS S., SMÝKAL P. (2019): Variation in wild pea (*Pisum sativum* subsp. *elatius*) seed dormancy and its relationship to the environment and seed coat traits. PeerJ 7: e6263.

Variation in wild pea (*Pisum sativum* subsp. *elatius*) seed dormancy and its relationship to the environment and seed coat traits

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ABSTRACT

Background. Seed germination is one of the earliest key events in the plant life cycle. The timing of transition from seed to seedling is an important developmental stage determining the survival of individuals that influences the status of populations and species. Because of wide geographical distribution and occurrence in diverse habitats, wild pea (*Pisum sativum* subsp. *elatius*) offers an excellent model to study physical type of seed dormancy in an ecological context. This study addresses the gap in knowledge of association between the seed dormancy, seed properties and environmental factors, experimentally testing oscillating temperature as dormancy release clue.

Methods. Seeds of 97 pea accessions were subjected to two germination treatments (oscillating temperatures of 25/15 °C and 35/15 °C) over 28 days. Germination pattern was described using B-spline coefficients that aggregate both final germination and germination speed. Relationships between germination pattern and environmental conditions at the site of origin (soil and bioclimatic variables extracted from WorldClim 2.0 and SoilGrids databases) were studied using principal component analysis, redundancy analysis and ecological niche modelling. Seeds were analyzed for the seed coat thickness, seed morphology, weight and content of proanthocyanidins (PA).

Results. Seed total germination ranged from 0% to 100%. Cluster analysis of germination patterns of seeds under two temperature treatments differentiated the accessions into three groups: (1) non-dormant (28 accessions, mean germination of 92%), (2) dormant at both treatments (29 acc., 15%) and (3) responsive to increasing temperature range (41 acc., with germination change from 15 to 80%). Seed coat thickness differed between groups with dormant and responsive accessions having thicker testa (median 138 and 140 μm) than non-dormant ones (median 84 mm). The total PA content showed to be higher in the seed coat of dormant (mean 2.18 mg g^{-1}) than those of non-dormant (mean 1.77 mg g^{-1}) and responsive accessions (mean 1.87 mg g^{-1}). Each soil and bioclimatic variable and also germination responsivity (representing

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synthetic variable characterizing germination pattern of seeds) was spatially clustered. However, only one environmental variable (BIO7, i.e., annual temperature range) was significantly related to germination responsivity. Non-dormant and responsive accessions covered almost whole range of BIO7 while dormant accessions are found in the environment with higher annual temperature, smaller temperature variation, seasonality and milder winter. Ecological niche modelling showed a more localized potential distribution of dormant group. Seed dormancy in the wild pea might be part of a bet-hedging mechanism for areas of the Mediterranean basin with more unpredictable water availability in an otherwise seasonal environment. This study provides the framework for analysis of environmental aspects of physical seed dormancy.

Subjects Plant Science, Spatial and Geographic Information Science
Keywords Dormancy, Seed coat, Proanthocyanidins, Testa, Pea, Niche-modelling, Temperature oscillations, Germination, Legumes

INTRODUCTION

The transition from seed to seedling is one of the most important stages in the plant life cycle (*Donohue et al., 2010*). This transition is a complex process influenced by structural and physiological characteristics of the seed, as well as by genetic and environmental factors. This stage constitutes one of the most dramatic changes of the plant life cycle, involving the switch from a robust, quiescent state of the protected seeds to one of extreme vulnerability, the young seedling. Therefore it is essential that germination takes place at the appropriate time and place (*Fenner & Thompson, 2005*). Seed dormancy provides the mechanism blocking germination of intact viable seeds under conditions when the probability of seedlings survival and growth is low (*Baskin & Baskin, 2004*; *Weitbrecht, Müller & Leubner-Metzger, 2011*; *Bewley et al., 2013*; *Long et al., 2015*). A diverse range of dormancy mechanisms have evolved as response to the diversity of climates and habitats (*Baskin & Baskin, 2004*; *Willis et al., 2014*). Physical dormancy (PY) occurs in at least 18 angiosperm plant families including Fabaceae (*Baskin & Baskin, 2014*; *Willis et al., 2014*) and is caused by a layer of water-impermeable palisade cells in the seed coat (reviewed in *Smýkal et al., 2014*; *Janská et al., 2018*). Phylogeny based analysis of world-wide observations of Fabaceae identified the evolution towards non-dormant seeds in climates with long growing seasons such as the ones found in the tropics (*Rubiode Casas et al., 2017*), suggesting that the seed dormancy might be favored as the bet-hedging strategy in temporally variable environments (*Venable, 2007*; *Rubiode Casas et al., 2017*). In such circumstances, dormancy may prevent inopportune germination by delaying germination until the onset of the autumn-winter rainy season as found in Mediterranean region (*Thompson, 2005*). Association between seed responsiveness to temperature and the thermic characteristics of their habitat range has been reported (*Fenner & Thompson, 2005*; *Bewley et al., 2013*; *Baskin & Baskin, 2014*; *Batlla & Benech-Arnold, 2015*; *Renzi, Chantre & Cantamutto, 2018*; *Thompson, 1970*; *Probert, 2000*; *Rosbakh & Poschlod, 2015*). These studies have shown that temperature determines the sensing of the time of the year and

depth of seed dormancy. Seeds of native plants in highly seasonal climates, such as the Mediterranean climate, experience both daily and seasonal fluctuations in temperature and moisture (*Ader, 1965*; *Norman, Cocks & Galwey, 2002*). Such temperature oscillations have been proposed to be one of the PY dormancy breaking mechanisms (*Baskin & Baskin, 2014*). The positive effect of temperature on seed dormancy release was confirmed experimentally in several legume species, including *Lupinus, Trifolium* and *Medicago* (*Quinlivan, 1961*; *Quinlivan & Millington, 1962*; *Quinlivan, 1966*).

In addition to temperature, the water availability is another important environmental factor, related to water potential of both the soil and seed, which also regulates dormancy (*Bradford, 2002*). Furthermore there are other soil factors that contribute to the complexity of water availability, these include physical (temperature, texture, gaseous environment, and seed burial depth), chemical (organic matter, pH, and nutrients) (*Long et al., 2015*) and biological soil (fungal and microbial activity) properties (*Sperber et al., 2017*).

Geographical gradients of environmental factors such as temperature and precipitation offer great opportunities for evaluating inter-population variability in plant traits and provide the possibility to analyze association between studied traits, environment and genetic constitution of the individual. Such studies have been conducted in species with sufficiently large geographical distribution and/or wide niche breath such as *Arabidopsis* (*Exposito-Alonso et al., 2018*; *Tabas-Madrid et al., 2018*; *Frachon et al., 2018*), *Medicago* (*Yoder et al., 2014*) or *Populus* (*Fitzpatrick & Keller, 2015*). With respect to seed dormancy, this was studied in *Arabidopsis* model (*Vidigal et al., 2016*; *Burghardt et al., 2015*; *Donohue et al., 2010*) and sea beet (*Beta vulgaris* subsp. *maritima*, *Wagmann et al., 2012*), but so far not on legume seeds with physical dormancy type.

Structural and compositional properties of the seed coat have been reported to affect the seed dormancy status (reviewed in *Smýkal et al., 2014*; *Hradilová et al., 2017*; *Janská et al., 2018*). Particularly polyphenols have been found in the seed coat, such as flavonoids, lignins and lignans and there is evidence that these influence both seed longevity and dormancy (*Lepiniec et al., 2006*). The relationship between proanthocyanidin (PA) content and dormancy was shown in *Arabidopsis* (*Appelhagen et al., 2014*), rapeseed (*Brassica napus*) and flax (*Linum usitatissimum*) mutants (*Diederichsen & Jones-Flory, 2005*; *Zhang et al., 2006*), faba bean (*Kantar, Pilbeam & Hebblethwaite, 1996*) and also pea (*Hradilová et al., 2017*). These PA insoluble polymers and other compounds (*Cechová et al., 2017*) result in the reinforcement of the seed coat as a barrier to water and oxygen permeation, mechanical damage and biotic and abiotic stresses (*Pourcel et al., 2007*). The association between the seed coat structure and composition in relation to dormancy and environmental factors across the species distribution range was not tested until now. Moreover, structural and compositional properties of the seed coat can be influenced by the intraspecific or even intra-individual variation in seed size (*Mandák, 1997*; *Baskin & Baskin, 2014*). In *Atriplex triangularis*, thickness of seed coat increases with decreasing seed size (*Khan & Ungar, 1985*). Consequently, larger mechanical resistance of testa of smaller seeds in *Atriplex* provides a resistance to germination and might induce dormancy in a smaller seeds (*Osmond, Björkman & Anderson, 1980*). The seed size also relates to water requirements during imbibition, presumably due to surface-to-volume ratio (*Wilson & Witkowski, 1998*).

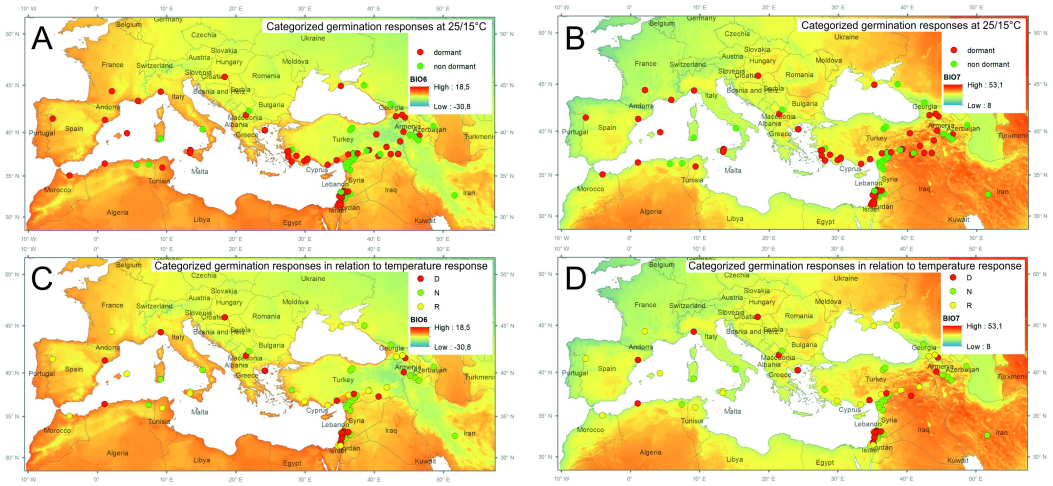

Figure 1 Spatial distribution of tested samples and their categorized germination pattern in relationship to selected bioclimatic variables. Categorized germination patterns of accessions tested at alternate temperature regimes 25/15 °C (A, B) and 35/15 °C (C, D) over smoothed bioclimatic variables BIO6 (Min Temperature of Coldest Month) and BIO7 (Temperature Annual Range) extracted from WorldClim 2.0 database. (D, dormant, N, non-dormant, R, responsive accessions. See Table S2 for details).

Full-size DOI: 10.7717/peerj.6263/fig-1

Indeed, it has been hypothesized that level of dormancy and seed size are interrelated and have adaptive values (*Venable & Brown, 1988*; *Rees, 1996*).

Wild pea (*Pisum sativum* subsp. *elatius*), with its wide ranging native distribution, spanning from the western Mediterranean, through the southeastern Europe to the Middle East (*Smýkal et al., 2017*; *Smýkal et al., 2018a*) offers an excellent model to study legume seed physical dormancy in an ecological context. Given the limited knowledge on regulation of legume seed dormancy (reviewed in *Smýkal et al., 2017*) this study addresses the gap in knowledge on association between the seed dormancy, seed properties and environmental factors. The present study had three objectives: (1) To describe germination response of pea seeds to various experimental temperature treatments. (2) To determine which ecological factors (climate, soil conditions) may act as adaptation drivers of seed dormancy level in wild pea. (3) To test if there is an association between seed dormancy level variation, seed coat anatomy and proanthocyanidin content.

MATERIALS & METHODS

Plant material

The investigated samples originated from various genebanks and contained 97 wild pea (*Pisum sativum* subsp. *elatius* (M. Bieb.) Asch. & Graebn) accessions. These span from the western Mediterranean, through the southeastern Europe to the Middle East (Table S1, Fig. 1). This material was selected based on passport data including GPS information and assessed for genetic diversity relationship (*Smýkal et al., 2017*; *Smýkal et al., 2018a*; *Trněný et al., 2018*). Plants were grown in 5 litre pots with peat-sand (90:10) substrate mix (Florcom Profi, BB Com Ltd. CZ), in glasshouse conditions (January–May 2016 and 2017) day/night

temperature within the ranges 35–20/15–12 °C, and a natural photoperiod increasing from 6 to 14 h without supplementary light (UP campus, Olomouc, CZ). Cultivated and wild peas are largely self-pollinating (*Smýkal et al., 2018a*) and as samples were of single seed descent they are expected to be genetically uniform. After harvest, mature seeds were cleaned from pods, dried at room temperature and packed in paper bags. The germination testing was performed within the month from harvest.

Germination testing

To assess the dormancy status, seed imbibition and germination was assessed (*Baskin & Baskin, 2014*). Based on temperature data from soil sensors monitored at several sites with wild pea populations (*Smýkal et al., 2017*; *Von Wettberg et al., 2018*) we tested the effect of two oscillating temperature regimes on dormancy level: (i) day/night temperatures of 35/15°C and (ii) 25/15 °C, both at 14h/10 h periodicity and in the dark. Intact seeds were placed on water saturated filter papers (Whatman Grade 1, Sigma CZ) in 90 mm Petri dishes (P-Lab, CZ) in temperature controlled chambers (Laboratory Incubator ST4, BioTech CZ). For each treatment, 25 seeds per accession with two replicas were incubated. To prevent fungal growth the fungicide (Maxim XL 035 FS; containing metalaxyl 10 g and fludioxonil 25 g) was applied in amount of 1 ml l^{-1} of tap water. Initial comparison of control and fungicide containing samples did not show any significant difference. Seeds were monitored at 24 h intervals for a total of 28 days (the longest time the seeds remained fungus-free in the 35/15 °C treatment) and water was added as needed usually every 3rd day. The plates were randomly rearranged during the scoring.

Germination data analysis

The number of imbibing seeds (i.e., swollen enlarged seeds upon the uptake of water) and also germinating seeds (i.e., radicle protrusion was the criterion for germination) was scored daily for the period of 28 days for each accession. Since we are primarily interested in study of physical dormancy (PY) executed by seed testa barrier of water entry (*Smýkal et al., 2014*) we have used the number of imbibed seeds as a measure of germination in all further calculations (thus our data contain both imbibed and fully germinated seeds, which can be separated by several days from each other, notably all imbibed seeds have germinated in course of experiment).

In order to capture the complexity of germination we have used a *spline function* (function defined piecewise by polynomials) fitted to the data (*Ramsay & Silverman, 2005*; *Van den Boogaart, Egozcue & Pawlowsky-Glahn, 2014*). This treats the original germination data (daily counts of germinated seeds) as discrete realizations of an asymptotically continuous process, i.e., for each sample the germination can be represented by a non-decreasing function called the *absolute germination distribution function* (AGDF) in the following text. The term *absolute* is used to distinguish AGDF from standard cumulative distribution function; unlike the latter one, AGDF deals with absolute values of cumulative germination. This function reflects well specific patterns related to germination of a given accession including final germination proportion and germination speed. In addition, we considered the area under AGDF, computed using the Riemann integral. From

its construction, increasing value of this coefficient indicates both higher germination proportion and/or germination speed. For its numerical computation we used numerical integration, composed trapezoidal rule (*Burden & Faires, 2011*). AGDFs were obtained by approximation of the original germination data by smoothing with spline functions (*De Boor, 1978*). We used a generalized functional (*Machalová, 2002*) with the smoothing spline of degree $k = 4$ (degree of the respective basis polynomials used for construction of the splines) and the first derivative in L_2 norm in Matlab (https://www.mathworks.com/) and further processed using R (*R Development Core Team, 2018*). Each spline is characterized by a set of real numbers, called B-spline coefficients. They represent coefficients of a linear combination of basis functions (so called B-splines) which are the same for all samples and each of them covers part of the domain of the germination function. Accordingly, a value of each specific coefficient indicates local behavior of the spline (and, consequently, of the original germination data). Moreover, B-spline coefficients can be also further tuned, like here to achieve better smoothing properties. B-spline coefficients for each accession (Table S2) were subjected to cluster analysis using UPGMA and Euclidean distance metric (*Legendre & Legendre, 2012*) separately for each temperature treatment, while areas under the curve were used in the case of combined temperature treatments dataset. Therefore, concrete values of B-spline coefficients were not important for interpretation purposes here, however, they were simply utilized for the above multivariate analyses. Cluster analyses were performed in the software PAST 3.21 (*Hammer, Harper & Ryan, 2001*).

Environmental variables

Environmental variables (climate: http://worldclim.org/version2; soil: https://soilgrids.org) were obtained using the ArcGIS Pro software (https://pro.arcgis.com). Species occurrence databases have often certain degree of uncertainty due to different spatial accuracy (*Stine & Hunsaker, 2001*; *Murphey et al., 2004*; *Meyer, Weigelt & Kreft, 2016*). In order to minimise the error caused by coordinates we developed a geoprocessing model to automate the calculation of mean values from 5 km buffer around the collection sites. Using this approach, we smoothed the uncertainty caused by imprecise localisation. Data were extracted using geoprocessing model from GeoTIFF rasters in the WGS-84 coordinate system (EPSG: 4326) with a spatial resolution of 30 arc-seconds (~1 km). The bioclimatic variables (BIO1–BIO19), are derived from the monthly temperature and rainfall values (*Fick & Hijmans, 2017*). These represent annual trends (e.g., mean annual temperature BIO1, annual precipitation BIO12), seasonality (e.g., annual range in temperature and precipitation BIO4 and BIO15) and extreme or limiting environmental factors (e.g., the temperature of the coldest and warmest month BIO5 and BIO6, and amount of precipitation in the wet and dry quarters BIO16 and BIO17). Soil data were extracted from SoilGrids collection of updatable soil property and class maps of the world (*Hengl et al., 2017*). We used values described in Table S1 from 5 cm depth and resolution 1 km (same as WorldClim). For details, including basic descriptive statistics of mean tendency and variation of each environmental variable and legend see Table S1.

Testing of relationships among germination pattern, geography and environment

Matrix of environmental variables was firstly checked for the presence of the multicollinearity. Because of strong covariation between several environmental variables (pairwise Pearson $r \geq |0.90|$), eight soil and nine climatic variables were removed from the dataset and all remaining analyses (except for niche analysis) were done with reduced matrix of environmental variables containing 10 climatic and nine soil variables (Table S1). To assess whether there is spatial correlation present in the data, Moran's I spatial correlation statistics (*Legendre & Legendre, 2012*) was calculated for each environmental variable in the reduced matrix using the software PASSaGE v. 2.0 (*Rosenberg & Anderson, 2011*). Ten distance classes with unequal widths were created and Moran's I was calculated for each distance class with largest class excluded. To test the global significance of each spatial correlogram, we check whether the correlogram contains at least one correlation statistics that is significant at the Bonferroni-corrected significance level (*Legendre & Legendre, 2012*).

The matrix of B-spline coefficients describing pattern of germination of the studied accessions in two temperature treatments (35/15 °C and 25/15 °C), considered as response variables, was analysed by Principal Component Analysis (PCA; *Legendre & Legendre, 2012*) to find the main gradients within the germination data-set. No standardization was applied on the matrix of B-spline coefficients prior to PCA. Moran's I spatial correlation statistics was used with the same settings as for environmental variables to test the presence of spatial structuring of accessions scores on the first two resulting ordination axes (PC1, PC2). Set of climatic and soil variables and geographic coordinates (latitude, longitude) were used as supplementary variables and correlated with the first two principal components. To control of possible spatial autocorrelation between each environmental variable and principal components representing germination pattern, we used both standard and modified version of the t-test to assess the significance of correlation between two spatial processes. Modified version of the t-test (*Dutilleul et al., 1993*) was performed in PASSaGE v. 2.0 and PCA was performed using Canoco 5.10 (*Ter Braak & Šmilauer, 2012*).

Constrained ordination (redundancy analysis, RDA; *Šmilauer & Lepš, 2014*) was used to partition the variation in the matrix of B-spline coefficients explained by space (spatial position of sampling sites) and primary predictors (environmental variables), using PCNM method (*Borcard & Legendre, 2002*) without and with forward selection also for primary predictors. To identify spatial explanatory variables, we used principal coordinates of neighbouring matrices (PCNM; *Borcard & Legendre, 2002*), resulting in the PCNM variables. Only those PCNM variables with an adjusted P lower than 0.05 during forward selection procedure were selected to enter RDA. Subsequently, pure and shared effects of environment (groups of soil and climatic variables were tested separately) and spatial variables (PCNM) on the matrix of B-spline coefficients were analysed using RDA. We used a specific partitioning algorithm that provides unbiased estimates of explained variation in RDA (*Peres-Neto et al., 2006*). Here, in the first step, all environmental variables (either soil or climatic) from reduced data-set entered analysis as primary predictors. In the second step, a stepwise procedure using forward selection was done resulting in the subset of environmental predictors, best explaining the matrix of B-spline coefficients. P values were

corrected using the False Discovery Rate adjustment (*Benjamini & Hochberg, 1995*). The significance of pure and shared (marginal) effects of the explanatory variables was tested by a Monte Carlo permutation test with 999 permutations. Analyses were performed in Canoco 5.10 (*Ter Braak & Šmilauer, 2012*; *Šmilauer & Lepš, 2014*) and the library *vegan* (*Oksanen et al., 2018*) in R.

Niche analysis

Using the geographic locations of the studied accessions and their categorized germination response based on 35/15 °C treatment, we constructed ecological niche models for each of the germination response categories. As the modelling approach, Maxent (version 3.4.1, *Phillips, Dudík & Schapire, 2017*), a maximum-entropy based machine learning method was used. Maxent was shown to perform better than other methods when samples sizes are small (*Elith et al., 2006*; *Hernandez et al., 2006*) and it estimates the potential niche instead of the realized distribution of the modelled entity (*Phillips, Anderson & Schapire, 2006*). A buffer zone of 300 km was defined as the background area for each group. As environmental predictors, 19 bioclimatic variables, extracted from WorldClim (*Fick & Hijmans, 2017*) at a resolution of 2.5 arc minutes, were used. The equivalency tests (*Warren, Glor & Turelli, 2008*) were performed with software ENMTools (version 1.4.4 *Warren, Glor & Turelli, 2010*). Two overlap indices were employed in these tests, Schoener's statistic, D (*Schoener, 1968*) and modified Hellinger distance, I (*Warren, Glor & Turelli, 2008*). Data manipulation and map creation were performed in the R with the packages sp (*Pebesma & Bivand, 2005*), raster (*Hijmans, 2017*) and dismo (*Hijmans et al., 2017*).

Quantification of soluble and insoluble proanthocyanidins in the seed coat

Seeds of 86 accessions (Table S3) were analyzed for soluble proanthocyanidins (PAs, in acetone and 4-dimethylaminocinnamaldehyde [DMACA] reagent) and insoluble PAs (in butanol-HCl) using the method described by *Pang et al. (2008)*. Briefly, separated seed coats were freeze dried and 20 mg was homogenized and extracted with 10 ml of acetone-acetic acid-water and 1 h sonication, followed by 5 min centrifugation at 10,000 rpm. To the 0.5 ml of extract, the 1.5 ml of 0.1% DMACA (Sigma-Aldrich, Karlin, Czech Republic) dissolved in HCl-ethanol-water solution, was added. The reaction was measured on spectrophotometer after 10 and 25 min at 640 nm using proanthocyanidin A (Sigma-Aldrich, Karlin, Czech Republic) as standard to calculate the amount of soluble PAs. The pellet after extraction of soluble PA was freeze dried and extracted with 5 ml of butanol-HCl ($x - yv/v$), sonicated for 1 h, centrifuged (5 min/10,000 rpm) and measured at 550 nm. Thereafter the extract was boiled in a water bath for another 1 h, cooled down and the absorbance measured again at 550 nm using proanthocyanidin A as standard to calculate the amount of insoluble PAs. The total content of PAs among dormancy categories was compared by ANOVA. Ratio soluble/insoluble PA among dormancy categories was compared by two AN(C)OVA models, one with and the second without using total PA as a covariate. Analyses were performed in NCSS 9 (NCSS, Kaysville, UT, USA).

Seed size and seed coat thickness measurements

Ten individual seeds per accession of all together 86 accessions (Table S3) were photographed using an Olympus SZ61 stereo microscope (Olympus Corp., Tokyo, Japan) equipped with an Olympus E-410 digital camera (Olympus Imaging Corp., Tokyo, Japan). The photographs were processed by QuickPHOTO MICRO 3.0, supplemented by the Deep Focus 3.3 module (PROMICRA s.r.o, Prague, Czech Republic). Image analysis of seeds was done by SmartGrain (*Tanabata et al., 2012*) recording seed area (AS), perimeter length (PL), width (W), length (L), length-to-width ratio (LWR), circularity (CS) and distance between intersection of length and width and centre of gravity (DS). The weight of twenty five seeds per accession with three replicas was recorded and converted to hundred seeds weight (HSW). Seed coat thickness of the stripped seed coat from five seeds per accession was measured in triplicate using a precision micrometer (0–25 mm, precision 0.01 mm, Hommel Herculer, Germany) and sectioned on cryo-microtome according to *Hradilová et al. (2017)*. For the analysis of dormancy with respect to morphological parameters of seeds we used ordinal logistic regression (*Kleinbaum & Klein, 2010*) implemented in R package MASS (*Venables & Ripley, 2002*). Regression parameters were estimated using the maximum likelihood principle. The relative quality of specific models was assessed by Akaike information criterion (*Akaike, 1974*).

RESULTS

Effect of temperature on pea seed germination

Ninety seven accessions were tested over a period of 28 days and the final percentage of germinated seeds ranged from 0% to 100% (Table S2). Cluster analysis of B-spline coefficients representing germination responses divided the accessions into two groups in the 25/15 °C temperature treatment (Fig. S1A). One cluster held 29 non-dormant (N) accessions with a mean germination of 92% and the second cluster had 68 dormant (D) accessions with a mean germination of 15% (Table S2). The non-dormant group was further divided into those that germinated quickly (N1, 13 accessions, with over 80% germinated at 10 days and 100% at 28 days) and more slowly (N2, 16 accessions germinating between 50 to 80% at 10 days and up to 80% at 28 days) (Table S2). The group of dormant accessions was further divided into D1 sub-group of 14 accessions with a mean germination of 31%, D2 subgroup of 47 with a mean germination of 6% and D3 subgroup of 7 accessions with a mean germination of 54%. In D2 subgroup there were 16 accessions which did not imbibed and germinated at all during 28 days of testing.

In the 35/15 °C temperature treatment, cluster analysis of B-spline coefficients resulted in two main clusters: one with 36 non-dormant (N) accessions with a mean germination of 95% and second with 61 dormant (D) accessions with a mean germination of 28%. The group of non-dormant accessions contained 29 accessions classified as non-dormant already at 25/15 °C, plus additional seven accessions (Fig. S1B).

When germination responses at 25/15 °C and 35/15 °C were analyzed together, UPGMA clustering identified three groups (Fig. 2). One, with 28 non-dormant (N) accessions largely corresponding to N group at 25/15 °C treatment. The R1 group with eight accessions

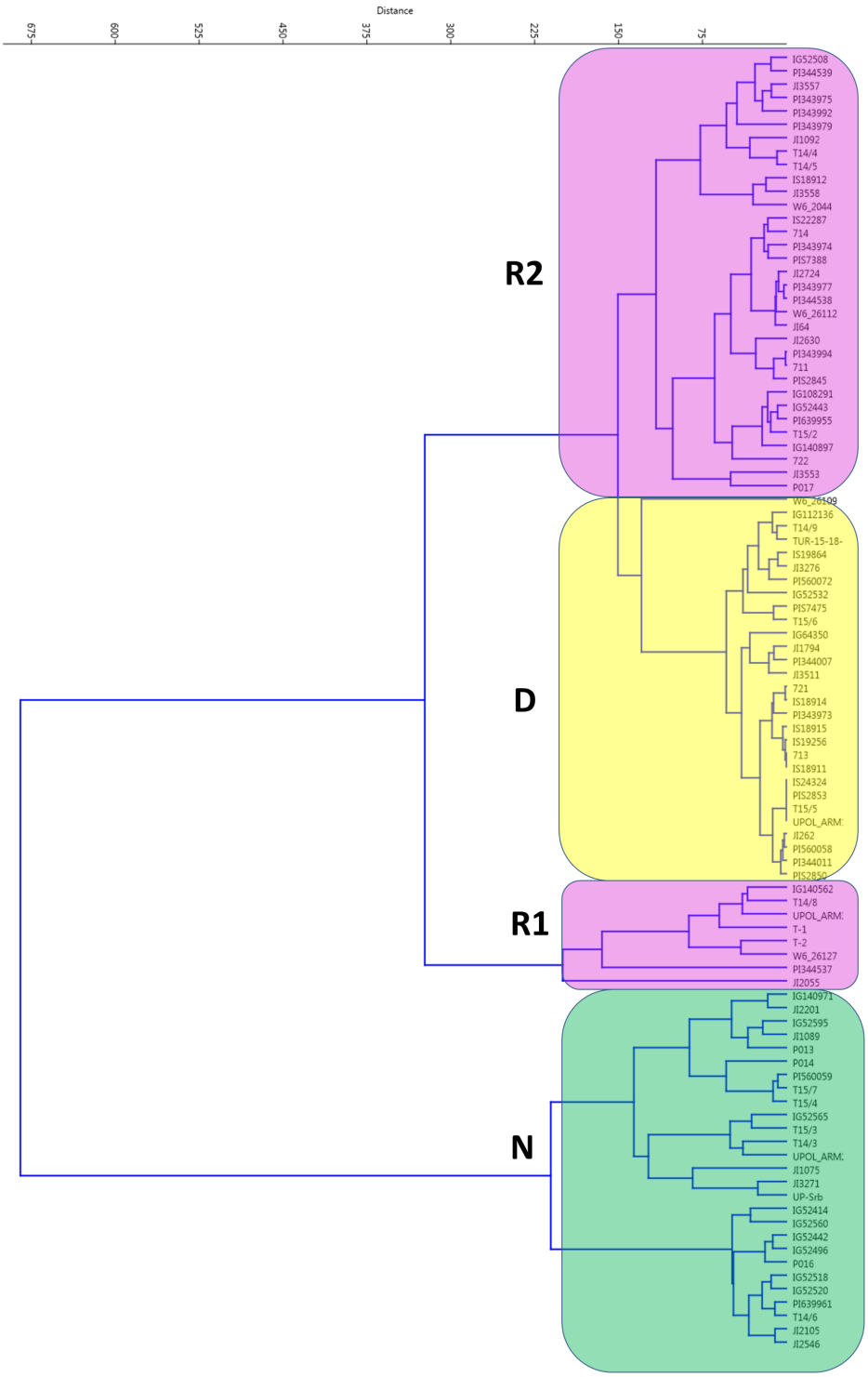

Figure 2 Cluster analysis of germination pattern of tested accessions in response to two alternate temperature regimes. Figure presents results of UPGMA of Euclidean distances of the area under the curves of calculated absolute germination distribution functions (AGDF) at 25/15 °C and 35/15 °C temperature regimes (See Table S2 for details). Clusters are coloured to visualise several categories of germination pattern (green: N, non-dormant, violet: R, responsive to temperature, red: D, dormant at both temperature regimes).

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having large positive response at 35/15 °C (with change from 15% at 25/15 °C to up to 80% germination at 35/15 °C). Second responsive R2 group had 33 accessions with moderate response from 16% at 25/15° C to up to 60% germination at 35/15 °C). Finally, there were 29 accessions which remained dormant (e.g., with final germination bellows 20% at 35/15 °C, with 19 accession having less than 10%).

Relationships between germination pattern, geography and environment

Each environmental variable was significantly spatially structured. Significant positive and negative spatial correlation coefficients were observed at small and large distance classes, respectively (Table S4).

The first principal component of the PCA captured 87.3% of the variation of the B-spline coefficients matrix used to describe the germination pattern under both experimental temperature treatments and showed a clear gradient with dormant accessions on the left through responsive ones in the middle to the non-dormant accessions on the right of the ordination diagram (Fig. 3). Scores of accessions along the first ordination axis were therefore considered to be an indicator of the germination response of these accessions to experimental germination treatments ('germination responsivity'). Germination responsivity was found to be significantly spatially structured. Significant positive spatial correlation coefficients were observed at two smallest distance classes (up to 100 and 200 km, respectively), negative spatial correlation was observed at the distance class 300–600 km, while nonsignificant spatial correlations were found for larger distance classes (Fig. S2).

Only subset of the environmental variables containing four bioclimatic variables (BIO1, BIO6, BIO7, BIO8) and one soil variable (CRFVOL) projected onto the two dimensional PCA diagram (PC1 and PC2 axes) were significantly correlated with at least one ordination axis after correction on spatial autocorrelation (Fig. 3, Table S5). However, only BIO7 was significantly correlated with PC1 axis representing germination responsivity (Fig. 3, Table S5).

The analysis of pure and shared effects of space (PCNM variables) and environmental variables (soil and climatic variables tested separately) on B-spline coefficient matrix representing germination pattern showed that all variables had an overall low explanatory power (space + soil variables: 13.6%; space + climatic variables: 17.4% of total variation in B-spline coefficient matrix; Table S6). PCNM variables accounted for more than a half of explained variation and always had significant effect on B-spline coefficients matrix. Shared effects between space and environmental variables (either soil or climatic) were non-significant on B-spline coefficients matrix. Overall pure effects of soil variables were non-significant on B-spline coefficients matrix, while climatic variables had significant overall pure effects on B-spline coefficients matrix. Using a forward selection procedure resulted in the stop of the selection process after the selection of BIO7 as the first and significant candidate term. Final RDA model thus included PCNM variables and BIO7 and explained 11.1% of total variation in B-spline coefficients matrix (Table S6). However, pure effect of BIO7 on B-spline coefficients matrix was weak (4.7% of total variation) and when three dormancy categories classified on the basis of cluster analysis were projected

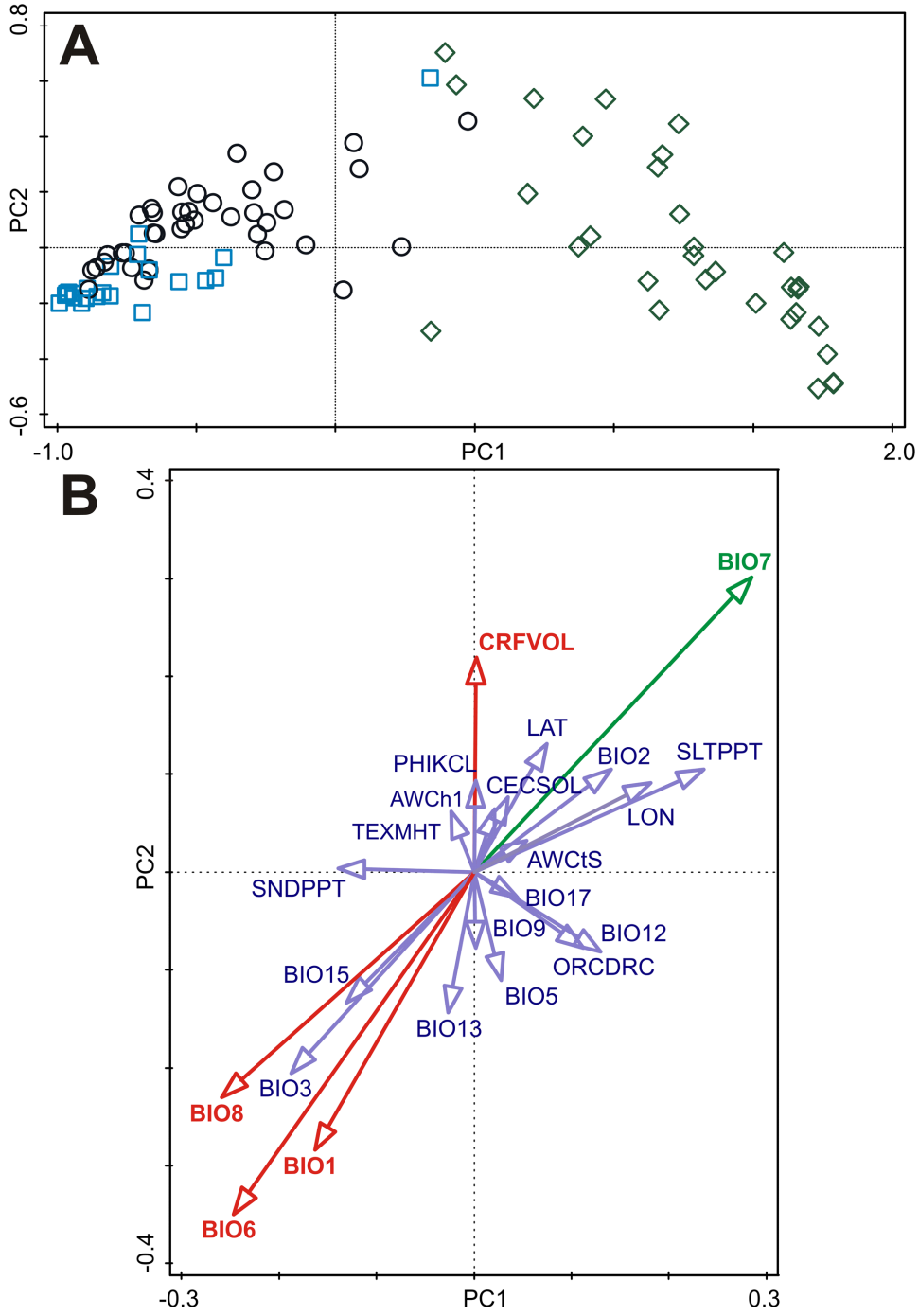

Figure 3 Principal component analysis (PCA) of the B-spline coefficients describing pattern of germination and multiple correlations of environmental variables with ordination axes. (A) The first and the second axes explain 87.3% and 5.6% of the total variation, respectively. Accessions are classified as either dormant (D, blue square), responsive (R, empty circle), or non-dormant (N, green diamond) according to the results of the cluster analyses (see Fig. 2). (B) Multiple correlations of environmental variables with the first and the second ordination axes of the PCA. Each arrow points in the (continued on next page…)

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Figure 3 (…continued)
direction of the steepest increase of the values for corresponding environmental variable. The angle between arrows indicates the sign of the correlation between the environmental variables. The length of the environmental variable arrows is the multiple correlation of that environmental variable with the ordination axes. Environmental variable in green is significantly correlated (spatial correlation) with both ordination axes. Environmental variables in red are significantly correlated (spatial correlation) with the second ordination axis. See Table S5 for Pearson correlation coefficients and corrected correlation coefficients (using Dutilleul method) of environmental variables with the first and the second ordination axes.

into resulting diagram, only poor separation among categories were visible (Fig. 4). Specifically, non-dormant and responsive accessions covered almost whole range of BIO7 while dormant accessions avoided occurring at sites with high values of BIO7, i.e., with larger temperature annual range.

The ecological niche modelling of potential distributions of the dormant (D), temperature responsive (R) and the non-dormant (N) groups obtained at 35/15 °C testing showed the differences in spatial patterns (Fig. 5, Fig. S3). The dormant group (D) shows a more localized potential distribution. Results of the niche equivalence tests (Fig. S3) further suggest that any observed geographical differences among dormancy groups are not produced by an underlying divergence in the niche.

Seed size and seed coat thickness relationship to dormancy level

Except for LWR having weak positive relationship towards non-dormancy, no clear relationships between categorized germination responses in the 25/15 °C treatment and seed morphological characters were observed (Fig. S4A). Similar results were obtained even when ordinal logistic regression with each morphological trait as covariate was employed (according to AIC simple logistic regression models were preferred over one multiple model); again the only significant covariate was LWR with $P = 0.008$. The ordinary logistic regression revealed a slight (positive) impact of the LWR covariate ($P = 0.064$) on dormancy release at 35/15 ° C treatment (Fig. S4B). Seed coat thickness differed between seed dormancy categories in the 25/15 °C treatment (Kruskal–Wallis test, $\chi^2 = 12.4$, $P < 0.001$) with dormant accessions having thicker seed coat (median 139 μm, 95% CI [128–146] μm) than non-dormant ones (median 97 μm, 95% CI [65–120] μm) (Fig. 6A, Fig. 6B, Fig. 7, Figs. S5A, Figs. S5B). Similar results were obtained in the 35/15 °C treatment (Kruskal–Wallis test, $\chi^2 = 11.7$, $P = 0.003$), with both dormant (median 138 μm, 95% CI [123–150] μm) and responsive accessions (median 140 μm, 95% CI [110–152] μm) having similar and thicker seed coat than non-dormant ones (median 84 μm, 95% CI [58–117] μm). However, non-dormant accessions showed more variable seed coat thickness, reaching also the high values typical of dormant accessions (>80 μm), while seed coat thickness of dormant and responsive accessions did not fall below ca 80 μm (Figs. 6A, 6B, 7, Figs. S5A, S5B).

Content of proanthocyanidins in the seed coat

The amount of soluble and insoluble PAs was measured in the isolated seed coat of mature dry seeds (Table S3). The total content varied from 1.21 to 4.70 mg g^{-1} of dry seed coat and showed weak tendency to be higher in the seed coat of dormant (mean ± SE;

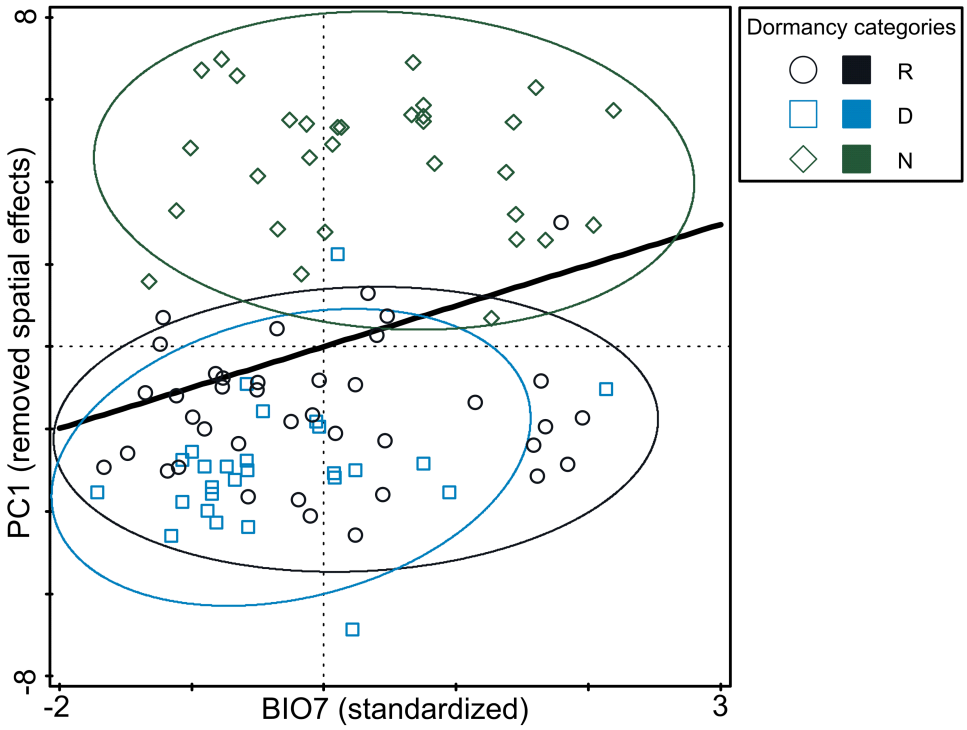

Figure 4 Relationship between germination responsivity of accessions in both temperature treatments and annual temperature range (BIO7). Germination responsivity is represented by residuals of scores along the first canonical ordination axis of RDA with one PCNM variable as an explanatory variable. Accessions are classified as either dormant (D; blue square), responsive (R; empty circle), or non-dormant (N; green diamond) according to the results of the cluster analyses (see Fig. 2). The ellipses were created based on a model of bivariate normal distribution of the dormancy class symbols (estimated from a variance-covariance matrix of their X and Y coordinates) to cover 95% of that distribution' cases. Black line represent fitted Generalized Additive Model (response variable: PC1, predictor: BIO7, distribution: normal, link function: identity, fitted model deviance: 1,113.01 with 94.996 residual DFs, null model deviance: 1,207.34 with 96 residual *df* s, model AIC: 518.23, model test: $F = 8.0$, $P = 0.0056$).

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2.18 ± 0.13 mg g^{-1}) that those of non-dormant (mean ± SE; 1.77 ± 0.14 mg g^{-1}) and responsive accessions (mean ± SE; 1.87 ± 0.12 mg g^{-1}; ANOVA, $F = 2.64$, $P = 0.079$). Soluble PA represented dominant component of total PA (94–99%) while insoluble PA were in minority (1–6%). However, ratio soluble/insoluble PA was not constant and increased with increasing total PA in all dormancy categories (Fig. S6). Correcting for total PA (covariate, $F = 26.26$, $P < 0.001$), dormancy categories recognized at 25/15 °C treatment significantly differed in sPA/inPA ratio ($F = 4.6$, $P = 0.036$) with dormant accessions having higher ratio (LS adjusted mean at mean total PA, 95% CI; 23.5, 20.8–26.4) than non-dormant ones (19.1, 16.3–22.2). Using the same model but with three dormancy categories (including responsive one; 35/15 °C treatment) showed similar trend to that of the previous model but the effect of dormancy category on sPA/inPA ratio was non-significant (total PA, $F = 15.52$, $P < 0.001$; dormancy categories, $F = 2.59$, $P = 0.086$) (Fig. S6). Custom comparison (D+R vs. N) showed that both dormant and responsive accessions have higher sPA/inPA ratio that non-dormant accessions ($F = 4.46$, $P = 0.039$).

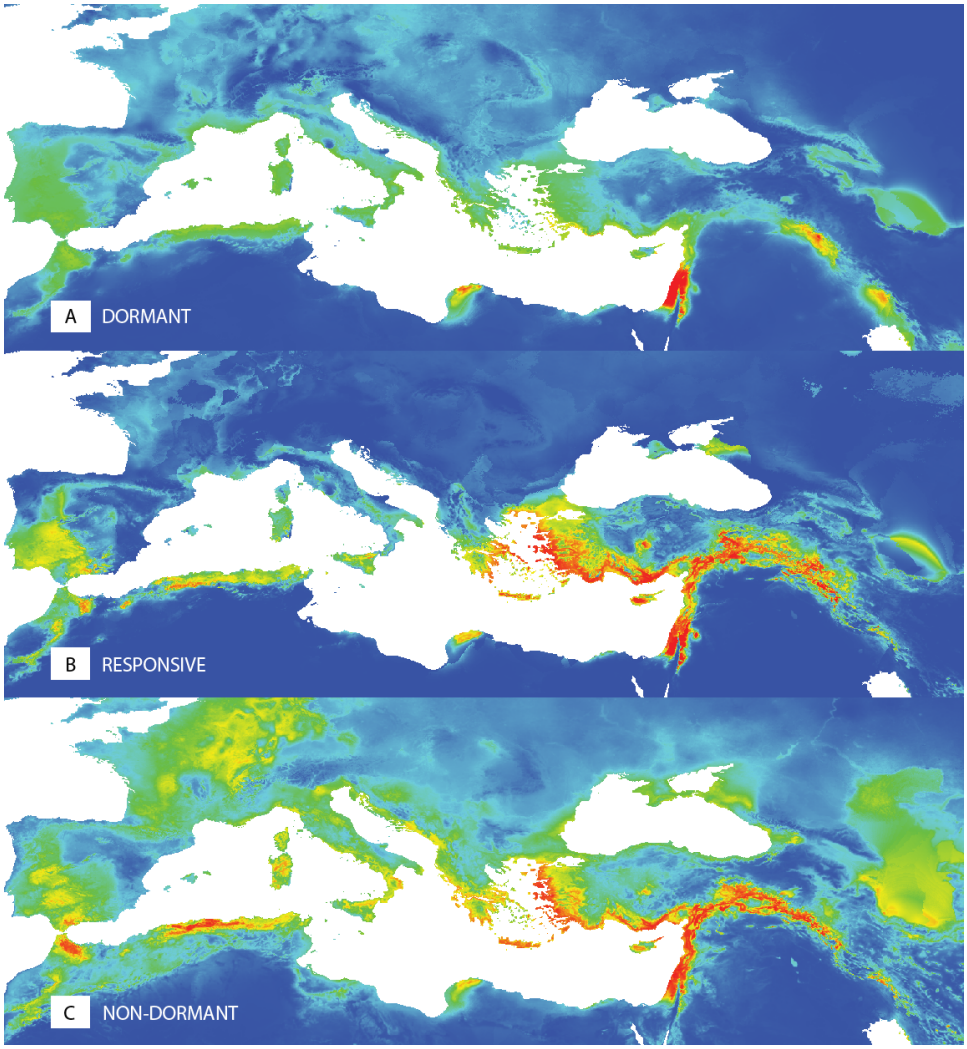

Figure 5 Predictions of niche models for the different dormancy groups. Colder colours represent areas of low probability of occurrence according to the models, while warmer colours correspond to areas of high probability, for dormant (A), responsive (B) and non-dormant (C) groups, respectively.
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DISCUSSION

We have tested the effect of temperature oscillation on wild pea seeds to mimic typical Mediterranean summer climate (*Thompson, 2005*) with often large diurnal temperature oscillations followed by main rainfall in the autumn season. Temperature and water availability are critical environmental factors regulating seed dormancy and germination (*Probert, 2000*; *Vleeshouwers & Kropff, 2000*). Temperature sensing determine the time of the year and depth of dormancy (*Fenner & Thompson, 2005*; *Bewley et al., 2013*; *Baskin & Baskin, 2014*; *Batlla & Benech-Arnold, 2015*; *Renzi, Chantre & Cantamutto, 2018*).

In the course of evaluation of germination data we have tested various commonly used coefficients (*Ranal & Santana, 2006*). It is known that there are difficulties in

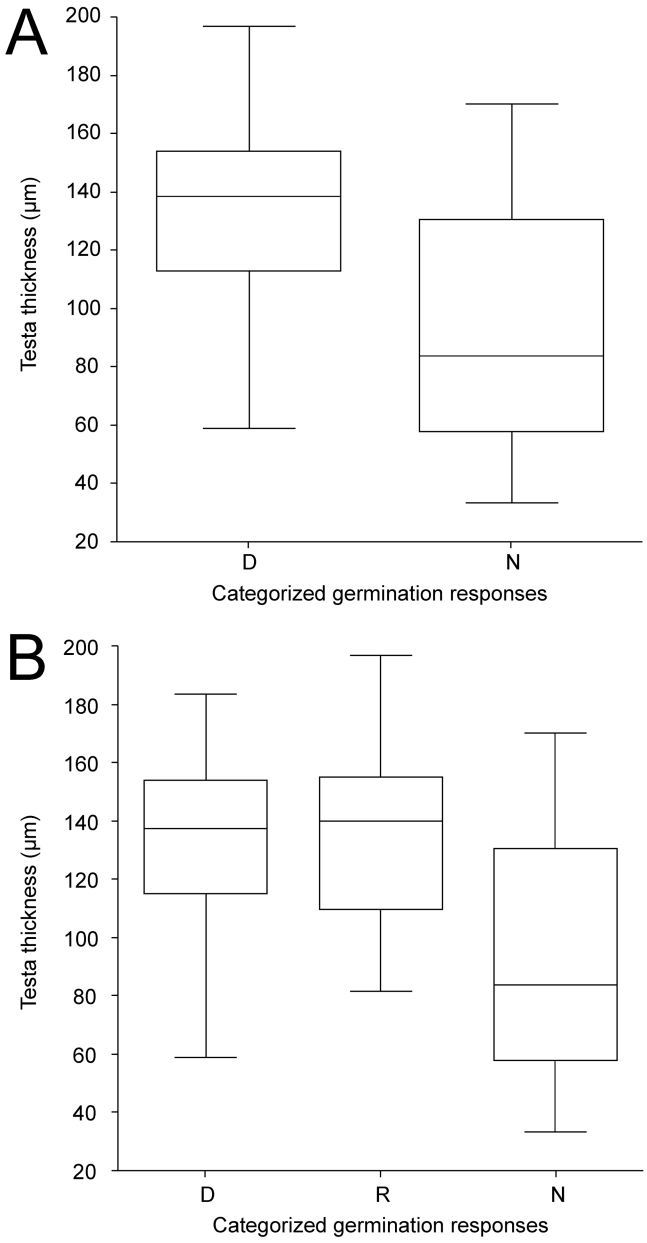

Figure 6 Box-plots of seed coat (testa) thickness of accessions classified into categories according to germination pattern under two temperature regimes. The panels compare dormant (D) and non-dormant (N) accessions tested under 25/15 °C temperature regime (A); and dormant (D), temperature responsive (R) and non-dormant (N) accessions under 35/15 °C temperature regime (B).

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their interpretation and statistical analysis (*Kader, 2005*; *Soltani et al., 2015*). None of the commonly used measures, such as: cumulative percentage which is the static expression of germination behaviour at given time-point, LT50—a time to 50% of final germination (*Soltani et al., 2015*), coefficient of velocity—as a measure of the rate and time-spread of germination, mean germination time—a weighted mean of the germination time

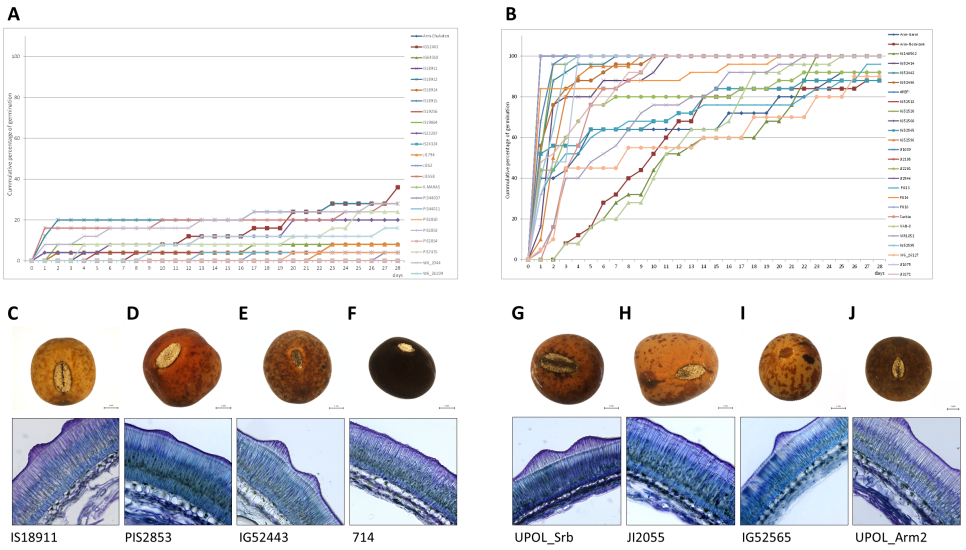

Figure 7 Comparison of dormant and non-dormant wild pea seed germination pattern, seed morphology and seed coat structure. The panels show the cumulative percentage over 28 days at 25/15 °C temperature regime of representative dormant (A) and non-dormant accessions (B) and seed micrographs and respective seed coat Toluidine stained sections at 40×magnification for dormant (C–F) and non-dormant (G–J) accessions.

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(*Ranal & Santana, 2006*) is able to differentiate between germination patterns (Table S2). Therefore in order to capture the complexity of germination we have used a *spline function* (function defined piecewise by polynomials) fitted to the data (*Ramsay & Silverman, 2005*; *Van den Boogaart, Egozcue & Pawlowsky-Glahn, 2014*). We have observed positive effect of higher temperature oscillation on release of wild pea seed dormancy (Fig. 2, Figs. S1A, S1B) similar to results obtained on some other legumes (*Lupinus*, *Trifolium* and *Medicago*) (*Quinlivan, 1961*; *Quinlivan, 1966*; *Quinlivan, 1967*; *Quinlivan, 1968*; *Quinlivan & Millington, 1962*) or *Phaseolus lunatus* (*Degreef et al., 2002*). However, detailed analyses of germination response to temperature treatments revealed marked differences in patterns of dormancy release among tested accessions. Intraspecific variation in germination patterns was little studied in legumes (reviewed in *Baskin & Baskin, 2014*); mostly only a few or even single accession was analysed. On the contrary, we have tested diverse wild pea accessions from the broad geographical area (*Smýkal et al., 2017*; *Smýkal et al., 2018a*; *Trněný et al., 2018*) originating from sites with markedly different climatic conditions. Such diverse environmental conditions might generate diverse selection pressures towards optimal germination strategies in specific environmental conditions (*Rubiode Casas et al., 2017*). Although the seed dormancy is highly heritable trait in pea (*Hradilová et al., 2017*; *Cechová et al., 2017*), the relationship between the environment and germination strategy is, however, largely unknown in pea.

Based on assessment of germination, we have obtained the range of germination response categories, from fully non-dormant (N) up to 100% germinating, through

positively temperature responsive (R) to dormant (D) accessions (Fig. 2). Some of the non-dormant accessions could potentially be domesticated peas escaped from cultivation, and/or introgression between wild and domesticated pea as suggested by genetic diversity analysis (*Trněný et al., 2018*). They could however also be genuine wild peas without substantial seed dormancy, which occurs as part of natural variation, and which was likely used during early stages of pea domestication process (*Trněný et al., 2018*; *Smýkal et al., 2018b*). We can speculate about the alteration of seed coat composition, including the oxidation of fatty acids (*Gamma-Arachchige et al., 2013*; *Cechová et al., 2017*) or polyphenolic compounds (*Hradilová et al., 2017*) as well as physical changes (*Janská et al., 2018*) in the seed coat to occur during the temperature treatment.

The relationship between environmental conditions and seed dormancy: patterns, potential limitations and alternative explanations

Despite expected relationships between pattern of seed dormancy release observed in our experiments and *in situ* environmental conditions of tested pea accessions, our results showed that germination responsivity (i.e., dormancy release) was weakly related to one climatic variable only: temperature annual range (BIO7). Specifically, dormant accessions does not occur at sites with large temperature annual range (Figs. 1, 3A, 3B, 4, Table S5). Using niche analysis we also showed that the dormant group (D) shows a localized potential distribution compared to the responsive (R) and non-dormant (N) groups (Fig. 5). This is a rather unexpected result, especially in the Mediterranean seasonal environment. This is further reinforced by the fact that the high probability areas of predicted occurrence constitute only a very small fraction of the high climate seasonality areas. Consequently, results of PCA, RDA and niche modeling in pea are not in agreement with metadata collected on wide range of legume species (*Rubio de Casas et al., 2017*) concerning the relationship between dormancy and seasonality. That study found that dormancy should increase in more seasonal environments. Of course, the niche patterns of both studies (*Rubio de Casas et al., 2017*; our study) could in part be attributed to scale differences (both spatial and taxonomic), but the need for an explanatory framework of wild pea dormancy in the Mediterranean region remains. Similar relationship between dormancy and environment as in pea was found by *Wagmann et al. (2012)* studying wild beet seed dormancy release. In contrary, two recent studies of Mediterranean wild lupines (*Berger, Shrestha & Ludwig, 2017*) and perennial woody legume (*Vachellia aroma*) along a precipitation gradient (*Ferreras, Zeballos & Funes, 2017*) showed no significant relationship between dormancy release and rainfall or temperature gradients. How could the dormancy-environment pattern we observed in pea be explained?

One set of explanations is related to (i) methodological constraints related to inaccurate estimations of *in situ* environmental conditions and (ii) specific conditions used in experimental germination treatments that might leave aside other important factors affecting *in situ* dormancy release in pea. Firstly, it can be speculated that the character of WorldClim data (average in term of time and also space) masks the micro-ecological pattern (*Smýkal et al., 2018a*). In geomorphologically complex regions, environmental conditions change considerably over short spatial scales, such that neighbouring populations can

be subject to different selective pressures. This was found in the study of seed dormancy of Swedish *A. thaliana* accessions, with general relationship to the latitude and climate variables, with northern lines generally being less dormant than southern ones (*Kerdaffrec & Nordborg, 2017*) but with detected exceptions related possibly to local scale. Secondly, in their original habitat the plants grow in very distinct environment (temperature and rainfall patterns) and consequently the dormancy status of produced seeds might differ from seeds we have been testing in our common garden experiments, as shown in *Arabidopsis* (*Edwards et al., 2017*). This would require *on site* seed collection and/or reciprocal common garden experiment (*Wagmann et al., 2012*; *Exposito-Alonso et al., 2018*), both being essentially prevented by current plant genetic resources handling methodology (Nagoya protocol, *Secretariat of the Convention on Biological Diversity United Nations Environmental Programme, 2011*). Since we have not tested seeds in natural environment, we do not know if there is a cyclic pattern of germination as reported for several legumes with physical dormancy (*Taylor, 1981*; *Taylor, 1996*; *Van Assche, Debucquoy & Rommens, 2003*; *Taylor, 2005*). Seasonal testing of germination capacity of 14 annual legumes (of *Trifolium, Medicago, Melilotus, Vicia*) suggested the importance of chilling (low winter temperatures), prior to the submission to oscillating temperatures (*Van Assche, Debucquoy & Rommens, 2003*). Most of these taxa are temperate species, while in the case of Mediterranean and middle East origin wild pea *Pisum* sp. (*Smýkal et al., 2017*) chilling is expected to play only minor role as seedling establishment in natural conditions occurs from early to late autumn (recorded observations).

It also remains to be elucidated how a combination of temperature and moisture treatments influence the water entry, as shown for *Ipomoea lacunosa* (*Jayasuriya et al., 2009*) and comparison of wild and cultivated pea (*Janská et al., 2018*). The effect of substrate moisture was far less studied, partly because of problems with its control and manipulation. In natural conditions, the oscillations of temperature are accompanied by soil moisture changes. These are not fully recapitulated in laboratory experiments. Commonly used PEG solution (*Michel & Kaufmann, 1973*) provides only static conditions. In our experimental system we did not manipulate the water potential, and tested only water saturated conditions. Moreover, we used fully matured dry seeds from glasshouse grown plants to minimalize the seed source variability and to allow the experimental testing of seed responses and analysis of seed coat properties. In our study we used fully mature dry seeds obtained from glasshouse grown plants to minimalize the influence of the environment (*Chen et al., 2014*; *Springthorpe & Penfield, 2015*; *Penfield & MacGregor, 2017*; *Renzi, Chantre & Cantamutto, 2018*). Finally, it was found that both seed-maturation temperature and the timing of dispersal, strongly influenced germination behaviour (*Edwards et al., 2017*). If such situation is present in dehisced pea seeds under natural conditions remains to be tested. In addition to abiotic environmental (such as tested temperature or moisture) also biotic factors can have significant role. Thus we cannot exclude the action of microbes and fungus as recently demonstrated for *Lepidium didymium* (Brassicaceae) seeds released from dormancy by fungal degradation of the pericarp (*Sperber et al., 2017*). However, pea seed coats contain numerous antimicrobial substances including

phenolic compounds (*Pourcel et al., 2007*; *Hradilová et al., 2017*; *Raviv et al., 2017*) which might prevent this.

Possible alternative explanation can be that pea seed dormancy is a bet-hedging mechanism in fluctuating (unpredictable) environments, in agreement with *Volis & Bohrer (2013)*. Most of the high probability areas of the dormant (D) group are restricted to regions such as Israel, which has high summer temperatures and low temperature seasonality, a pattern also supported by the PCA and RDA results. These areas are very close to semi-desert environments and are characterized by higher unpredictability compared to the wet edge seasonal Mediterranean environment, which is more predictable. This is in agreement with models of *Holst, Rasmussen & Bastiaans (2007)* where seasonal fluctuations in temperature are considered the main regulatory factor of annual dormancy cycling (*Bewley et al., 2013*), while in semiarid and arid regions with high inter-annual rainfall variability, soil moisture is a major determinant for seedling emergence. As such, it could be concluded that for wild pea, dormancy is a bet-hedging mechanism developed to overcome the most unpredictable seasonal environments of the Mediterranean region. This explanation corresponds with the view that if conditions at germination are predictive of future survival and reproduction, then the value of bet-hedging decreases (*Clauss & Venable, 2000*). However, there is an area of overlap between the predicted distributions of the dormant (D), responsive (R) and the non-dormant (N) groups. It seems that this overlap is a mechanism of risk spreading, obtaining a long term selective advantage, which is a very successful strategy to adapt rapidly to changing environments. According to *Philippi & Seger (1989)*, bet-hedging strategies are of two main forms: conservative and diversified. Conservative bet-hedging strategies tend to produce ever safer phenotypes, while diversifying bet-hedging strategies distribute the risk among two or more phenotypes. If seed dormancy is considered as a bet-hedging mechanism, then it can be viewed as the conservative form, producing the safest phenotypes; if we consider the niche overlap of the three groups, then the diversified, risk-spreading form is also apparent. Furthermore, the absence of strong environmental determination of environment-dormancy relationship, which is in consistency with the results of the niche equivalency tests, suggests that this bet-hedging mechanisms is functioning to cope with fine unpredictable fluctuations in similar environmental conditions.

The high probability areas of the non-dormant (N) group coincide with the Middle East and the Fertile Crescent, where the agricultural revolution and pea domestication took place (*Trněný et al., 2018*). Two hypotheses can be formulated with regard to this pattern. According to the first, mentioned above, the non-dormant group could be potentially domesticated pea escaped from cultivation, and/or an introgression group between wild and domesticated pea (*Trněný et al., 2018*). However, genomic analysis has revealed that only nine non-dormant accessions might be indeed genetically related to cultivated pea (IG52518, 52520, IG52442, IG52596, JI1089, JI2105, JI2201, JI2546 and P014) while the remaining 20 non-dormant accessions have clear genetic relationship to various clusters of wild *P. elatius* and are distant to cultivated pea (*Trněný et al., 2018*). On the other hand, if we assume that non-dormancy was a pre-domestication trait of wild pea even in very low frequencies within a bet-hedging diversified mechanism, our data support the view that during pea domestication such genotypes were selected and fixed in the pea crop.

Seed size, seed coat thickness and proanthocyanidins content relationships to the dormancy

In our study we have found differences in total PA content and ratio of soluble to insoluble PAs in relationship to dormancy levels (Table S3, Fig. S6). Inhibitory effect of PAs in the seed coat on germination was found in *Sapium sebiferum* (Euphorbiaceae) where the seed coat extract or PAs significantly inhibited germination via alteration of gene expression (*Afzal et al., 2017*). This would be certainly the conclusion when non-pigmented cultivated pea varieties were compared to wild accessions (*Hradilová et al., 2017*). Moreover also in legumes, pigmented seeds imbibe slower than non-pigmented ones and also germinate later (*Wyatt, 1977*; *Werker, Marbach & Mayer, 1979*; *Kantar, Pilbeam & Hebblethwaite, 1996*). Notably, insoluble PAs were shown to contribute to *Rubus* seed dormancy (*Wada, Kennedy & Reed, 2011*). Variation in PA content in the cultivated pea seeds has been reported (*Troszynska & Ciska, 2002*) but not in comparison to wild peas. Our data of PA content (Fig. S5) are in agreement with early studies (*Marbach & Mayer, 1974*; *Werker, Marbach & Mayer, 1979*) reporting positive correlation in content of phenolics, activity of catechol oxidase and pea seed dormancy, although they compared only wild versus domesticated accessions. We can speculate about the alteration of seed coat composition, including the oxidation of fatty acids (*Gamma-Arachchige et al., 2013*; *Cechová et al., 2017*) or polyphenolic compounds (*Hradilová et al., 2017*) during the temperature treatment (*Janská et al., 2018*). Indeed in *Medicago truncatula* cells of the outer integument showed abundant accumulation of polyphenolic compounds which may impact seed permeability (*Moïse et al., 2005*). Our analysis was based on spectrophotometric measurements of total content and we cannot exclude that specific PAs can be more specifically associated with dormant seed coat, as revealed by detailed analysis of pea seed coat (*Hradilová et al., 2017*) as well as soybean (*Zhou et al., 2010*). It remains to be tested if there are any specific PA compounds related to dormancy levels in wild pea.

Intraspecific variation of seed size in relation to dormancy was so far only rarely tested. Comprehensive study of legume species (*Rubio de Casas et al., 2017*) showed that smaller, dormant seeds are favoured in the environments where the growing season is short, whereas in the tropics, large non-dormant seeds dominate. In our study, we have found no relationship between seed weight or seed size parameters and dormancy, except for length-to-width ratio (LWR) being slightly positively correlated with non-dormant seed types (Figs. S4A, S4B). There might be some influence of the shape of seeds (Fig. 7) but obviously not the size of seeds themselves. During crop domestication (*Smýkal et al., 2018b*) including the legumes (*Berger, Shrestha & Ludwig, 2017*), selection acted toward seed size increase. At intraspecific level, in the case of bird vetch (*Vicia cracca*) the positive relationship between seed size and germination (*Eliášová & Münzbergová, 2014*) was observed but in relation to the ploidy level. This is not a case of diploid *Pisum*. From a reproductive point of view, seed size trades off with seed number, small-seeded species clearly have the advantage in fecundity. The advantage of large seeds appears to be their tolerance of stresses such as shade or drought (*Rees, 1996*). If this operates in wild pea, it remains to be tested. Positive correlation between testa thickness and seed dormancy is

in agreement with several reports (reviewed in *Baskin & Baskin, 2014*) and confirms our previous findings in pea (*Hradilová et al., 2017*; *Janská et al., 2018*).

CONCLUSIONS

This study provides the framework for the study of environmental aspects of physical seed dormancy exploring also the observed germination pattern of seed coat structure and composition. New approach based on functional data analysis was successfully used and this can be recommended as a new analytical tool in seed dormancy/germination studies. In our experimental set up, pea seed germination responsivity in the *ex-situ* germination experiment (dormant → responsive → non-dormant) was weakly related with the increase of *in-situ* temperature annual range (BIO7). The significant differences between dormant and non-dormant seeds were found in the seed coat thickness and marginally also in the amount of proanthocyanidins in the seed coat, while the seed size was not related to the dormancy categories. Although it is established that temperature is the main driving force of dormancy release, a mechanistic and molecular mechanism are lacking. Dormant accessions are found in the environment with generally high annual temperature, smaller temperature variation, seasonality and milder winter. In general, dormant accessions are typical of narrower temperature profiles. Responsive accessions originate from the environment with lower annual temperature, colder winter and higher diurnal and seasonal temperature fluctuations. Ecological niche modelling showed a more localized potential distribution of dormant group and supports the view that the wild pea dormancy is part of a bet-hedging mechanism to cope with fine unpredictable environmental fluctuations in seasonal environments.

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Author Contributions

- Iveta Hradilová, performed the experiments, approved the final draft.
- Martin Duchoslav, Stergios Pirintsos and Petr Smýkal conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Jan Brus analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Vilém Pechanec, Pavel Kopecký and Nikola Štefelová analyzed the data, approved the final draft.
- Miroslav Hýbl contributed reagents/materials/analysis tools, approved the final draft.
- Lucie Smržová performed the experiments, approved the final draft.
- Tadeáš Vaclávek analyzed the data, approved the final draft.
- Michael Bariotakis and Jitka Machalová analyzed the data, prepared figures and/or tables, approved the final draft.
- Karel Hron analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw measurements are provided in Tables S1–S6.

Supplemental Information

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Palacký University Olomouc

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SEED DORMANCY AS THE KEY DOMESTICATION TRAIT IN LEGUMES

Summary of the Ph.D. thesis

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Content

1. Introduction

Seed dormancy had played a significant role in the evolution and adaptation of plants, since timing of seed germination is one of the key steps in plant life. It determines when plant enters a natural or an agricultural ecosystem and it is also a basis for crop production. In general, seed dormancy is the inability of a viable and healthy seed to germinate under favourable conditions (Bewley, 1997; Baskin and Baskin, 2004, 2014). These conditions include enough water, oxygen and suitable temperature. Several dormancy classes were defined among plant species, which can be divided into morphological, physiological, morphophysiological, physical and combinational dormancy (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). This study focuses on the physical dormancy (also called hardseededness), which is caused by one or more water-impermeable layers of palisade cells in seed coat or fruit. In legumes this type of dormancy is particularly common and in laboratory conditions can be overcome by mechanical or chemical disruption of the seed coat. Under natural conditions, it may be broken by factors such as temperature, or even fire, alternating high and low temperatures and humidity, abrasion by soil particles, by an action of fungi and microorganisms.

Seed dormancy is a complex quantitative trait which is influenced by many both endogenous and environmental factors acting during the seed development, seed storage and germination. The transition from seed to seedling is one of the most important stages in the plant life cycle (Donohue *et al*., 2010). Therefore, it is essential that germination takes place at the appropriate time and place. Seeds act as environmental sensors and adjust their dormancy status as a response to a range of environmental factors. In regions where are seasonal climate, seasonal and daily fluctuations in temperature are considered the main regulatory factor of dormancy, whereas in tropics, soil moisture is a major determinant for seedling emergence (Holst *et al*., 2007; Bewley *et al*., 2013). Even though physical dormancy has been relatively well studied in agricultural conditions, much remains unknown about the mechanisms and reasons for inter- and intraspecific variations of PY in natural ecosystems (Hudson *et al.*, 2015). Studies considering both sources of variability together are rare. One example is the work in which were compared two tropical Fabaceae tree species from two distant populations differing in their degree of disturbance but with similar precipitation regime (Lacerda *et al*., 2004).

Since Mendel's work, pea has been an important model plant. For study seed dormancy conducted in this study, various contrasting genotypes of pea and 126 recombinant inbred lines (RILs) of F6 generation, established by crosses of JI64 (dormant wild parent) and JI92 (non-dormant cultivated parent) was selected. Owing to a wide geographical distribution and occurrence in diverse habitats, pea (*Pisum sativum*) offers an excellent model to study physical type of seed dormancy also in an ecological context.

2. Aims of the thesis

This dissertation thesis focuses on the study of pea seed dormancy with typical physical dormancy type. At the centre of interest there were the following aims:

- To compare seed dormancy in wild and cultivated pea genotypes (*Pisum sativum* subsp. *elatius* and subsp. *sativum)*.
    a) Analyse structure of seed coat and other phenotypic characteristic of pea seeds.
    b) Detect differences in gene expression between wild and domesticated pea seed coats.
- To study seed dormancy in an ecological context.
    a) Describe germination of seeds at various experimental temperature settings.
    b) Determine which ecological factors may drive seed dormancy level (and germination response) in wild pea (*Pisum sativum* subsp. *elatius*).
    c) Test if there is an association between seed dormancy, seed coat anatomy and content of proanthocyanidins.

3. Material and Methods

3.1. Plant material

For study comparing wild and domesticated pea four parental genotypes (JI64, VIR320, JI92 and Cameor), plus 126 recombinant inbred lines (RILs) of F6 generation of JI64 x JI92 and their reciprocal cross (North *et al*., 1989) were used. Set of 97 wild peas (*Pisum sativum* subsp. *elatius*) was selected for the study of seed dormancy in the context of ecological conditions. This material spans from the western Mediterranean, through the southeastern Europe to the Middle East.

Plants were grown in 5 litre pots in glasshouse. After harvest, mature seeds were cleaned from pods, packed in paper bags and stored in room temperature. The germination testing was performed within the month from harvest. Seeds for transcriptomic analysis were harvested at several developmental stages (2, 3, 4 weeks and older) in case of parental genotypes, and in case of RIL lines, there were approximately one month old. The seed coat was separated from embryo, immediately frozen in liquid nitrogen and stored at – 70 °C until analysis.

3.2. Transcriptomic part

RNA isolation

Total RNA was isolated from separated seed coats by BioTeke Plant Total RNA Extraction Kit (China) according to the manufacturer's protocol. Yield and purity of RNA was determined by using NanoDrop 2000 spectrophotometer (Thermo Scientific). Isolated RNA was stored at – 70 °C. Before MACE analysis and cDNA synthesis, residual DNA was removed from samples by Baseline-ZERO^{TM} DNase kit (Epicenter) according to manufacturer's instruction.

Massive Analysis of cDNA Ends (MACE)

Since the developmental stage when relevant genes are expressed is unknown, the bulk of four developmental stages (2, 3, 4 weeks and older) for every parental genotype was created. In addition, two bulks of contrasting RILs (dormant and non-dormant) were made, each containing 7 independent lines. Each sample of bulk contained 5 µg of DNase treated RNA. The samples were sent to GenXPro GmbH (Frankfurt, Germany) which carried out MACE analysis. The procedure was described in Zawada *et al*. (2014). In total, 6 cDNA libraries were prepared and sequenced, each providing around 10 million Illumina reads.

cDNA synthesis and quantitative real time PCR

Reverse transcription was carried out with Oligo(dT)$_{15}$ primer (Promega) in a two steps reaction in final volume 40 µl according to the manufacturer's protocol.

The gene expression analysis was conducted accurately as described in Hradilová *et al*. (2017). The expression of selected genes was studied at 2 developmental stages in 4 contrasting parental genotypes (JI64, JI92, Cameor and VIR320). The analysis was run on CFX96^{TM} Real-Time Detection System (Bio-Rad) using SensiFast SYBR® No-ROX kit (Bioline). The specific primers were designed by FastPCR program (Kalendar *et al*., 2014). Primers specificity was verified by observing melting curve and in addition 2% agarose gel.

Every PCR reaction included 2 µl cDNA (1:10 diluted cDNA), 5 µl 2x SensiFast SYBR and 400 nM of each primer mix in final volume 10 µl. Quantification of transcript level was determined by CFX Manager Software (Bio-Rad). All samples were investigated in triplicates and normalized to the expression of actin (Ferraro *et al.*, 2014) reference gene. Changes in transcript were estimated as fold change relative to the expression in the genotype Cameor (younger stage).

3.3. Seed germination tests

Study of seed germination variation in wild pea

Seed were tested the in two oscillating temperatures of 25/15 °C and 35/15 °C in dark at 14h/10h (day/night) regimes. Intact seeds (25 seeds per experiment in 2 replicates per treatment) were placed on water saturated filter paper (Whatman Grade 1, Sigma) in Petri dishes (P-Lab,) in incubator (Laboratory Incubator ST4, BioTech). Due to prolong testing there was necessity to apply fungicide (Maxim XL 035 FS; containing metalaxyl 10 g and fludioxonil 25 g) to prevent fungal growth. Seeds were monitored at 24h intervals for 28 days and scored number of imbibing and germinating seeds. Seeds were considered imbibed, when there was visible swelling and germinated when radicle protruded from testa. After 28 days seed coat of non-imbibing seeds was mechanically disrupted by sandpaper to test for seed viability. The data were analysed using spline function conducted by the colleagues from Department of Mathematical Analysis and Applications of Mathematics, UP Olomouc as described in Hradilová *et al*. (2019).

Comparative study of wild and domesticated pea seed germination

The seeds of four parental genotypes (JI64, VIR320, JI92 and Cameor) and 126 RILs of F_6 generation of JI64 x JI92 and reciprocal cross were tested. In this case, Petri dishes with samples (25 seeds per line or with minimum 10 seeds in event some RIL lines) were incubated at 25 °C for 7 days in dark and filter papers was saturated with water. Seed imbibition and germination was scored daily. After testing, percentage of germination, coefficient of velocity, Timpson index and mean germination time were calculated according to (Kader, 2005; Ranal and Santana, 2006).

3.4. Weight of seeds and seed coat thickness measurements

The weight of 25 seeds per accession with three replicas was recorded and converted to hundred seeds weight (HSW). Testa was peeled off from dry seed and thickness was measured by micrometer (0-25 mm, Hommel Hercules, Germany) and scanning electron microscope (VEGA3 LMU, Tescan) without the need for metal coating.

3.5. Quantification of proanthocyanidins in seed coat

Seed coats were separated from dry seeds and freeze before use. There were analysed both soluble proanthocyanidins (PAs, in acetone and 4-dimethylaminocinnamaldehyde reagent) and insoluble PAs (in butanol-HCl) using the method described by Pang *et al.* (2008). The procedure was exactly same as described in Hradilová *et al*. (2019).

4. Summary of the results

The data of this thesis were published in three research papers. The first two publications, Hradilová *et al.* (2017) and Cechová *et al.* (2017), summarize differences in gene expression, seed coat structure, and metabolites composition between wild dormant and non-dormant pea genotypes and recombinant inbred lines. Third article, Hradilová *et al.* (2019) focuses to test association between the seed dormancy, seed properties and environmental factors.

4.1. Paper I

HRADILOVÁ I., TRNĚNÝ O., VÁLKOVÁ M., CECHOVÁ M., JANSKÁ A., PROKEŠOVÁ L., AAMIR K., KREZDON N., ROTTER B., WINTER P., VARSHNEY R. K., SOUKUP A., BEDNÁŘ P., HANÁČEK P. AND SMÝKAL P. (2017): A combined comparative transcriptomic, metabolomic and anatomical analyses of two key domestication traits: pod dehiscence and seed dormancy in Pea (*Pisum sp*.). Frontiers in Plant Sciences 8: 542.

Abstract

The origin of the agriculture was one of the turning points in human history, and a central part of this was the evolution of new plant forms, domesticated crops. Seed dispersal and germination are two key traits which have been selected to facilitate cultivation and harvesting of crops. The objective of this study was to analyze anatomical structure of seed coat and pod, identify metabolic compounds associated with water-impermeable seed coat and differentially expressed genes involved in pea seed dormancy and pod dehiscence. Comparative anatomical, metabolomics, and transcriptomic analyses were carried out on wild dormant, dehiscent *Pisum elatius* (JI64, VIR320) and cultivated, indehiscent *Pisum sativum* non-dormant (JI92, Cameor) and recombinant inbred lines (RILs). Considerable differences were found in texture of testa surface, length of macrosclereids, and seed coat thickness. Histochemical and biochemical analyses indicated genotype related variation in composition and heterogeneity of seed coat cell walls within macrosclereids. Liquid chromatography–electrospray ionization/mass spectrometry and Laser desorption/ionization–mass spectrometry of separated seed coats revealed significantly higher contents of proanthocyanidins (dimer and trimer of gallocatechin), quercetin, and myricetin rhamnosides and hydroxylated fatty acids in dormant compared to non-dormant genotypes. Bulk Segregant Analysis coupled to high throughput RNA sequencing resulted in identification of 770 and 148 differentially expressed genes between dormant and non-dormant seeds or dehiscent and indehiscent pods, respectively. The expression of 14 selected dormancy-related genes was studied by qRT-PCR. Of these, expression pattern of four genes: porin (MACE-S082), peroxisomal membrane PEX14-like protein (MACE-S108), 4-coumarate CoA ligase (MACE-S131), and UDP-glucosyl transferase (MACE-S139) was in agreement in all four genotypes with Massive Analysis of cDNA Ends (MACE) data. In case of pod dehiscence, the analysis of two candidate genes (*SHATTERING* and *SHATTERPROOF*) and three out of 20 MACE identified genes (MACE-P004, MACE-P013, MACE-P015) showed down-expression in dorsal and ventral pod suture of indehiscent genotypes. Moreover, MACE-P015, the homolog of peptidoglycan-binding domain or proline-rich extensin-like protein mapped correctly to predicted *Dpo1* locus on PsLGIII. This integrated analysis of the seed coat in wild and cultivated pea provides new insight as well as raises new questions associated with domestication and seed dormancy and pod dehiscence.

4.2. Paper II

CECHOVÁ M., VÁLKOVÁ M., **HRADILOVÁ I.**, JANSKÁ A., SOUKUP A., SMÝKAL P., BEDNÁŘ P. (2017): Towards better understanding of pea seed dormancy using laser desorption/ ionization mass spectrometry. International Journal of Molecular Sciences 18: 2196.

Abstract

Seed coats of six pea genotypes contrasting in dormancy were studied by laser desorption/ionization mass spectrometry (LDI-MS). Multivariate statistical analysis discriminated dormant and non-dormant seeds in mature dry state. Separation between dormant and non-dormant types was observed despite important markers of particular dormant genotypes differ from each other. Normalized signals of long-chain hydroxylated fatty acids (HLFA) in dormant JI64 genotype seed coats were significantly higher than in other genotypes. These compounds seem to be important markers likely influencing JI64 seed imbibition and germination. HLFA importance was supported by study of recombinant inbred lines (JI64xJI92) contrasting in dormancy but similar in other seed properties. Furthermore, HLFA distribution in seed coat was studied by mass spectrometry imaging. HLFA contents in strophiole and hilum are significantly lower compared to other parts indicating their role in water uptake. Results from LDI-MS experiments are useful in understanding (physical) dormancy (first phases of germination) mechanism and properties related to food processing technologies (e.g., seed treatment by cooking).

Keywords: pea, fatty acid, seed coat, seed dormancy, seed hardness, laser desorption-ionization mass spectrometry, imaging mass spectrometry, multivariate statistics

4.3. Paper III

HRADILOVÁ I., DUCHOSLAV M., BRUS J., PECHANEC V., HÝBL M., SMRŽOVÁ L., ŠTEFELOVÁ N., VÁCLAVEK T., BARIOTAKIS M., MACHALOVÁ J., HRON K., PIRINTHOS S., SMÝKAL P. (2019): Variation in wild pea (*Pisum sativum* subsp. *elatius*) seed dormancy and its relationship to the environment and seed coat traits. PeerJ 7: e6263.

Abstract

Seed germination is one of the earliest key events in the plant life cycle. The timing of transition from seed to seedling is an important developmental stage determining the survival of individuals that influences the status of populations and species. Because of wide geographical distribution and occurrence in diverse habitats, wild pea (*Pisum sativum* subsp. *elatius*) offers an excellent model to study physical type of seed dormancy in an ecological context. This study addresses the gap in knowledge of association between the seed dormancy, seed properties and environmental factors, experimentally testing oscillating temperature as dormancy release clue.

Seed total germination ranged from 0% to 100%. Cluster analysis of germination patterns of seeds under two temperature treatments differentiated the accessions into three groups: (1) non-dormant (28 accessions, mean germination of 92%), (2) dormant at both treatments (29 acc., 15%) and (3) responsive to increasing temperature range (41 acc., with germination change from 15 to 80%). Seed coat thickness differed between groups with dormant and responsive accessions having thicker testa (median 138 and 140 μm) than non-dormant ones (median 84 mm). The total PA content showed to be higher in the seed coat of dormant (mean 2.18 $mg.g^{-1}$) than those of non-dormant (mean 1.77 $mg.g^{-1}$) and responsive accessions (mean 1.87 $mg.g^{-1}$). Each soil and bioclimatic variable and also germination responsivity (representing synthetic variable characterizing germination pattern of seeds) was spatially clustered. However, only one environmental variable (BIO7, i.e., annual temperature range) was significantly related to germination responsivity. Non-dormant and responsive accessions covered almost whole range of BIO7 while dormant accessions are found in the environment with higher annual temperature, smaller temperature variation, seasonality and milder winter. Ecological niche modelling showed a more localized potential distribution of dormant group. Seed dormancy in the wild pea might be part of a bet-hedging mechanism for areas of the Mediterranean basin with more unpredictable water availability in an otherwise seasonal environment. This study provides the framework for analysis of environmental aspects of physical seed dormancy.

Keywords: dormancy, seed coat, proanthocyanidins, testa, pea, niche-modelling, pea, temperature oscillations, germination, legumes

5. Conclusions

The work focuses on the legume seed dormancy using pea as model. Comparative anatomical, metabolomic and transcriptomic analyses of wild dormant *Pisum elatius* (JI64 and VIR320) and domesticated non-dormant *Pisum sativum* (JI92 and Cameor) genotypes and recombinant inbred lines, significant differences in texture of testa surface, length of macrosclereids and seed coat thickness were found. The dormant genotypes had considerable thicker testa as compared to non-dormant, which might contribute to water impermeability of seed coat. However, thickness or length of macrosclereids alone does not necessarily cause water impermeability. Dimer and trimer of gallocatechin, quercetin, myricetin rhamnosides and hydroxylated long chain fatty acids ranging from C26 to C28 were detected in higher concentration in dormant peas compared to non-dormant. Similarly, higher signals of hydroxylated fatty acids with long chain were found in dormant genotypes. Seeds of cultivated genotypes germinated quickly (JI92 within 24 h and Cameor 48 h), while wild pea seeds remained highly dormant and imbibed and germinated only to a certain level (in case of JI64 at 8% and VIR320 at 30%). Bulk Segregant Analysis (BSA) coupled to high throughput RNA sequencing using Massive Analysis of cDNA Ends (MACE) approaches resulted in finding of altogether 770 differentially expressed genes (DEGs) between wild and cultivated genotypes. To validate MACE results, expression levels of selected genes was analysed by qRT-PCR. From 14 genes, expression pattern of 4 genes was in agreement with MACE data. The most of DEGs belonged to phenylpropanoid and flavonoid biosynthetic pathways.

Second part of the thesis deals with an association between the seed dormancy, seed properties and environmental factors. Seed dormancy, which is manifested by impermeable seed coat, is typical of wild pea, but it is variable. Ninety-seven pea accessions were monitored over a period of 28 days, the final percentage of germinated seeds ranged from 0 to 100%. The responses at two temperature regimes (25/15 °C and 35/15 °C) were used to cluster samples into three main groups: dormant with 29 accessions, non-dormant with 28 representatives and group responsive to increasing temperature range. Furthermore, relationships between germination and environmental conditions (bioclimatic and soil variables) at the site of source were studied. As result, only one environmental variable was found to be in relation to the dormancy level, annual temperature range (BIO7). There was dependence of seed coat thickness on seed dormancy using a statistical analysis. Dormant and responsive accessions had thicker testa (median 138 and 140 μm) than non-dormant ones

(median 84 mm). In the case of seed pigmentation, it also appears that there is a correlation between the seed dormancy and quantity of pigments in the seed coat (proanthocynidins, PAs). The total PA content showed to be lower in the seed coat of non-dormant (mean 1.77 $mg.g^{-1}$) than those of dormant (mean 2.18 $mg.g^{-1}$). The amount of proanthocynidins in responsive accessions was between the content of dormant and non-dormant (mean 1.87 $mg.g^{-1}$), with value closer to the amount of total proanthocynidins in non-dormant pea seed coat. Nevertheless, in analysis Hradilová *et al.* (2019) only total content of PAs was measured and it remains to be tested if there are any specific PA compounds related to dormancy levels in wild pea. There were no relationship between seed size and the level of seed dormancy.

6. References

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7. List of author's publications

1) **HRADILOVÁ I.** (30%), DUCHOSLAV M., BRUS J., PECHANEC V., HÝBL M., SMRŽOVÁ L., ŠTEFELOVÁ N., VÁCLAVEK T., BARIOTAKIS M., MACHALOVÁ J., HRON K., PIRINTSOS S., SMÝKAL P. (2019): Variation in wild pea (*Pisum sativum* subsp. *elatius*) seed dormancy and its relationship to the environment and seed coat traits. PeerJ 7: e6263. Doi: 10.7717/peerj.6263 (IF: 2,35).

2) CECHOVÁ M., **HRADILOVÁ I.** (5%), SMÝKAL P., BARTÁK P., BEDNÁŘ P. (2019): Utilization of atmospheric solids analysis probe mass spectrometry for analysis of fatty acids on seed surface. Analytical and Bioanalytical Chemistry 411: 1169–1180. Doi.org/10.1007/s00216-018-1551-3 (IF: 3,307).

3) TRNĚNÝ O., BRUS J., **HRADILOVÁ I.** (5%), RATHORE A., DAS R. R., KOPECKÝ P., COYNE C. J., REEVES P., RICHARDS C., SMÝKAL P. (2018): Molecular evidence for two domestication events in the pea crop. Genes 9: 535. Doi:10.3390/genes9110535 (IF: 3,16).

4) **HRADILOVÁ I.** (30%), TRNĚNÝ O., VÁLKOVÁ M., CECHOVÁ M., JANSKÁ A., PROKEŠOVÁ L., AAMIR K., KREZDON N., ROTTER B., WINTER P., VARSHNEY R. K., SOUKUP A., BEDNÁŘ P., HANÁČEK P., SMÝKAL P. (2017): A combined comparative transcriptomic, metabolomic and anatomical analyses of two key domestication traits: pod dehiscence and seed dormancy in Pea (*Pisum* sp.). Frontiers in Plant Sciences 8: 542. Doi: 10.33.89/fpls.2017.00542 (IF: 3,68).

5) SMÝKAL P., **HRADILOVÁ I.** (5%), TRNĚNÝ O., BRUS J., RATHORE A., BARIOTAKIS M., DAS R. R., BHATTACHARYYA D., RICHARDS C., COYNE C. J., PIRINTSOS S. (2017): Genomic diversity and macroecology of the crop wild relatives of domesticated pea. Scientific Reports 7: 17384. Doi:10.1038/s41598-017-17623-4 (IF: 4,609).

6) CECHOVÁ M., VÁLKOVÁ M., **HRADILOVÁ I.** (5%), SOUKUP A., JANSKÁ A., SMÝKAL P., BEDNÁŘ P. (2017): Towards Better Understanding of Pea Seed Dormancy using Laser Desorption/Ionization Mass Spectrometry. International Journal of Molecular Sciences 18: 2196. Doi:10.3390/ijms18102196 (IF: 3,687).

8. Published abstracts – poster presentations

1) **Hradilová I.**, Brus J., Pechanec V., Duchoslav M., Hýbl M. and Smýkal P. (2017): Wild pea (*Pisum sativum* ssp. *elatius*) and *Medicago truncatula* seed dormancy as adaptation to environment. 18th International Conference on Legume Genetics and Genomics (ICLGG2017), Siófok, Hungary.

2) **Hradilová I.**, Trněný O., Khan W. A., Krezdorn N., Varshney R., Winter P. and Smýkal P. (2016): Comparative transcriptomic analysis of wild and cultivated pea (*Pisum sativum* L.) seed coat in relation to seed dormancy. Plant Biology Europe, EPSO/FESPB Congress, Prague.

3) **Hradilová I.**, Ponížilová M., Cechová M., Bednář P. and Smýkal P. (2015): Genetic and chemical approaches to study seed dormancy. Czech Society of Experimental Plant Biology, Student conference, Brno.

4) **Hradilová I.**, Procházková L., Ponížilová M., Cechová M., Smutná L., Trněný O., Soukup A., Hanáček P., Bednář P. and Smýkal P. (2015): Anatomical, chemical and genomic approaches to dissect two key domestication traits in pea: seed dormancy and pod dehiscence. Mendel´s legacy 150 years of the genus of genetics, Brno.

9. Participation on projects

1) Czech Science Foundation: *Ecological genomic approaches to uncovering the adaptive significance of seed dormancy in legumes* (Project number 16-21053S).

2) Czech Science Foundation: *Seed dormancy and pod dehiscence as the key domestication traits in legumes* (Project number 14-11782S).

10. Souhrn (Summary in Czech)

Název práce: Dormance semen jako klíčový domestikační znak bobovitých rostlin

Autor: Mgr. Iveta Hradilová

Disertační práce je zaměřena na studium semenné dormance u planých a kulturních forem hrachu (*Pisum sativum*). První část je věnována srovnání anatomických, chemických a transkriptomických odlišností mezi kulturními nedormantními (JI92, Cameor), planými dormantními (JI64, VIR320) genotypy hrachu a rekombinatními inbredními liniemi. Nalezeny byly významné rozdíly v povrchové struktuře osemení, jeho tloušťce i délce makrosklereidních buněk. Pozoruhodná je absence zvrásněného povrchu (Gritty) u planého genotypu VIR320. Tloušťka nebo délka makrosklereidů však sama o sobě nemusí nutně znamenat nepropustnost osemení pro vodu. Určitou roli hraje také odlišné chemické složení. Jako významný chemický marker dormance byl identifikován galokatechin (konkrétně dimer a trimer), rhamnosid kvercetinu, myricetin a hydroxylované mastné kyseliny s dlouhým alkylovým řetězcem (v rozmezí od C26 až C28). Nejvíce diferenciálně exprimovaných genů bylo nalezeno v biosyntetické dráze fenylpropanoidů a flavonoidů.

Druhá část práce se zabývá spojitostí mezi dormancí, vlastnostmi semen a faktory prostředí. Dormance byla hodnocena na základě bobtnání a klíčení semen v rozdílných teplotních režimech. Na základě reakce ve dvou teplotních režimech byly jednotlivé genotypy rozděleny do tří hlavních skupin – na dormantní, responzivní a nedormantní. Pouze u ročního teplotního rozmezí (BIO7) byl prokázán vztah mezi klíčením a podmínkami prostředí. V osemení dormantních zástupců byl naměřen vyšší obsah proanthokyanidinů, naopak nebyla nalezena žádná spojitost mezi velikostí semen a dormancí.

Získané výsledky mohou posloužit při srovnávací analýze s ostatními nezávisle domestikovanými luštěninami. Transkriptomická data umožní zpřesnění informací o vývoji semen hrachu a dalších bobovitých rostlin. Vyvinuté metodické postupy chemické analýzy mohou být aplikovány při studiu osemení dalších druhů rostlin, stejně jako přístup použitý při hodnocení klíčení lze využít pro jiné studie zabývající se klíčivostí semen/dormancí.