Palacký University Olomouc

Master thesis

OLOMOUC 2023 **GLORY EBHOSETALE OKOJIE**

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Genetic mapping of seed dormancy as a key domestication trait in pea (*Pisum sativum* L.)

Master thesis

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Study program: Plant biology

Form of study: Full-time

Olomouc 2023

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Bibliographical identification

Author's first name and surname: Glory Okojie

Title of work: Genetic mapping of seed dormancy as a key domestication trait in pea (*Pisum sativum* L.)

Type of thesis: Master thesis

Workplace: Department of Botany, Faculty of Science, Palacký University Olomouc

Supervisor: prof. Ing. Petr Smýkal, Ph.D.

Year of presentation: 2023

Abstract:

In this research, I focused on the analysis of candidate gene(s) encoding calcineurin and testing its association with seed dormancy in pea. To identify the gene locus conditioning seed dormancy in wild pea (*Pisum elatius* L.), I selected two genetically divergent parents of wild (JI64) and cultivated (JI92) pea which show clear difference in the dormancy for genetic mapping based on genome - wide association mapping on RIL population (Hradilová *et al*. 2017). This analysis resulted in identification of two loci, one of them placed at chromosome 2. Searched at current pea genome (Cameor version 1.0) indicated 49 gene of which two were homologues genes encoding calcineurin-like protein.

The rate of recombination of these two genes (Psat2g013240 and Psat2g024960) was compared between RILs of JI64xJI92 and its reciprocal JI92xJI64 using PCR and restriction analysis. Also comparison of the testa thickness measurement, anatomic and phenotypic analysis between cultivated (JI92) and wild (JI64) pea as well as their RILs. Furthermore, statistical analysis was used to evaluate the dormancy status between parental alleles in the recombinant inbred lines and also to test the relationship between the seed coat thickness and level of imbibition.

Based on genetic diversity analysis panel, it has shown a strong support for Psat2g013240 gene of wild genotype (JI64) being related to dormancy. However, sequence variability detected in set of domesticated, non-dormant pea genotypes excluded domestication based selection as expected for key genes. Thus respective gene might be associated but not causative with dormancy status in pea.

Key words: Pea (*Pisum elatius* L), Dormancy, Domestication, Genetic mapping, Anatomic and Phenotypic analysis, Seed coat, RILs population, genotype, genetically divergent, Allele, Calcineurin, Homologues gene.

Number of pages: 93.

Language: English.

DECLARATION

I claim that I wrote this work independently with the assistance of my supervisor, prof. Ing. Petr Smýkal, Ph.D., and that I used all the sources listed below.

In Olomouc,…….. ……

Glory Okojie

ACKNOWLEDGMENT

I would like to express my gratitude and appreciation to my supervisor, prof. Ing. Petr Smýkal, Ph.D., for his kindness and understanding. He spent hours explaining everything to me, put up with my repeated mistakes, and was always glad to help. I also want to thank all the staffs of the department of botany PřF UPOL for their supports during my work in the laboratory. Finally, I'm grateful to my family and friends for being patient, caring, and loving on my way of getting this work done. It is fact that without them it wouldn't have been complete.

CONTENT

ABBREVIATIONS

ABA	Abscicic acid
AtCBL	*Arabidopsis thaliana* B-like protein
ac.	Accession
Bp	Base pair
BCE	Before the common era
BLAST	Basic local alignment search tool
CBL	Calcineurin b-like protein
CIPKs	CBL-interacting kinase
CEL	Carboxyl ester lipase
CAMK	Calmodulin-dependent protein kinase
^{0}**C**	Celsius degree
cv.	Cultivated
C>T	Cytosine>Thymine
CAPS	Cleaved amplified polymorphic sequence
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
F	Forward
GA	Giberrellins
GH	Glycosyl hydrolase family
Kb	Kilo base
LPWG	Legume taxonomy working group
Mbp	Mega base pair
NF-ATp	Nuclear factor-activated T cells

nm	Nanometer
ng	Nanogram
P.	genus *Pisum*
PCR	Polymerase chain reaction
PG	Polygalactoron
QTL	Quantitative trait locus
rpm	Revolution per minute
R	Reverse
RILs	Recombinant inbred lines
RNAseq	Ribonucleic acid sequence
SNP	Single nucleotide polymorphism
Subsp.	Subspecies
SNP	Single nucleotide polymorphism
TBE	Tris + boric acid + EDTA
UV	Ultraviolet
µl	Microliter

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1. INTRODUCTION

Legumes are members of the Fabaceae (also called the leguminous) family of flowering plants. A legume is a simple, dry fruit that develops from a simple carpel and usually dehisces on two sides releasing the seeds that are attached to one or both seams. All members of this family have five-petaled flowers in which the superior ovary attached to the receptacle above the attachment of other floral parts, ripens to form a "pod," technically called a legume. The Leguminosae is the third largest angiosperm family in terms of species numbers after Asteraceae and Orchidaceae. The Leguminosae consists of about 750 genera and 19,000 species of herbs, shrubs, trees, and climbers. (Stevens 2008). This large family is divided into four subfamilies—Mimosoideae, Caesalpinoideae, Swartzioideae, and Papilionoideae (Lee *et al*. 2001). Legumes are multipurpose and economically important food crops providing highly nutritious sources of protein and micronutrients that can greatly benefit health and livelihoods. They have been domesticated alongside grasses in different regions of the world since the beginning of agriculture and have played a key role in its early development. Legumes are also important as fodder and green manure in both temperate and tropical regions, and are used for their wood, tannins, oils and resins, in the manufacture of varnishes, paints, dyes and medicines, and in the horticultural trade. The family is morphologically, physiologically and ecologically exceptionally diverse (Merga *et al*. 2019). It is one of the most spectacular examples of evolutionary diversification in plants.

Legumes are cosmopolitan in distribution, representing substantial ecological constituents in almost all biomes across the globe, and occur even in the most extreme habitats. They constitute important elements in terms of both species diversity and abundance in lowland wet tropical forests, especially in the Neotropics. They also dominate dry forests and savannas throughout the tropics, and are also very diverse and abundant in the Mediterranean and other temperate regions, up to high latitudes and at high elevations (Stevens *et al*. 2008). They can be emergent tropical trees, small ephemeral annual herbs, climbing annuals or perennials with tendrils, desert shrubs, geoxylic subshrubs, woody lianas and less commonly, aquatics. Floral is radially symmetric (actinomorphic) to bilaterally symmetric (zygomorphic) and asymmetric flowers, which are in turn adapted to a wide range of pollinators such as insects, birds and bats. The ability of the majority of legume

species to fix atmospheric nitrogen in symbiosis with soil rhizobia is perhaps the best known ecological attributes of the family. However, not all legumes form associations with nitrogen-fixing bacteria (Bruneau *et al*. (LPWG) 2019).

Legumes were among the earliest plants cultivated by humans. Reason is due to the large seeds of most of its species which makes them easy to gather and store and they provide good nutritional value. Cultivated legumes fall into two classes: grain and forage. Grain legumes are cultivated for their seeds (pulse). The seeds are used for human and animal consumption or for the production of oils for industrial uses (Kurlovich and Repyev 1995). Grain legumes include soybeans, faba beans, lentils, lupines, chickpeas, peas, and peanuts. While Forage legumes includes alfalfa, acacia, clover and vetch, are sown in pasture and grazed by livestock or harvested as hay. Grain legumes were first cultivated in the Middle East, perhaps as long as 10,000 years ago. The first five species cultivated are considered "Neolithic founder crops," along with flax, barley, and two species of wheat. These five founder legume species are lentil (*Lens culinaris* Medik.), pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), bitter vetch (*Vicia ervilia* L.) and the broad or faba bean (*Vicia faba* L.) (Zohary and Hopf 2000). The use of forage legumes is estimated to be as old as 11 000 years, with some species first being used as grain for human consumption and more recently only used for fodder or pasture, or vice versa (Mathison 1983). Although tropical forage legumes have been grazed in natural habitats for many years, they have only been under cultivation for as little as 75 years, while some like *Centrosema pubescens*, *Lablab purpureus* and *Pueraria javanica* were used as cover crops and green manure at least a century earlier. Tropical legumes may have originated from tropical forests and natural grasslands and were later adapted to a variety of environments. Currently grasslands can be found from the tropics to the Arctic regions, from high rainfall regions to deserts and from rocky slopes and sand dunes to swamps (Williams 1983). Alfalfa (*Medicago sativa* L.) was one of the first cultivated forage legumes, It is native to Iran (ancient Persia) and was cultivated there soon after the introduction of the horse, around 3000 to 4000 years ago. However the domestication history of alfalfa is poorly known (Muller *et al*. 2006). It was spread to Europe when the Persian Empire invaded Greece around 490 BCE. Alfalfa is now an important crop in the United States being harvested as hay to feed cattle and horses.

Clover or trefoil are common names for plants of the genus *Trifolium*, comprises of about 300 species of flowering plants in the legume family, originated in Europe (Ellison *et al*. 2006). They are mainly planted along with grasses to provide forage for grazing animals, although, they are sometimes harvested as hay, boiled and consumed in times of famine and emergency. Clover has a cosmopolitan distribution with highest diversity in the temperate Northern Hemisphere, but numerous species also occur in South America and Africa, including at high altitudes on mountains in the tropics. They are small annual, biennial, or short lived perennial herbaceous plants, which typically grow up to 30 cm tall. Species belonging to this genus often includes *Trifolium repens* L. (White clover) and *Trifolium pretense* L. (Red clover), *Trifolium incarnatum* L. (Crimson clover) (McGraw-Hill *et al*. 2005).

Acacias (genus *Acacia*), an important wooden leguminous plant, is one of the largest tree and shrubs genera in the world. They mostly live in dry tropical and subtropical regions, with Australia having the most species. Some species form symbiotic relationships with ants in which the trees provide the ants with food from sap and special fruits. Hollow thorns act as nesting places and the ants protect the trees from grazing animals, other insects, and competing plants. *Senegalia brevispica* (Harms.), an example of Acacia specie is mainly used as forage tree and its foliage, pods and seeds are easily eaten by goat. Also it can be used as firewood and live fence while its roots are used for ethno medicinal purpose (Bekele – Tesemma 2007). Many other acacia species provide valuable hardwoods, including the *Acacia koa* commonly known as Koa tree of Hawaii and *Acacia seyal* (Del.) from which it is said that the Ark of the Covenant was made. Acacia seeds are also used as food in Africa and also in Australia, where seeds gathered from about 120 species are an important part of the traditional diet of the native people (Armstrong 1998).

The soybean (*Glycine max* (L.)Merrill.) was domesticated from its wild relatives (*Glycine soja* (L.)Merrill.) in China, perhaps 5,000 years ago, resulting in a diversity of landraces with permeable seed coats (Carter *et al*. 2004; Kim *et al*. 2010). Soybeans were introduced to java in Malay Archipelago circa 13th century or probably earlier. By the 17th century through their trade with Far East, soybeans and its products were traded by European traders (Portuguese, Spanish, and Dutch) in Asia, and reached Indian subcontinent by this period. By the 18th century, soybeans were introduced to the Americas and Europe from china in the late 19th century, and are now widespread across the continent. It is now the most

important world's legume crop. The common bean (*Phaseolus vulgaris* L.) and the peanut (*Arachis hypogea* L.) were first cultivated in the new world. There are so many other grain legume species cultivated around the World, and also many wild ones that are harvested in times of need by local people. Some of these are being investigated for their potential cultivation (Hymowitz 1990). Chickpea (*Cicer arietinum* L.) is an annual, self-pollinated legume crop grown mainly in arid and semi-arid regions all over the world (Wang *et al*. 2019). It is the second most important legume grain crop and is a source of high quality nutrients. It forms an essential part of a diet in developing countries (Kudapa *et al*. 2018). *Cicer reticulatum* L. is the wild progenitor of chickpeas and currently grows only in southeast Turkey, where they are believed to have been domesticated, which can be dated to around 7000 BC. Chickpea spread to the Mediterranean region around 6000 BC and India around 3000BC (Pearman and Georgina 2005). Lentil (*Lens culinaris* Medik.) is also an important grain legume. It is an annual plant known for its lense shaped seeds, and the seed grows in pods, usually with two seeds in each. Lentil is a cool seasonal pulse crop and can be cultivated on any soil from sandy loams to heavy black cotton soils, provided drainage is adequate (Erskine and William 2009). Lentil is the fourth most important pulse crop of the world after beans, pea and chickpea. Besides being a valuable source of protein, it's also an important component of local or international trade (Erskine and William 2009). Lentil was domesticated in the Fertile Crescent of the Near East and then spread to Europe, the Middle East, North Africa and the Indo-Gangetic plain. The primary center of diversity for the domestic *Lens culinaris* as well as its wild progenitor *L. orientalis* is considered to be in the Middle East. The oldest known carbonized remains of lentil from Greece's Franchthi Cave are dated to 11,000 BC. In archaeobotanical excavations, carbonized remains of lentil seeds have been recovered from widely dispersed places such as Tell Ramad in Syria (6250-5950 BC), Aceramic Beidha in Jordan, Hacilar in Turkey (5800-5000 BC), Tepe Sabz (Ita. Tepe Sabz) in Iran (5500-5000 BC) and Argissa-Magula Tessaly in Greece (6000-5000 BC), among other places (Jain *et al*. 2019). Faba bean (*Vicia faba* L.), commonly known as broad bean is another cool season annual legume (Bilalis *et al*. 2003) that forms coarse, upright, hollow, and unbranched stem(s) from the base, and grows between 0.1 and 2 m tall (Bond *et al*. 1985). The leaves are alternate, pinnate, and consist of two to six leaflets, which are up to 8 cm long without tendrils (Bond *et al*. 1985). The flowers have a typically papilionaceous structure and are grouped in inflorescences; they are either pure white in color or with

diffuse anthocyanin pigmentation on all petals, while black spots are often present on the wing petals (Bond *et al*. 1985; Duc *et al*. 2015a). Faba bean is a self-pollinated plant with significant levels of cross-pollination (Suso *et al*. 1996; Chen 2009). The main pollinating insects are honeybees (*Apis* spp.) and bumblebees (*Bombus* sp.).

Knowledge of the wild progenitor and area of origin of the genus, and subsequent steps in the domestication of its most important member species, *V. faba* L., is and disputed (Shiran *et al*. 2014). The Near East is considered a center of origin for faba bean (Cubero 1974), while China seems to be a secondary center of faba bean genetic diversity (Zong *et al*. 2009, 2010). In support of Cubero's findings, Caracuta *et al*. (2016) have identified seeds of a potential ancestor of faba bean adjacent to Mount Carmel, Israel – the remains were C-dated to 14,000 years BP (before present). Moreover, Caracuta *et al*. (2015) have determined that faba bean was already domesticated about 10,200 years BP in the Lower Galilee, Israel. In any case, faba bean can be considered one of the earliest domesticated crops in light of numerous archeological findings in Eurasia and Africa which date back to the early Neolithic (Duc *et al*. 2015a). *Vicia faba* has a large genetic diversity. According to Duc *et al*. (2010, 2015a), more than 38,000 accessions of faba bean germplasm are conserved globally in numerous gene banks, as well as at the International Center for Agricultural Research in Dry Areas (ICARDA).

Field pea (*Pisum sativum* L.) is an annual and a cool season crop grown in many parts of the world, belonging to the leguminous (Fabacea) family, it has a protein content of about 25% making it beneficial for animal feed and human subsistance worldwide (Graham and Vance 2003). Pea belongs to the world's oldest domesticated crops and still a globally important grain leguminous crop (Smýkal *et al*. 2012, 2015). Through a symbiotic relationship with nitrogen fixing bacteria, peas as most of the other legumes play a role in the conversion of atmospheric nitrogen into a form the plants can utilize. This harmonious, mutually beneficial relationship between diverse species is also utilized in agriculture for the replenishing of soils depleted of nitrogen due to the planting of non-leguminous crops. As other wild legume species, also the wild pea exhibits full pod dehiscence upon maturity, while cultivated pea has indehiscent pods that allow all the seeds to be retained at maturity (Abbo *et al*. 2014). The wild plant is native to the Mediterranean region, and ancient remains dating to the late Neolithic period have been found in the Middle East. European

colonization introduced the crop to the new world and other regions throughout the globe (Zohary and Hopf 2000). In the mid-1800s, peas in a monastery garden in Austria were famously used by the monk Gregor Mendel in his pioneering studies of the nature of heredity.

The process of domestication is broad and comes with conscious and unconscious artificial selections that result in often morphologic and physiologic changes between the domesticated plants and wild progenitors known as domesticated syndrome (Hammer 1984) which is underplayed by genetic changes (Smýkal *et al*. 2018). As in many other crops, there are two key traits such as the loss of seed dormancy and seed dispersal executed by pod dehiscence.

Seed dormancy is an important mechanism that allows delay of germination until conditions for seedling survival are optimal. Dormancy is an acquired trait during evolution based on the selection abilities to survive unfavorable conditions. Change of temperature can affect seed dormancy and serves to reduce the risk of premature death in seed. (Bewley *et al*. 2013). Dormant seeds are common iin wild plants, while domesticated species are selected for their fast and uniform germination, providing good crop establishment and thereby achieving a greater yield. Although non-dormant individuals may have reduced lifespan seeds, they are used for commercial purposes including faster cooking and improved taste. Seed dormancy is divided into 5 types: physiological, morphological, morpho-physiological, physical and combined (Baskin and Baskin 2000). For leguminous seeds, the most important dormancy is physical, which caused seed coat mediated water and likely gases impermeability caused by macrosclereid cells in the seed coat (Smýkal *et al*. 2014). The role of plant hormones, especially abscisic acid (ABA) and gibberellins (GA) in the regulation of vegetative rest and germination has long been recognized. Several more have been identified in the past decade on factors that influence dormancy including specific genes. In the case of leguminous seed plants, this is a physically conditioned dormancy mediated by impermeability seedling for water. Dormancy has been removed for cultural forms, allowing for quick germination after sowing and at the same time swelling of the seeds in connection with boiling and subsequent digestibility.

2. AIM OF THE WORK

1. The aim of this thesis was to test the candidate gene encoding two homologous calcineurin genes in the loci that condition the dormancy of the wild ancestor of pea (*Pisum elatius*) by genetic mapping and phenotypic analysis of the recombinant inbred lines (RILs).
2. In the theoretical part of the work, on the basis of literary sources, described the development of seed and mechanism of dormancy in selected legume and respective crops.
3. In the practical part, experiments were performed to test for seed dormancy in mapping population of recombinant inbred lines (RIL) of wild (*P. elatius*) and cultivated (*P. sativum*) pea segregating for seed dormancy. PCR amplification of selected genes-specific markers, sequencing analysis, identification of polymorphism necessary for mapping (SNPs) and applying these polymorphic markers to a set of RIL lines. Finally to conduct statistical data analysis.

3. LITERATURE OVERVIEW

ORIGIN OF GARDEN PEA (*Pisum sativum* L.)

Pea belongs to the oldest cultivated crops together with cereals and other legumes such as soybean, chickpea and common bean. It belongs to a legumes family (Fabaceae) and is grown throughout the temperate zone mainly as a spring crop. Pea has the ability to fix atmospheric nitrogen and sequester carbon there by improves soil fertility providing sustainability in crop production systems (Erskine *et al.* 2009). It has specific position in the history of science as it represents an important experimental plant in the field of genetics (Bastianelli *et al.* 1998; Ellis *et al.* 2011; Smýkal 2011, 2015; Smýkal *et al.* 2012).

Three pea species are currently distinguished in the genus *Pisum* - ***P. sativum* L.**, including the cultivated **P. *sativum* subsp. *sativum***, which originates from wild ***P. sativum subsp. elatius*** Asch. et Graebm, wild ***P. fulvum* Sm.** and cultivated ***P. abyssinicum* A. (Br)**,

probably domesticated independently of ***P. sativum*** in Ethiopia and Yemen (Maxted and Ambrose 2001; Smýkal *et al.* 2011, 2012, 2015; Trněný *et al*. 2018).

The process of gradual domestication of wild plant forms into cultivated ones took place in geographical places, the centers of origin of cultivated plants, which were defined by the Russian botanist N.I. Vavilov (1926). These centers of origin include China, Northeast Asia, India, Turkey, Iraq, Mediterranean, Ethiopia, Central America and South America (see **Figure 1**). Like the region of origin and primary domestication of the pea is considered to be the Middle East and especially the Near East (Smýkal *et al*. 2017). However, due to the early cultivation of peas, it is difficult to determine the exact location of the site of origin of genetic diversity and domestication, primarily because much of the Mediterranean and the Middle East has been significantly altered by human activity and changing climate conditions. Furthermore, reliable and conclusive data are often missing or incomplete.

Distribution of the wild peas **(*P. sativum* subsp. *elatius)*** are scattered over the Mediterranean basin such as in Southern Europe, Western Asia, including Asia Minor, and its area extends to North Africa, While the distribution of ***P. fulvum*** occurs only in the Middle East, namely in Jordan, Syria, Lebanon and Israel (Smýkal *et al*. 2011). ***P. abyssinicum*** currently grows only as cultivated or wild species in Yemen and Ethiopia and was probably domesticated independently of ***P. sativum*** (Maxted and Ambrose 2001, Trněný *et al*. 2018). Thousands of varieties have evolved over centuries of selection and cross-breeding (genotypes) of pea, maintained in many gene banks around the world (Smýkal *et al.* 2012).

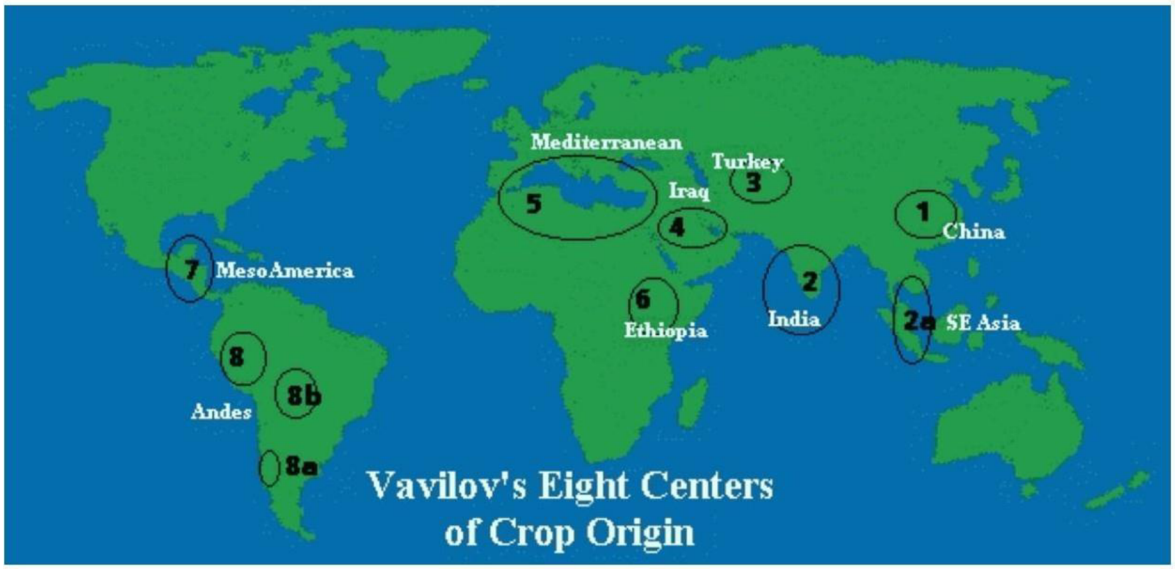


Figure 1: Centers of origin of cultivated plants (NI Vavilov, 1926) (adapted from http://oregonstate.edu/instruct/css/330/two/) (1) Chinese Centre, (2) India Centre, (2a) Indo – Malayan Centre, (3) Central Asiatic Centre, (4) Asia Minor Centre, (5) Mediterranean Centre, (6) Abyssinian Centre, (7) South Mexican and Central American Centre, (8) South American Centre, (8a) Chilean Centre, (8b) Brazilian – Paraguayan.

3.1. DOMESTICATION SYNDROME

The emergence of agriculture was one of the key points in human history and a major part of it was the development of new plant forms known as domesticated crops (Meyer *et al.* 2012; Fuller *et al.* 2014). Early farmers used only a small number of selected individuals, and as a result of these current cultivated plants have lower genetic diversity. It is generally stated that cultivated crops on average have 60% genetic diversity compared to their wild counterparts. Domestication of wild plants into cultivated crops can be seen as accelerated evolution, the result of both human and natural selection (Abbo *et al.* 2012, 2014). The process of domestication produced changes representing adaptation to cultivation and harvesting accompanied by genetic changes such as shattering/fruits abscission, shorter height, large fruits size, easier threshing, synchronous flowering, an increase yield, and changes in color, taste and texture. (Brown 2010; Lenser and Theissen

2013; Olsen and Wendell 2013; Shi and Lai 2015). Among all the domestication characters, two points stand out of central position, as they were decisive for the success of cultivation from the beginning. These characters are the loss of seed dormancy during cultivation and the second is the loss of free seed dispersal (Fuller *et al.* 2009). A common set of domestication characters was found in unrelated crops of geographically distant areas and was called "domesticated syndrome" (Hammer 1984; Harlan 1992). This genetic modification led in many cases of high or complete dependence on human, especially loss of natural ability of reproduction. From a genetic point of view, there are several factors influencing the process of domestication such as the phenotypic variability of a given trait, the number and range of acting genes, heredity, strength selection, mutation rate, outcrossing rate, population size and recombination rate or conversely, the degree of linkage between selected genes (Abbo *et al.* 2014).

The evolutionary trajectory from wild species to domesticated crops is a complex process. The archaeological record suggests that there was a pre-domestication period when humans first began the deliberate planting or freeing up of space for the growth of wild vegetation that possess advantageous properties. Later with crops diversified, as were cultivated in new areas (von Wettberg *et al.* 2018; Abbo *et al.* 2014). It is commonly believed to have occurred first to the harvesting of wild plants followed by conscious and unconscious selection to modify traits plants, and finally to consciously select vegetable material for specific location and use, thereby crop plants in general lost ability to survive without human care (Harlan 1992).

3.2. AGRICULTURAL IMPORTANCE OF LEGUMES

Legumes belong to the family Fabaceae, a diverse family with worldwide distribution and a broad range of plant forms (Lewis 2005). They are the second most important crop family after Poaceae and grain legumes (pulses) account for 27% of global crop production providing nutrition for millions of people (Vance *et al*. 2002). Legumes are considered to be multipurpose crop and can be used directly as food or in processed forms as a feed for livestock and can be used as a rotation crops to improve soil health through nitrogen fixation, thereby reducing the use of fertilizers and energy in arable systems and

consequently lowering the greenhouse gas emissions (Merga *et al.* 2019; Reckling *et al*. 2014). Although domestication traits are favorable in agricultural ecosystems, they impose a trade-off for fitness in natural habitats (Simpson *et al*. 2017). In the natural environment, the timing at which seeds break dormancy is crucial, as germination at the wrong time can result in reduced survival and fitness (Finch-Savage *et al*. 2017). Besides synchronization with the environment, seed dormancy is involved in seed dispersal and in the reduction of resource conflicts between mother and offspring (Penfield 2017). Despite being advantageous in natural environments, extended seed dormancy is not desirable traits for crops (Purigganan *et al*. 2019). In cultivated legumes, seed dormancy reduces the rate of germination, which results to uneven germination and consequently decreased yields (Abbo *et al*. 2008).

Legume seeds also contain lectins, a protein that is harmful to health which recognize and bind sugars, whether free or bound, with a high degree of specificity. Lectin belongs to the anti-nutritional substances that inhibit the action of amylase and other digestive enzymes. They can cause a slowdown in the growth of the organism by affecting the metabolism of red blood cells, reduce the level of insulin in the blood, can cause insufficient utilization of vitamins, glucose, fats and amino acids, digestive disorders can also occur and to lose weight. Lectin is soluble in water, so it is advisable to soak legumes before cooking. These substances cease to be dangerous for soaked legumes after only 10 minutes of boiling, unlike non-soaked legumes, where these substances are destroyed only after 90 minutes of boiling (Dahl 2012). The vitamin and mineral components of peas can play an important role in the prevention of diseases related to a deficiency, especially of selenium or folic acid. Pea contains various substances that act as anti-nutritional factors. Including polyphenols, which however, also have antioxidant and anticarcinogenic effects, as well as saponins that reduce blood cholesterol and have anticarcinogenic effect, and galactose oligosaccharides, which have beneficial prebiotic effects in the large intestine (Dahl 2012).

3.3. SEED DORMANCY

Seed dormancy is the main physiological adaptation to the heterogeneity of life environment that affects natural population dynamics (Bewley *et al.* 2013). Dormancy provides a strategy to develop germination over time to reduce the risk of plant death and

eventual extinction of species in an unfavorable period. Dormancy has been defined as: *"the property of a seed, degree determining what conditions should be met for the seed to germinate" (*Vleeshouwers *et al.* 1995). This strategy is regulated by various factors such as seed heterogeneity at the level of individual plants (Matilla *et al.* 2005). Depending on age and maternal nutrition of plants during seed maturation, the position of the seeds on the mother plant, the size and shape of the seeds, the time of seed harvesting and the time of their storage (Probert 2000). Despite all these variations, dormancy has a clear genetic basis (Graeber *et al.* 2012). The classic concept of seed dormancy was formulated by Harper (1957) distinguishing three types. Plants whose seeds do not germinate immediately have **primary innate dormancy** after ripening on the mother plant. To end this type of dormancy, the seeds must be exposed for a certain period of time to the special conditions that the given species needs to overcome dormancy. The seeds of many species need a special stimulus to germinate, without which they will not germinate, even when dormancy is over. This is often exposure to light or regular rotation temperature that occurs in the surface layers of the soil during the day and night. **Secondary dormancy** occurs in germinating seeds lying in the soil as a reaction to certain, mostly unfavorable conditions. A seed is in a state of forced dormancy maintained by the action of external conditions, when these conditions pass away, it will soon occur to end dormancy. **Induced dormancy** is a state physiologically similar to primary dormancy. Secondary dormant seeds do not germinate immediately after the onset of favorable conditions, but they need to go through a period of conditions suitable for termination to end dormancy. Dormant classes were defined by Nikolaev (1969, 1977) and more recently reviewed by Finch-Savag and Leubner-Metzger (2006). **Physiological dormancy,** the most widespread form, probably includes metabolism of abscisic acid (ABA) and gibberellin (GA). These growth hormones depend on external conditions such as light, heat and humidity. ABA is thought to induce dormancy, whereas GAs is responsible for the initiation of germination. If the seed is weakly dormant, and therefore contains a low dose of ABA, it also requires a low dose of GA for germination. Conversely, if a greater amount of ABA was produced in the seed during its development, greater amounts of GA are also required for germination (Baskin and Baskin 2004). ABA prevents premature germination of the seed during its development, it has the same effects of osmotic pressure on the surrounding environment or a combination thereof (Berry and Bewley 1992). Hormonal regulation of dormancy is also affected by ethylene. It was in many

plant species shown to terminate dormancy and stimulate germination (Kÿpczynski *et al.* 1997). Ethylene interacts with ABA and apparently reduces seed sensitivity to it (Baskin and Baskin 2004). **Morphological dormancy** refers to seeds that have an undeveloped embryo and they require time to grow and germinate. Under favorable conditions, this period lasts several days, no more than two weeks. The seeds then germinate within a month (Baskin and Baskin 2004). **Morpho-physiological dormancy** is found in seeds that have little developed embryos and in addition have physiological conditions for their dormancy. These seeds require ending dormancy, scarification, or GA application and significantly longer development time (Baskin and Baskin 2004). **Physical dormancy** involves the development of palisade cell layers of sperm impermeable to water, the so-called hard seed (Baskin *et al.* 2000). This impermeability can be broken by mechanical or chemical scarification (Finch-Savage *et al.* 2006). While in nature the factors likely includes the effect of microbes and soil properties, temperature and moisture oscillations. The so-called hard seed is a typical phenomenon in most of the legumes (Smýkal *et al*. 2014). This attribute provides better possibilities for survival in nature (Hradilová *et al*. 2019; Renzi *et al*. 2020). The presence of hard seeds not only prevents rapid uniform germination as required for cultivation, but also prolongs germination time (Quinlivan 1971; Maas 2005). This type of dormancy is found in at least 17 plant families, including agronomical important families such as *Fabaceae, Malvaceae, Cannaceae, Geraniaceae,* and *Convolvulaceae* (Baskin *et al.* 2000) and is present in wild ancestors of cultivated legumes (Dueberrn de Sousa and Marcos-Filho 2001; Zohary and Hopf 2012; Abbo *et al.* 2014; Smýkal *et al*. 2014). **Combination dormancy** is found in water impermeable seeds in combination with physiological dormancy (Baskin and Baskin 2004).

3.4. LEGUME SEED ANATOMY

Seed anatomy comprises a seed coat, endosperm and embryo, the seed coat develop solely from maternal tissues while the endosperm and embryo are products of fertilization (Le *et al*. 2007). Seed starts their development with the double fertilization process. In the first stage of the process, the future embryo pattern and symmetry are established (Capron 2008). Accompanied by the development of the endosperm (Olsen

2004). At the following stage, the seed synthesizes the necessary nutrient storage pool and acquire the potency to germinate. The number of ovule integuments varies depending on the species; legumes have two integuments (Bitegmic ovules). The inner integument largely vanishes during development (Esau 1965) while the outer one produces several distinct cell layers and establishes the "typical" seed coat structure. The chalazal region is an important part of the testa where connections of the vascular tissues of the maternal funiculus terminate. The scar where the funiculus was attached is called hilum. Current seed identification criteria are based upon morphological including characteristics seed size, general shape, surface shape, color, pattern, hilum length and width. These are often used in taxonomical classifications (Lestern and Gunn 1981; Chernoff *et al*. 1992; Günes 2013) and archeobotany (Zohary *et al*. 2012). Legume seed characters support the concept of one family (Fabaceae) as advocated already by de Candolle (1825). Although the seed coats of different species vary greatly in structure and composition, they undergo similar phases of development in relation to the embryo and endosperm (Butler 1988). In legumes, the seed coat and endosperm develop first, followed by development of the embryo (Weber *et al*. 2005). In spite of some known exceptions such as peanut (*Arachis hypogaea*) with lignified pod or *Archidendron* and *Pithecellobium*, which have a partly pulpy and edible testa (Gunn 1981), there is a rather common blueprint of seed coat structure for the Fabaceae family (Lush and Evans 1980). Interspecific variation comprises mainly the patterns of differentiation, dimensions, and modifications of cell walls of individual layers. Their shapes determine the structure of the seed surface (Güneÿ 2013).

The seed coat surrounds the embryo and endosperm, performs a protective function against physical and ultraviolet damage by light and in some seeds it helps in propagation and in the area of germination control. They consist of four layers from the surface, they are the waxy cuticle, epidermis, hypodermis and internal parenchyma (see **Figure 2**). The outer cell walls are usually covered with waxy cuticle, are irregularly thickened and are called end caps.

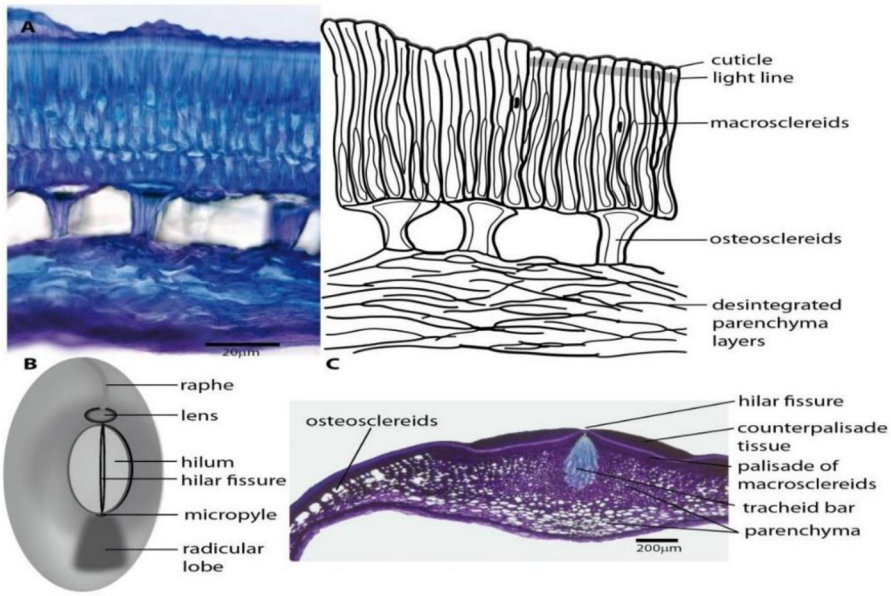


Figure 2: Seed structure of legumes (Fabaceae) (adapted from Smýkal *et al.* 2014). (A) Transversal section of the seed coat of wild Pisum sativum subsp. elatius (left), with a schematic drawing (right), (B) Generalized scheme of the seed coat morphology commonly found in Fabaceae seeds showing the most important structural features, including the hilum, lens differentiated on the raphe and micropylar pore. (C) Transversal section of a Pisum sativum seed coat in the area of the hilum. The macrosclereids of the hilar scar are covered with counter palisade tissue with a central fissure above the tracheid bar, which is surrounded by star-shaped parenchyma interconnected to intercellular spaces of a layer of osteosclereids.

3.5. SEED DORMANCY RELATED GENES IN LEGUMES

Physical seed dormancy is an important trait in legume domestication. Although seed dormancy is beneficial in wild ecosystems, it is generally considered to be an undesirable trait in crops due to reduction in yield and/or quality (Abbo *et al*. 2014). Physical dormancy appears to be the most prevalent cause of dormancy in legumes, while physiological dormancy is a factor in some legume and non-legume species (Martin 1946). While the underlying genetic mechanism is largely unknown, changes in the level of dormancy are controlled by a few loci in some legume species (Koinange *et al*. 1996;

Isemura *et al*. 2012). From a physiological perspective, impermeable seeds often have a hard, pertinacious outer layer of palisade cells (Werker *et al*. 1979) or higher lignin content in the seed coat (Kannenberg and Allard 1964). Seed impermeability can also be associated with fatty acid composition of the cutin layer of the seed coat (Shao *et al*. 2007). Saio (1976) also states that insemination impermeable seeds contain a large amount of calcium compared to permeable seeds.

3.5.1. CALCINEURIN GENES

Calcineurin (CaN) is a protein phosphatase 2B (PP2B), a serine/threonine phosphatase under the control of Ca^{2+}/calmodulin (Klee *et al.* 1979; Stewart *et al.* 1982; Klee *et al.* 1998). Although CaN is a member of a family of protein phosphatases, it is structurally and functionally distinct from alkaline and acid phosphatases (Cohen 1989; Guerini and Klee, 1991). Calcineurin is a heterodimer of a ~60-kDa catalytic subunit, calcineurin A (CNA) and a 19-kDa regulatory subunit, calcineurin B (CNB; Klee *et al.* 1988), and the two-subunit structure is well conserved from yeast to human. CaN is abundantly expressed in the animal brain and broadly distributed in nonneural tissues as well (Kincaid 1993). Among its several functions in controlling intracellular Ca^{2+} signaling, CaN participates in gene regulation and external signal-mediated biological responses in many organisms and in many cell types (Crabtree 1999).

CaN functions have been extensively studied in the yeast (*Saccharomyces cerevisiae*). To investigate the biological role of CaN, immunosuppressant drugs Cyclosporin A (CsA) and FK506 were used to inhibit CaN function (Cyert 1993). Calcineurin has been shown to regulate Ca^{2+} pumps and exchangers to maintain Ca^{2+} homeostasis (Stark 1996). Calcineurin is also known to regulate adaptation to high salt stress (Hirata *et al.* 1995). However, in higher animals, it is known to regulate the transcription of the T-cell growth factor, interleukin-2 (Schreiber and Crabtree 1992). Dephosphorylation of the transcription factor NF-ATp (nuclear factor–activated T cells) by CaN is required for the translocation of NF-AT from the cytoplasm to the nucleus, in response to an increased intracellular Ca^{2+} level. Calcineurin also plays a role in programmed cell death (Shibasaki and McKeon 1995) and in hippocampal long-term depression (Mulkey *et al.* 1994). Furthermore, studies

reveal that CaN play a critical role in the pathogenesis of hypertrophic cardiomyopathy (Molkentin 1998). Thus, calcineurin as a key signaling molecule has been shown to be involved in diverse types of physiological processes.

Caenorhabditis elegans has been an ideal model to study gene functions especially at the organism level using a genetic approach. Study conducted on this organism, identified and characterized the *C. elegans* homologue of calcineurin B, which binds calcium and functions together with calcineurin A as a heterodimeric protein phosphatase. Null mutants of calcineurin B showed multiple adverse phenotypes including defects in locomotion and egg laying. Interestingly, these phenotypes are quite similar to those observed in gain of function mutants of *unc-43*, which encodes the Ca^{2+}-calmodulin–dependent protein kinase CaMKII. Recently, a G-protein signaling pathway regulated by *unc-43* has been found to be involved in locomotory and egg-laying functions (Robatzek and Thomas 2000). Results describe the relationship between the *cnb-1* null mutant and mutants of *unc-43* indicating that calcineurin and CaMKII may have opposing and complementary functions in this G-protein signaling pathway in *C. elegans.*

3.5.2. CALCINEURIN B-LIKE (CBLs) GENES IN PLANTS

In plant, calcineurin B-like protein(CBL) family represents a unique group of calcium sensors and play a key role in decoding calcium transients by specifically interacting with and regulating a family of protein kinases (CIPKs). Calcium (Ca2+) is an important inorganic nutritive element and a ubiquitous second messenger (Xiong *et al*. 2002). Ca2+ not only plays a vital role in maintaining the stability of the cell wall, cell membrane, and membrane binding proteins, but also is widely involved in the regulation and control of plant growth and development, as well as response to external environmental stimuli (Hepler 2005). In plants, intracellular Ca2+ sensors accurately recognize specific Ca2+ signatures that are generated in response to different external stimuli (Allen *et al*. 2000). Under adverse conditions, cell signal transduction receptors on the plant cell membrane recognize the stimulus signal, and then activate the Ca2+ channel protein through phosphorylation, thereby leading to an instantaneous increased in the Ca2+ concentration in the cytoplasm, which produces the “Ca2+ signal” (Chinnusamy *et al*. 2004). Ca2+ sensors in plants detect

this stress-induced Ca2+ signal and deliver it to downstream effectors, activating a signal cascade reaction that regulate resistance and tolerance (Reddy and Reddy 2004). Ca2+ sensors can be divided into two main types according to their structural features. One type is the sensor responders, including calcium-dependent protein kinases and calmodulins, which have all the functions of Ca2+ sensor relay proteins, as well as the kinase activity (Xi *et al*. 2017). The other type is the sensor relays, including calmodulin-like proteins and CBL, which do not have kinase activity. Sensor relays can specifically target downstream proteins to transfer the perceived Ca2+ signals in response to various environmental stimuli (Xi *et al*. 2017). CBL, a Ca2+ sensor relay protein that is expressed in a wide range of plants, can interact with a family of serine-threonine protein kinases known as CBL-interacting protein kinases (CIPKs) (Shi *et al*. 1999). CBLs were initially identified in *Arabidopsis thaliana* and are closely related to both the regulatory B subunit of calcineurin and neuronal calcium sensors in animals (Kudla *et al*. 1999). CBLs have been found in many terrestrial plants, such as mosses, ferns, gymnosperms, monocots, and dicots (Today-Kennedy *et al*. 2015). CBLs are a multigene family. The *A*. *thaliana*, *Oryza sativa*, and *Populus trichocarpa* genomes are each predicted to contain approximately 10 CBL family members (Batistič and Kudla 2009; Kolukisaoglu *et al*. 2004; Zhang *et al*. 2008). Eight CBL genes have been identifed in Sorghum biocolor (Li *et al*. 2010; Zhang *et al*. 2011). In addition, CBL genes have been investigated in *Brassica napus* (Zhang *et al*. 2014), *Solanum melongena* (Li *et al*. 2016) and other plant species (Kabir and Wang 2010; Mahajan *et al*. 2010). CBL proteins contain a classical EF-hand helix-loop-helix motif with a 12-residue loop (Lewit-Bentley and Réty 2000). Different CBL proteins have different degrees of variation in the EF-hand structure, but the number of EF-type regions and the distance between them is the same in all known CBL proteins (Kolukisaoglu *et al*. 2004). The function of CBL genes has been studied in *A*. *thaliana*, *O*. *sativa*, and other plant species. In *A*. *thaliana*, AtCBLs play a role in the response to multiple abiotic stresses (Yong *et al*. 2003, 2010; Pandey *et al*. 2004). For instance, AtCBL1 functions as a positive regulator in response to salt and drought but as a negative regulator in response to cold (Yong *et* al. 2003). Abscisic acid (ABA) is a signaling molecule that plays a role in the plant response to aging and stress (Shen *et al.* 2014). AtCBL9 is a common element in the ABA signaling and stress-induced ABA biosynthesis pathways (Pandey *et al*. 2004). Ten OsCBL genes in rice are expressed in various organs at the adult stage and have also been found to respond to different stress conditions [sodium chloride (NaCl),

polyethylene glycol (PEG) and cold] (Gu *et al*. 2008). In addition, OsCBL8 overexpressing transgenic rice seedlings showed more tolerance to salt stress than non-transgenic seedlings (Gu *et al*. 2008). S. bicolor CBL genes are thought to regulate sodium carbonate stress-specific cellular adaptation responses and influence the plant growth and developmental patterns (Zhang *et al*. 2011). Analysis of CBL transcripts in *Populus euphratica* under abiotic stress suggested that seven CBL (PeCBL1, 2, 3, 4, 5, 9, and 10) members may play important roles in responding to specific external stimuli (Zhang *et al*. 2008).

Sugarcane (*Saccharum spp*.) is an economically attractive polyploid C4 grass that is used not only to produce approximately 60% of the world's sugar but also to produce ethanol, a low-carbon-emission fuel (D'Hont *et al*. 2008). To date, there have been few reports on CBL genes in sugarcane (Ling *et al*. 2018; Farani *et al*. 2015; Zhang 2013). Zhang (2013) cloned five CBL genes and found that CBL5 and CBL6 may play key roles in adaptation to low temperatures (Zhang 2013). Using real-time quantitative polymerase chain reaction (qRT-PCR) analyses, Ling *et al*. (2018) found that SsCBL1 and SsCBL6 play important regulatory roles in response to a variety of stresses (low potassium, drought, and salt). Yeast two-hybrid assays showed that ScCIPK8 interacts with ScCBL1, ScCBL3, and ScCBL6 (Farani *et al*. 2015).

3.5.3. SOYBEAN GmHs1-1 GENE INVOLVED IN SEED COAT PERMEABILITY

Leguminous seed coat impermeability such as in wild soybean (*Glycine soja*) is controlled by a single gene *GmHs1-1*, which encodes a **calcineurin like metallophosphoesterase transmembrane protein** (Foley *et al*. 2001). *GmHs1-1* is expressed in the maphighian layer of the seed coat and it's associated with calcium content of soybeans, offering a genetic target for enhancing the nutrition of soy food products (Sun *et al*. 2015). The transition from impermeability to permeability in domesticated soybean was caused by artificial selection of a point mutation in *GmHs1-1.*

In a study conducted by Jianxin Ma, and fellow researchers found that a mutation in the gene *GmHs1-1* causes the tough seed coats of wild soybeans to become permeable (Sun *et al*. 2015; Liu *et al.* 2007; Zhang *et al*. 2008). Previous studies mapped a common quantitative trait locus (QTL) underlying hard-seededness to an overlapping region on

soybean chromosome 2 (Liu *et al*. 2007; Zhang *et al.* 2008; Watanabe *et al.* 2004). To understand the molecular basis of hard-seededness, they crossed the permeable soybean cultivar Williams 82 with each of two hard-seeded wild soybean *G. soja* accessions, PI 468916 and PI 479752, and obtained two F2 populations. The F1:2 seeds, whose coats developed from the maternal tissues of the F1 plants, were hard-seeded and phenotyping of F2:3 seeds from individual F2 plants from the two populations revealed 3:1 ratios of hard-seededness to permeability, suggesting that the former is dominant over the latter, and showed that the trait was controlled mainly by a single locus, designated *GmHs1-1.* An initial scan revealed a linkage between *GmHs1-1* and the markers defining the common QTL region on chromosome 2, suggesting that the hard-seededness investigated in this and previous studies is likely to be controlled by the same locus. Finally to fine map *GmHs1-1* to a 22-kb region harboring two genes, Glyma02g43700.1 and Glyma02g43710.1, according to the Williams 82 reference genome (Schmutz *et al*. 2010), they sequenced Glyma02g43700.1 and Glyma02g43710.1 genes in the *G. soja* parents. Seven polymorphic sites that each resulted in amino acid changes between PI 468916 and Williams 82 were detected in Glyma02g43700.1. Only one of the seven polymorphisms, a (C>T) point mutation in Glyma02g43700.1 was detected as a difference between PI 479752 and Williams 82. This C>T mutation, which resulted in a change from threonine to methionine, is also the only mutation in this gene, including in its introns and exons and its flanking ~2.5-kb and ~1.5-kb regions, that could be used to distinguish Williams 82 from the two *G*. *soja* parents and eight additional *G. soja* accessions previously sequenced (Kim *et al*. 2010; Li *et al*. 2014), among which 88 variant sites were found. By contrast, no nucleotide differences associated with amino acid changes were observed between the *G. soja* parents and Williams 82 in Glyma02g43710.1. Glyma02g43700.1 was primarily expressed. These observations suggest that Glyma02g43700.1 is most likely to be the *GmHs1-1* locus. Orthologs or homologs of Glyma02g43700.1 have been found in many other plants, but none of them has been shown to have any known functions. Nevertheless, Glyma02g43700.1 was predicted to encode a calcineurin-like metallophosphoesterase transmembrane protein localized to cellular membranes. The amino acid switch resulting from the C>T mutation was predicted to be located outside of membranes and to affect the α-helix of the protein structure. The transcripts of Glyma02g43700.1 were predominantly abundant in the Malpighian layer of the seed coat, particularly in the lucent region of Malpighian cell walls separating

Malpighian terminal caps from their basal parts (Lush and Evan 1980; Miller 1999). This so-called light line is thought to be essential for hard-seededness (Harris 1987).

3.5.4. ENDO-1,4-B-GLUCANASE

Endo-1,4-β-glucanase was also predicted to be responsible for seed dormancy in soybean, it makes seed coat more adaptable to dehydration stress during maturation by accumulating β-1,4-glucan derivatives on the outer layer of palisade cells and reinforcing the seed coat impermeability in soybean (Jang *et al*. 2015; Sun *et al*. 2015). The endo-1,4-β-glucanases belong to glycosyl hydrolase family 9 (GH9) (Cantarel *et al*. 2009). It possess a broad substrate specificity, for example, OsCe19 hydrolyzed 1,4-β-glycosyl linkages of carboxymethyl cellulose, phosphoric acid-swollen cellulose, β-(1,3),(1,4)-glucans, arabinoxylan, xylans, glucomannan, and cello oligosaccharides (Yosida and Komae 2006). Fine-mapping and subsequent expression and sequencing analyses revealed that *qHS1,* a quantitative trait locus for hardseedness in soybean, encodes an endo-1,4-β-glucanase (Jang *et al*. 2015). A single-nucleotide polymorphism (SNP) introduced an amino acid substitution in a substrate-binding cleft of the enzyme, possibly reducing or eliminating its affinity for substrates in permeable cultivars. *qHS1* is involved in the accumulation of β-1,4-glucan derivatives such as xyloglucan and/or β-(1,3) (1,4)-glucan that reinforce the impermeability of seed coats in soybean.

Endo-1,4-β-D-glucanases exist in many different organisms including bacteria, fungi, termites, and plants (Libertini *et al*. 2004), and are involved in the degradation of cellulose to its monomeric subunits as a carbon source. Generally, these endoglucanases contain a cellulose binding domain (CBD) that aids in the degradation of crystalline cellulose. In contrast, most plant endoglucanases lack a CBD, and are therefore thought to act on the para-crystalline (amorphous) regions of cellulose or non-crystalline polysaccharides such as xyloglucan (Wilson and Urbanowicz 2010). Plant endoglucanases belong to glycosyl hydrolase family 9 (GH9) (Cantarel *et al*. 2009), which contains enzymes capable of breaking β-1,4 glycosidic bonds within a glycan chain (Wilson and Urbanowicz 2010). In *Arabidopsis*, there are currently 25 genes coding for endoglucanases, and according to Urbanowicz *et al*. (2007), these endoglucanases can be divided into three structural subclasses: A, B and C.

Given the various chemical properties of the plant endoglucanases, they have been hypothesized to play a variety of roles in cell wall biosynthesis and modification, including cell elongation and differentiation, cytokinesis, organ abscission and fruit ripening (Libertini *et al*. 2004).

For example, the class A endoglucanases is membrane-bound and include a long N-terminal extension, linked to a catalytic domain. In *Arabidopsis*, there are three genes that code for membrane-bound endoglucanases, termed *KORRIGAN (KOR) KOR2* and *KOR3* (Nicol *et al*. 1998; Molhoj *et al*. 2001). There is evidence of *KOR* playing a role in both primary (Lane *et al*. 2001) and secondary cell wall development (Szyjanowicz *et al*. 2004). Although the phenotypic effects of *KOR* are unmistakable, the specific role it plays in cell wall biosynthesis is still under debate. It has been suggested that *KOR* is involved in removing a sitosterol-β-glucoside precursor molecule from the elongating cellulose chain (Peng *et al*. 2002). However, several studies have reported results to the contrary (as discussed in Maloney and Mansfield 2010).

Class B endoglucanases are predicted to be secreted enzymes, and consist of a signal peptide, followed by a catalytic domain. These enzymes make up the bulk of the Arabidopsis GH9 family with 19 members, and have been linked to a variety of different developmental roles. One such endoglucanase, *CEL1* (At1g70710), was found to be highly expressed in young developing tissues, particularly in thickening cell walls of xylem (Shani *et al*. 2006). Additionally, Tsabary *et al*. (2003) showed that *cel1* antisense plants had mechanically weaker stems and a significant decrease in cellulose and lignin content. Research examining *Eucalyptus globulus* secondary growth has provided evidence of the up regulation of *EG1* (*Arabidopsis* ortholog At2g32990) and *EG3* (*Arabidopsis* ortholog At4g39010) in the phloem of juvenile wood (Goulao *et al.* 2011). Furthermore, several microarray studies have alluded to the potential roles for Class B endoglucanases, including the up regulation of At1g22880, At2g44570, and At2g32990 in response to IAA application (Goda *et al*. 2004) or the high level of expression of At4g39000 in stamen, pistil, seeds, and ovules (Xie *et al*. 2011).

Finally, class C endoglucanases are structurally similar to class B endoglucanases, but also contain a carbohydrate binding module (CBM) at their C-terminus. This CBM is unique

to plants and has been classified as CBM49 (Urbanowicz *et al*. 2007). Many CBMs are hypothesized to facilitate the binding of these enzymes to crystalline cellulose, but it has yet to be shown what role, if any, the plant CBM49 plays in cell wall synthesis (Shoseyov *et al*. 2006). The three members of class C have not been well studied in *Arabidopsis*, however, the tomato and rice orthologs of At1g64390 (AtGH9C2) have been examined. For example, Urbanowicz *et al*. (2007) provided evidence for the binding of the tomato SlCel9C1 CBM to crystalline cellulose, as well as hydrolysis of artificial cellulosic polymers, and a variety of plant cell wall polysaccharides by the catalytic domain. A similar study was performed on the orthologous rice endoglucanase, further confirming that the catalytic domain is capable of hydrolyzing a suite of polysaccharides. However, contrary to the tomato study, the authors provided evidence that the CBM is post-transnationally cleaved (Yoshida and Komae 2006b). Therefore, the role of CBM49 is still uncertain, and the other two members of class C do not provide any clues because they remain largely unstudied.

3.5.5. POLYGALACTURONASE GENE (PG031)

Polygalacturonase (PG) belongs to a large family of hydrolases that can degrade pectin during plant growth and development (Hadfeld and Bennett 1998; Markovic and Janecek 2001; Xiao *et al*. 2014). Silencing PG homologs decreased intracellular spaces with more cellular adhesion, leading to increased storage-life of ripe tomato or firmness of ripe apple and strawberry (Smith *et al*. 1988, 1990). In contrast, overexpression of PGs reduced cell adhesion and pectin content in rice and apple (Atkinson *et al*. 2002; Liu *et al*. 2014). Polygalacturonase (PG) gene, *PG031* controls seed coat permeability in soybean, it implements the function likely through the reduction of cell adhesion in the cell wall of the seed coat tissue by reducing the level of pectin. As such, increased cell separation permits penetration of more water through the multiple layers of cells in the seed coat. *PG031* exhibited tissue expression specificity in flowers while it was strongly induced in the seed coat and radical upon imbibition (Wang *et al*. 2016). Subcellular localization localized *PG031* to the cell wall, suggesting its role specific to the cell wall of the seed coat. Natural variation analysis reveals three haplotypes ($PG031^{289H}$, $PG031^{289Y}$ and $PG031^{Hap3}$) (Zhou *et al*. 2015) and the single nucleotide polymorphism (SNP) variation for H289Y may explain the variation

in permeability in cultivated soybean population. *PG031* was also located within a quantitative trait locus (QTL) (Chandra *et al*. 2020) explaining ~ 15% of total phenotypic variation in permeability, nominating it as the QTL gene controlling permeability.

3.5.6. PECTIN ACETYLESTERACE

Pectin acetylesterase 8 (**PAE8**) has been identified in common bean (*Phaseolus vulgaris* L) where it influences pectin acetylation in the seed coat and subsequently impacts seed imbibition and dormancy. Pectin is a major component of soluble dietary fiber in legumes, which is abundant in the seed coat. Pectin substances are a constituent of the cell wall representing a mixture of heterogeneous, highly branched and hydrated polysaccharides rich in D-galacturonic acid (Carpita *et al*. 2015). They are present in the middle lamella adjacent to the primary walls of neighboring cells. In the seed coat of common bean, pectin is found in the palisade layer, where it forms an impermeable barrier to seed hydration (Esau 1977). Pectin is synthesized as a highly esterified polymer in the Golgi apparatus, which increases its solubility. O-Acetylation occurs primarily at the C-2 and C-3 positions of D-galacturonic acid in homogalacturonan. Upon secretion, it can be de-esterified by methylesterases and acetylesterases. De-esterification favors interactions with cations like calcium and boron, which is associated with water impermeability.

There is strong experimental evidence linking pectin with an important environmentally induced negative attribute of common bean called the hard-to-cook defect. The “phytase-phytate- pectin” hypothesis explains this defect by stating that seeds stored at high temperature and humidity have increased phytase activity, leading to the release of Ca^{2+} ions which bind carboxyl groups in pectin, forming insoluble pectate complexes in the middle lamella of the cell wall, thus interfering with seed water uptake (Galiotou-Panayotou *et al*. 2008). Beans carrying the *low phytic acid 1* (*lpa1*) mutation display an approximately 90% reduction in phytate levels (Panzeri *et al*. 2011). This mutation was originally selected to increase the bioavailability of iron. The presence of the *lpa1* allele increased cooking time in a series of different genetic background (Cominelli *et al*. 2020). In combination, the *lpa1* allele and the lectin leucoagglutining phytohemagglutinin (PHA-L) resulted in decreased lectin hydrolysis during cooking as compared to wild-type. This reduced lectin digestibility

was associated with an increased seed Ca^{2+} concentration, and enhanced localization of Ca^{2+} to cell walls. These results strongly suggest that the increased cooking time conferred by *lpa1* was due to increased interaction between pectin and Ca^{2+}.

3.5.7. KNOX4-LIKE GENE

KNOX4 belongs to the class II *KNOX* family of transcription factors in plants. Different from class I *KNOX* genes, which are well known for their roles in maintenance of shoot apical meristem (Zhou *et al*. 2014; Hake *et al*. 2002, 2004; Hay and Tsiantis 2010), class II *KNOX* genes show diverse expression patterns and few known functions (Liu *et al*. 2014; Gao *et al*. 2015). *KNOX4* controls physical dormancy in (*Medicago truncatula G*.)**.** The seed of barrel medic *M. truncatula*, a legume plant belong to a classic hardseededness type and possess strict physical dormancy and do not germinate unless the dormancy is disrupted by scarification. This together with available genome and mutant resources makes *M. truncatula* an ideal model to study seed physical dormancy (Tadege *et al*. 2008). Class 11 *KNOX* (KNOTTED- like homeobox) transcription factor, *KNOX4*. *KNOX4* is a key regulator for seed coat development and controls seed physical dormancy by affecting downstream cuticle genes (Chai *et al*. 2016). Loss of function of *KNOX4* resulted in abnormal cuticle composition that led to altered seed coat structure and permeability, *KNOX4* caused dysfunctional palisade cuticles in the mutant seeds, allowing the seeds to absorb water easily (Zhou *et al*. 2014). *KNOX4*-like genes exist widely in seed plants but are lacking in nonseed species, indicating that *KNOX4* may have diverged from the other *KNOXII* genes during the evolution of seed plants (Chai *et al*. 2016).

Microarray and ChIP-PCR analysis identified *CYP86A* as one of the downstream target genes of *KNOX4* (Chai *et al*. 2016). *CYP86A* is a cytochrome P450 mono-oxygenase and is associated with extracellular lipid polyester biosynthesis (Gallardo *et al*. 2007). The *CYP86A* family plays an important role in the cuticle biosynthetic pathway and the *CYP86A* is critical for seed coat permeability in *M*. *truncatula* (Quinlivan 1961).

4. MATERIAL AND METHODS

4.1. PLANT MATERIAL

Two parental genotypes included wild *P. elatius* JI64 from Turkey and cultivated Afghan land races *P. sativum* JI92 both from the John Innes Pisum Collection (Norwich, UK). Furthermore, 130 recombinant inbred lines (RILs) of F_6 generation derived from JI64 and JI92 cross (North *et al*. 1989). Seeds of two parental and 130 recombinant inbred lines (RILs) were harvested from greenhouse-grown plants (period of spring, 2022). Seeds from each recombinant inbred line were placed one by one in pots filled with seedling substrate (Profi seeding substrate, Florcom, Czech Republic). Experimental plants were grown in the grounds of the Department of Botany, Faculty of Science, Palacky University in Olomouc, in greenhouse conditions in the period January - June 2022. Upon harvest the pods were left to dry at room temperature in paper bags and subsequently seeds were cleaned manually. Cleaned seeds were stored in paper bags at room temperature in dark until testing.

4.2. SEED DORMANCY TESTING

4.2.1. SEED WATER UPTAKE AND GERMINATION ASSAYS

To test the dormancy level, the harvested dry seeds were used within 3 months since harvest. Twenty five seeds per line except line A59 (having 19 seeds) 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 seeds coat.

4.2.2. METHOD

1. Filter papers (Whatmann, Grade 1) and 25 seeds each of parents and RILs were placed in Petri dishes.

2. Each plate was described.
3. The seeds were covered with 3 ml of water and incubated at 25°C.
4. Dormant seeds were recorded at 24-hour intervals for one week.
5. Seeds that did not germinate after seven days were scarified with sandpaper to mechanically disrupt the seed for better water permeability and subsequent germination.

4.3. DNA ISOLATION AND ANALYSIS

Leaf samples were taken from all plants, from which genomic DNA was isolated, necessary for subsequent molecular analyses. Young fresh leaves were either directly processed or kept frozen at -8 ^{0}C until isolation.

Genomic DNA from parents and RILs was isolated from two young leaves each time, i.e. approximately 100 mg of plant material using a commercially available kit (Invisorb Spin Plant Mini Kit) using silicate adsorption. After isolation, the DNA samples were stored for a long time at -20 ^{0}C.

However in my work analysis, the DNA samples were provided to me.

Procedure according to the manufacturer's instructions (Invisorb Spin Plant Mini Kit, STRATEC Molecular GmbH, Germany).

1. All tubes were labeled with a description.
2. 400 µl of lysis was added to the sheet Buffer P and 20 µl Proteinase K (100 µg/ml).
3. The samples were homogenized for 60 seconds.
4. The samples were incubated for 30 min at 65°C.
5. The homogenate was poured onto the PREFILTER column.
6. The tubes were centrifuged for 1 min at 12,000 rpm.
7. 10 µl RNAase A (10 µg/ml) was added to the tubes and left at room temperature for 10-15 min.
8. 200 µl Binding was added to the tubes Buffer P and was vortexed.
9. The samples were poured onto the SPIN FILTER column and left to stand for 1-2 min.
10. The tubes were centrifuged for 1 min at 12,000 rpm.
11. The filtrate was removed, the columns were returned, and the columns were pipetted 550 µl Wash Buffer I.

12. The tubes were centrifuged for 1 min at 12,000 rpm.
13. The filtrate was removed; columns were returned and pipetted 550 µl Wash Buffer II.
14. Step 12 and 13 were repeated.
15. The tubes were centrifuged for 1 min at 12,000 rpm.
16. The filtrate was removed, the columns were returned, and the tubes were centrifuged for 2 min at 12,000 rpm.
17. 50 µl was pipetted into the test tubes Elution Buffer D preheated to 65 °C and the samples were incubated for 3 min.
18. The tubes were centrifuged for 1 min at 12,000 rpm.
19. Steps 17 and 18 were repeated.

4.3.1. SPECTROPHOTOMETRIC ANALYSIS OF DNA (NANODROP)

The concentration of isolated genomic DNA was determined spectrophotometrically on the NanoDrop 2000 (Thermo Fischer Scientific, USA.). The results were obtained from the ratio of DNA absorbance at wavelengths of 260 and 280 nm, indicating the purity of preparation.

1. 2 µl of distilled water was pipetted onto the NanoDrop to read the blank.
2. Subsequently, 2 µl of the DNA sample was pipetted and the absorbance values at wavelengths of 260 nm and 280 nm, the value of 260/280 nm and the concentration in ng/µl were measured.
3. After each measurement, the sensor of the device was cleaned with a cotton swab.

4.4. PCR AMPLIFICATION

Two candidate genes were selected for polymerase chain reactions (PCR) amplification: Psat2g013240, Psat2g024960, based on the reference pea genome Cameor v1.0 (Kreplak *et al*. 2019 https://urgi.versailles.inra.fr/Species/Pisum/Pea-Genome-project). For PCR amplification was designed primers using program FastPCR (Primer Digital Ltd., Helsinki, Finland) and were synthesized at Generi

Biotech (Hradec Králové, Czech Republic). Primers were set to preferentially start at conserved exons and to span intron region with expected higher amount of nucleotide polymorphism (**Table 1)**.

Table 1: Primers used

Gene	Forward/Reverse position of the primer	Primer combination	Sequence (3'-5')	Product length [bp]
Psat2g013240	F115	A	AGATCGGAAGTGGAAGTACTTTGT	945
	R1060		CATGATTTCCTTGCGTTACCATCC	
Psat2g013240	F980	B	AACATCTTTGGGACTCGTTTGGTA	1451
	R2431		TGTATTTGTATGGCGTGTCATACTC	
Psat2g024960	F25	C	GGACAAGGGAGTAACTGTTAGTGT	960
	R985		GTCGTAGTTTAACTGTGCCAACAC	
Psat2g024960	F961	D	TGTGTTGGCACAGTTAAACTACGA	1196
	R2157		CCAAAATGGTTCCGTCACTTCCTA	
Psat2g024960	F1867	E	AAGATGTCAAGGTATTGACCGAGT	1949
	R3816		GAAGGTTTGCTGCCTGGTATCAT	

4.4.1. PCR AMPLIFICATION METHOD

1. The work was performed in a sterile flow box.
2. DNA samples were diluted 1:7 with water to have an approximate concentration of 20-80 ng/µl.
3. PCR chemicals were placed in a cooling rack after thawing.

4. The necessary amount of reaction mixture without DNA sample was prepared in a 2 ml microtube according to the number of samples (for one sample: 4 µl 5x buffer, 13 µl distilled water, 1 µl primer mix (5 mM F/R), 0.1 µl My*Taq* Red polymerase (5 units / µl)
5. 18µl of the reaction mixture was pipetted into marked 0.2 ml tube strips or wells of a 96-well plate.
6. 2 µl of DNA sample was pipetted into 18 µl of the reaction mixture.
7. The 96-well plate or tube strips were loaded into the PCR thermocycler and the CAPS_55 program was run (**Table 2**).

Table 2: PCR program profile

Step	Temperature	Time	Cycle
Initial denaturation	95^0C	5 min.	1 time
Denaturation	95 ^{0}C	30 sec.	35 times
Annealing	55 ^{0}C	45 sec.	
Extension	72 ^{0}C	45 sec.	
Final extension	72 ^{0}C	5 min.	1 time
Final hold	4 ^{0}C	Forever	

4.4.2. GEL ELECTROPHORESIS

4.4.3. HORIZONTAL AGAROSE ELECTROPHORESIS OF DNA, PCR PRODUCTS AND RESTRICTION DIGESTION PRODUCTS

Agarose gel was used to check the integrity of the obtained genomic DNA, to verify the correctness of the subsequent polymerase chain reactions (PCR) and to visualize the results of restriction digestion. Gels with electrophoresis results were evaluated using a UV transilluminator (FireReader, Uvitec Cambridge) at a wavelength of 302 nm and documented using a digital

camera with an orange filter (EDAS 290, Kodak). The images were computer processed in the graphics program IrfanView.

4.4.4. METHOD

1. Depending on the size of the electrophoretic bath (80 ml or 250 ml), in this work, 80 ml was used.
2. 1.2 g of agarose was weighed into a 1.5% agarose solution in a 500 ml Erlenmayer flask.
3. 80 ml of 0.5 x TBE buffer was poured into the flask and everything was microwaved.
4. The bottle was removed from the microwave, mixed and returned until the agarose was completely dissolved.
5. After cooling to about 50 - 60 ^{0}C, 1.5 µl of EurX dye stock solution was added and the agarose was poured into the gel electrophoresis mold.
6. Combs were placed in the gel electrophoresis mold and the gel was allowed to solidify for 30 min.
7. After solidification, the combs were removed and the gel was placed in an electrophoresis bath with 0.5 x TBE buffer.
8. 10 µl of PCR or genomic DNA samples and 5 µl marker were pipetted into the wells.
9. Electrophoresis was run for 45-60 min at 100 V.
10. The gel was then documented using a UV transilluminator (wavelength 302 nm) and a digital camera with an orange filter.
11. The resulting image was processed on a PC in the graphics program IrfanView.

4.5. SEQUENCING ANALYSIS

To check the PCR products and to identify the polymorphism needed for RIL mapping, the Sanger sequencing was performed. Prior to sequencing, residual primers were removed according to the Alkaline Phosphatase-Exonuclease I (AP-EXO) protocol using exonuclease I, which cleaves single-stranded primers, and alkaline phosphatase, which dephosphorylates

residual dNTPs. Before sending the sample for sequencing, the presence of the insert was verified by PCR amplification and horizontal gel electrophoresis.

4.5.1. CLEANING PROTOCOL OF PCR PRODUCTS FOR SEQUENCING ANALYSIS

1. The necessary amount of reaction mixture without PCR product was prepared in a 2 ml microtube according to the number of samples (for one sample: 1 µl of 10x Fast AP buffer, 3.45 µl of distilled water, 0.15 µl of AP phosphatase, 0.4 µl Exo I).
2. 5 µl of the reaction mixture was pipetted into marked 0.2 ml tube strips or wells of a 96-well plate.
3. 5 µl of PCR product was pipetted to 5 µl of the reaction mixture.
4. The 96-well plate or tube strips were loaded into the PCR thermocycler and the Exo -AP program was run (**Table 3**).

Table 3: Exo -AP PCR temperature program for PCR product purification

Step	Temperature	Time
Incubation	37 ^{0}C	15 min.
Thermal inactivation	85 ^{0}C	15 min.

4.5.2. SEQUENCING, FINDING POLYMORPHIC RESTRICTION SITES

Sequencing was carried out as a service in the Laboratory of DNA Analysis at the Faculty of Science, Charles University in Prague (https://www.natur.cuni.cz/biologie/servisni-laboratore/laborator-sekvenace-dna), where a purified PCR reaction (approximately 100 - 200 ng DNA) together with the corresponding sequencing primer (5 pmol) in a total volume of 8 µl (4 µl PCR product, 1 µl F primer and 3 µl distilled water). Sequencing was performed using the BigDye ® Terminator v3.1 Cycle method Sequencing Kit (Applied Biosystems, UK) according to the protocol on the 3130xl Genetic sequencer Analyzer (2010) by Applied Biosystems. Obtained

reads were processed using Geneious 7.2 software. Sequences were edited and alignment to Cameor reference sequence to check correct amplification, thereafter the polymorphism between JI92 and JI64 parental genotypes were search using command “find restriction sites”.

4.5.3. RESTRICTION ANALYSIS

4.5.4. RESTRICTION OF THE PCR PRODUCT (CAPS-PCR, CLEAVAGE AMPLIFIED POLYMORPHIC SEQUENCE)

Table 4 lists the restriction enzymes (Biogen, CZ) used in this work. The restriction cleavage products were electrophoretically separated, the gels with the results of the separation properly documented.

Table 4: Restriction enzymes with buffers used and digestion temperatures

Gene	Primer combination	Restriction enzyme	Buffer	Cleavage temperature
	A or B	*Taq1*	Taq buffer	65 °C
Psat2g024960	D	*BsuR1/Hae111*	R buffer	37°C

4.5.5. METHOD

1. The restriction chemicals were placed in a cooling rack after thawing.
2. The necessary amount of reaction mixture without PCR product was prepared in a 2 ml microtube according to the number of samples (for one sample: 1.5 µl of 10 x buffer, 8.4 µl of distilled water, 0.1 µl of restriction enzyme).
3. 10 µl of the reaction mixture was pipetted into the marked tube strips or wells of the plate.
4. 5 µl of PCR product was pipetted into 10 µl of the reaction mixture.

A 96-well plate or tube strips were placed for 2-3 hours in a PCR thermocycler or incubator with the required cleavage temperature according to the enzyme (**Table 4**).

4.6. ANATOMICAL ANALYSIS – MICROSCOPY

4.6.1. SEED COAT THICKNESS MEASUREMENT

Seed coat thickness of the stripped seeds 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 a cryo-microtome according to Hradilová *et al*. (2017).

4.6.2. PREPARATION OF UNDERLYING GLASSES

1. Glass (Microscope slides, Knittel glass) was washed with a drop of detergent and rinsed with water.
2. Glass was degreased with 96% ethanol for a period of 24 hours and cleaned gauze.
3. The glasses were immersed in water solution of gelatin for 10 sec. Then they were placed on a filter tray paper and left at 40-50 ^{0}C for 12 hours or 24 hours at laboratory temperature.

4.6.3. PREPARATION OF SAMPLES

1. Samples of seeds coat (from parental genotype and RILs) at least five seeds per genotype were dissected from dry seed and saturated with 2% sucrose solution under vacuum. Equal volume of cryo-gel (Shandon Cryomatrix) was added to samples and shake overnight.
2. Saturated samples were mounted into cryo-gel on the alum chuck.
3. Frozen down to -25 ^{0}C and cut in cryotome into 12 µm transversal section (Leica

Bio systems, Nossloch, Germany).

4.6.4. SAMPLE SECTIONING ON CRYOMICROTOME

1. Turned on cryomicrotome at least 1-5 hours before cutting and with targets on sample.
2. Temperature chambers must be chilled on -19 ^{0}C and temperature sample -21 ^{0}C.
3. Placed brushes to chambers and on targets was given basic layer cryogel.
4. Samples were placed on cryogel with tweezers and arrange in layer.
5. Targets with sample were laid on freezing plate in cryotome and then freeze for about 5 minutes.
6. A sample target was clamped to the head of the cryotome. The cryomicrotome cover was closed and sample was trimmed with knives (trim 40 μm).
7. Cutting was adjusted to 20 μm.
8. The sample was moved using a brush. It was necessary to check the orientation of the cut on under lay glass on Olympus BX 51 microscope, (Olympus Corp., Tokyo, Japan).
9. Samples were put together on underlying glass.
10. Used sample on targets was cut down scalpel and was left behind basic layer cryogel.
11. Work was terminated by removing cryogel of targets and by removing leftover cryogel from the chamber. The manifold was pulled from the freezer counter and was covered.
12. Cleaned and properly dried all equipment used and turned off cryomicrotome.

4.6.5. HISTOCHEMICAL STAINING WITH TOLUIDINE BLUE O

1. Dropped 0.01% Toluidine Blue O. on microsections samples on slide.
2. It was necessarily to dye in horizontal position on Petri dish.
3. Dye worked 0.5-1 minutes, after that was rinsed away with water.

4. Poured 20% glycerol on sample and cover with a glass.
5. Dyed preparation was viewed under a 3D digital microscope VHX 7000 (Keyence International, Mechelen, Belgium), and was attached to counting with program on photographing and image editing.

4.7. STATISTICS ANALYSIS OF DATA

Statistical analysis of samples data was done using Rstudio software (Posit team 2022; version 4.2.2) and excel package.

4.8. BIOINFORMATICS ANALYSIS

For sequence alignment and editing GENEIOUS version R7 (Biomatters Ltd.) was used. Sequences were compared to sequences obtained by BLAST (Altschul *et al*. 1997) analysis, which was carried out on the NCBI website: http://www.ncbi.nlm.nih.gov/ to verify the identity of the gene sequence and, above all, between the parental genotypes to find the necessary polymorphism for mapping.

The website (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_multalinan.html) was used to check insertions, sequence alignment and proposed or accepted primers. The primers themselves were designed in the Fast PCR program (Primer Digital, Finland).

4.9. LIST OF USED LABORATORY EQUIPMENT:

Petri dishes (diameter 9 cm), filter paper (Whatman, grade 1), sandpaper, tweezers, 3D digital microscope VHX 7000 (Keyence International, Mechelen, Belgium), Olympus BX 51 microscope (Olympus Corp., Tokyo, Japan), caliper micrometer (0 – 25 mm, accuracy 0.01 mm, Dept of Biophysics, UP vortex (MS2 Minishaker, IKA), thermoblock (Thermo Block MB - 102, BIOER.), centrifuge (Centrifuge 5415 R, Eppendorf; MPS 1000 Mini PCR Plate Spinner, Labnet), minicentrifuge (Spectrafuge Mini, Labnet), automatic pipette (Eppendorf Research plus, Biohit),

tips, homogenizer (FastPrep-24, MP Biomedical), NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, USA), beaker, cellulose wadding, 500 ml Erlenmeyer flask, counterweights (572, Kern), electrophoretic bath and gel mold (Owl A6, Thermo Scientific; Wide Mini-Sub Cell GT, Bio-Rad), electrophoretic voltage source (PowerPac ™ Basic, Bio-Rad), combs, spoon, silicone glove, UV transilluminator (FireReader, Uvitec Cambridge), digital camera with orange filter (EDAS 290, Kodak), sterile flowbox (PV – 102, TelSTAR), 2 ml test tubes (Eppendorf), test tube strips (Treflab, Switzerl), 96-well plate for PCR (4titude), PCR thermocycler (PTC- 200 Peltiers Thermal Cycler, MJ. (Research).

4.10. LIST OF USED CHEMICALS AND SOLUTIONS:

Distilled water, agarose (SERVA, Germany), molecular weight standard (marker) (GeneRuler 100bp Plus DNA Ladder, cat. no. SM0241, Thermo Scientific , Biogen, USA), 10 x TBE (for 1 litr 5.40 g TRIS base; 2.75 g boric acid; 2 ml 0.5 mol/l EDTA at pH 8.0; Sigma Aldrich , Czech Republic), bromophenol blue in 30% of glycerin (Promega), fluorescent dye for DNA Simply Safe ™ (EurX), *MyTaq* ™ RED Polymerase (Bioline), 5x *MyTaq* ™ Reaction Buffer Concentrate Buffer containing 15 mmol/l MgCl2, 5 mmol/l dNTPs and application dye (Bioline , UK), primers (see **Table 1**) (Generi-Biotech, CZ), Exonuclease I (20 U/ µl, Thermo Scientific, USA), Thermosensitive Alkaline Phosphatase (1 U/µl, Thermo Scientific, USA), Fast Alkaline Phosphatase buffer (10x, Thermo Scientific, USA), restriction enzyme (see Table 4) (Thermo Scientific , Biogen, CZ, enzymes tested for parental polymorphism), corresponding buffers for restriction enzymes 10x (see **Table 4**) (Thermo Scientific, Biogen), Invisorb Spin Plant Mini Kit (STRATEC Molecular GmbH, Germany).

5. RESULT

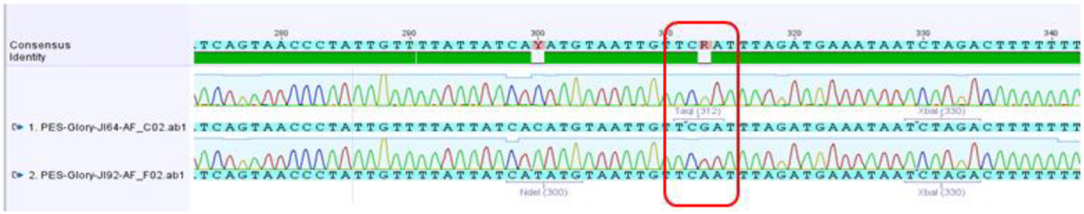
5.1. PCR AMPLIFICATION OF GENES ENCODING CALCINEURIN-LIKE GENES

Previous genetic mapping analysis based on genome-wide mapping on RIL population indicated locus at chromosome 2 spanning region of around 16 million bases in current pea genome Cameor v1.0. This search has revealed 49 predicted genes and among them, there were 2 homologues genes encoding calcineurin-like protein. Along with their differential gene expression based on seed coat RNAseq analysis and in line with published paper on soybean (Sun *et al*. 2015), these were selected as putative candidate genes. These are positioned at 12,022,981 nt (Psat2g013240) and 28,971,472 nt (Psat2g024960) at reference Cameor v1.0 genome. The predicted gene is 2,489 nt long and consists of 5 exons. Coding region is 1,317 nt long and encodes protein of 321 amino acids, with theoretical pI 5.79 and molecular weight of 49.84 kDa with homology to calcineurin-like phosphoesterase.

Similarly, the predicted Psat2g024960 gene is 3,821 nt long and consists of 9 exons. Coding region is 1,395 nt long and encodes protein of 336 amino acids, with theoretical pI 8.66 and molecular weight of 52.42 kDa with homology to putative acid phosphatase.

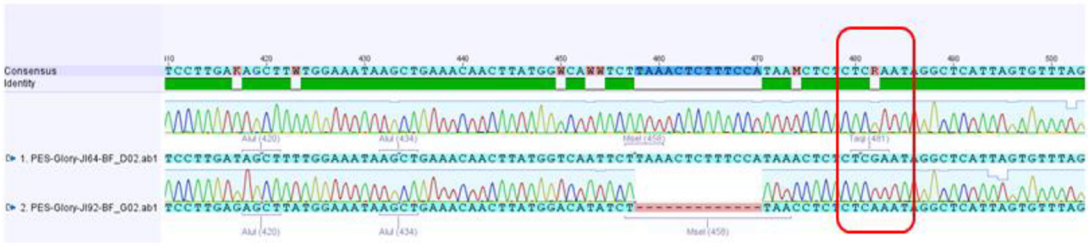
Several primer pairs were designed to start at more conserved exon region and spanning typically more polymorphic intron. For verification of tested primers functionality on parental genotypes using CAPS-PCR method, forward and reverse primer combination A (Psat2g013240-F115-R1060) and B (Psat2g013240-F980-R2431) were effective on Psat2g013240 gene while forward and reverse primer combination C (Psat2g024960-F25-R985) and D (Psat2g024960-F961-R2157) were affective on Psat2g024960 gene, whereas primer E (Psat2g024960-F1867-R3816) showed no visible result. The successfully amplified 945 bp and 960 bp regions from both JI64 and JI92 parental genotypes were subjected to sequencing and then searched for polymorphism suited for restriction enzyme based analysis applicable to entire RIL mapping population derived from JI92 and JI64 cross (**Figure 3-5)**.

(A) Psat2g013240



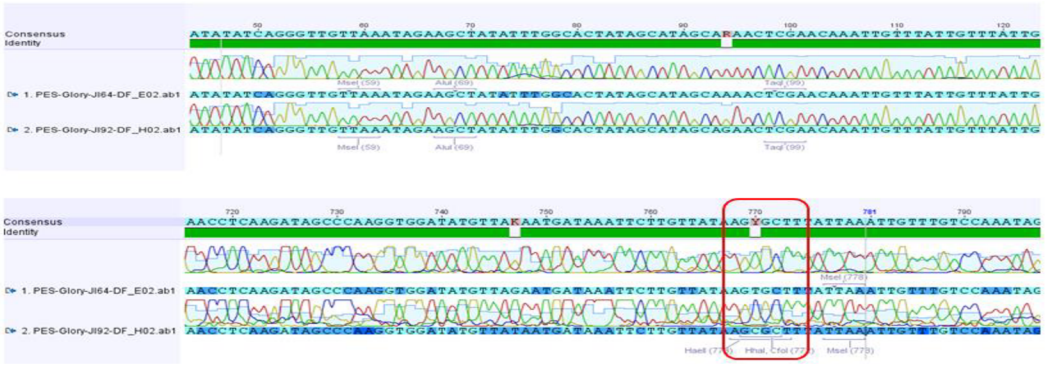
TaqI restriction enzyme with primer combination A (Psat2g013240-F115-R1060), showing difference between parental genotypes (in RED box), cuts at JI64 and not in JI92.

(B) Psat2g013240



TaqI restriction enzyme with primer combination B (Psat2g013240-F980-R2431), showing difference between parental genotypes (in RED box), cuts at JI64 and not in JI92, there is an addition length polymorphism, but too short to be visualized at simple agarose gel.

(C) Psat2g024960



HhaI restriction with primer combination D (Psat2g024960-F961-R2157), showing difference between parental genotypes (in RED box), cuts at JI92 and not in JI64.

Figure 3 (A-C): Sample of sequence polymorphism marker of parental genotype (JI64 and JI92) with respective restriction enzyme and primer combination.

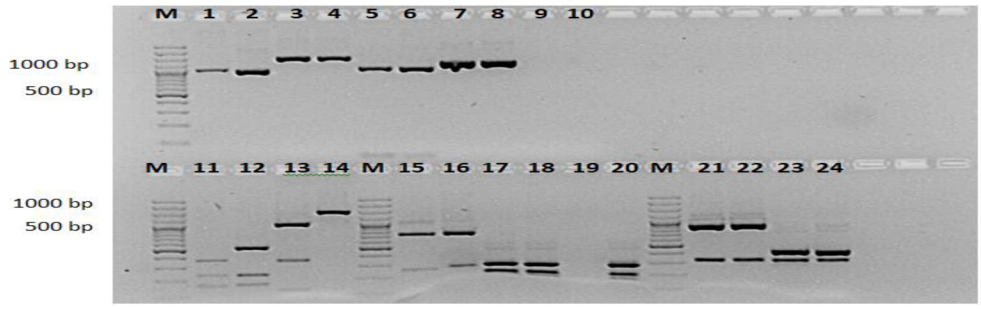


Figure 4: PCR amplification on Psat2g013240 and Psat2g024960 genes of parental genotypes, JI64 and JI92 with respective primer combinations and restriction enzymes. M (molecular marker), lane 1-10 (PCR amplification of Psat2g013240 gene and Psat2g024960 gene with forward and reverse primer combination A-E), lane 10-24 (restriction of the PCR products with respective restriction enzymes: *TaqI*, *AluI*, *HpaIII* and *HaeIII*).

As shown in **Figure 4**, JI64 (lane 1) and JI92 (lane 2) was successfully amplified with primer combination A (Psat2g013240-F115-R1060), JI64 (lane 3) and JI92 (lane 4) amplified with primer combination B (Psat2g013240-F980-R2431), JI64 (lane 5) and JI92 (lane 6) amplified with primer combination C (Psat2g024960-F25-R985), JI64 (lane 7) and JI92 (lane 8) amplified with primer combination D (Psat2g024960-F961-R2157), JI64 (lane 9) and JI92 (lane 10) amplified with primer combination E (Psat2g024960-F1867-R3816). Based on the result shown in **Figure 4**, forward and reverse primer combination A – D amplified the respective gene, while forward and reverse primer combination E did not. Subsequently PCR products of JI64 and JI92 parental genotypes with respective tested primers were digested with selected restriction enzymes *TaqI*, *AluI*, *HpaII* and *HaeIII*. The sequence of amplified Psat2g013240 gene with primer combination A and B showed variant polymorphism in the region digested with *TaqI* restriction enzyme (lane 11-14), the sequence of amplified Psat2g024960 gene with primer combination C and D showed variant polymorphism in the region digested with *HaeIII* restriction enzyme (lane 15-24) while the sequence of amplified Psat2g024960 gene with primer combination E could not be tested (lane 19-20).

The use of different restriction enzymes (*MboII*, *TaqI*, *HhaI* and *RsaI)* for cleavage of different genotypes, (Cameor, JI64, JI92 and JI1794) of PCR products with respective primer combination C (Psat2g024960-F25-R985) and D (Psat2g024960-F961-R2157) was demonstrated as shown in **Figure 5**.

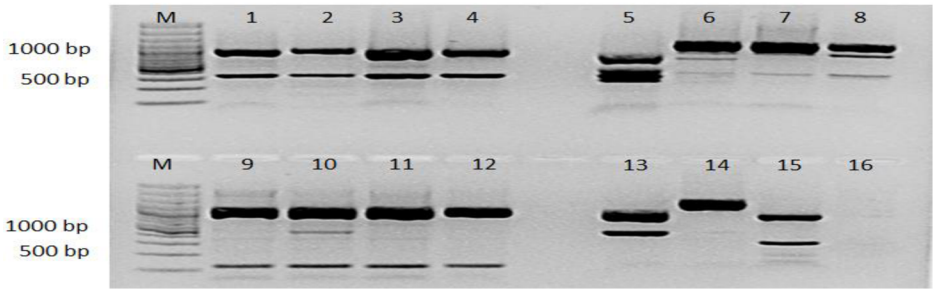


Figure 5: PCR amplification and restriction analysis of Psat2g024960 gene of different genotypes (Cameor, JI64, JI92, and JI1794) with respective restriction enzymes (*MboII*, *Taq1*, *HhaI* and *RsaI*). M- Molecular marker. Lane (1- Cameor, 2- JI64, 3-JI92, 4- JI1794) with primer combination C using *MboII* restriction enzyme, (5- Cameor, 6- JI64, 7-JI92, 8- JI1794) with primer combination C using *RsaI* restriction enzyme, (9- Cameor, 10- JI64, 11- JI92, 12- JI1794) with primer combination C using *TaqI* restriction enzyme, (13- Cameor, 14- JI64, 15- JI92, 16- JI1794) with primer combination D using *HhaI* restriction enzyme.

This restriction analysis demonstrated that JI64 genotype showed variant polymorphism on site digested with *TaqI* and *RsaI* enzyme restriction, while for Camoer genotype showed variant polymorphism on site digested with primer *RsaI* restriction enzyme, JI174 genotype showed variant polymorphism on site digested with *RsaI* enzyme, while variant polymorphism could not be identified in JI92 genotype. Since *TaqI* and *HaeIII* restriction enzyme proved to be effective with parental genotype JI64 and JI92, they were selected and applied on entire set of recombinant inbred lines.

5.2. PCR ANALYSIS OF RIL POPULATION

Application of respective primers and respective enzymes on set of recombinant inbred lines. For JI64 x JI92 RILs, Primer combination A (-F115-R1060) and *TaqI* restriction enzyme was used. While for JI92 x JI64 RILs, primer combination D (Psat2g024960-F961-R2157) and *HealII* restriction enzyme were used. As shown in **Figure 6-9** of the PCR restriction analysis, out of the 71 RILs tested of JI64 x JI92, 30% recombined and 3.9% lines were heterozygues. While for JI92 x JI64 RILs, 60% lines recombined out of 79 lines tested and 1.2% was heterozygues.

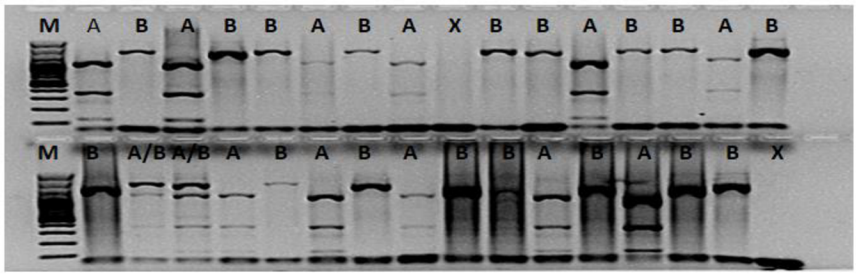


Figure 6: Sample of an electrophoretic gel showing segregation patterns of JI64 x JI92 RILs amplified by primer combination A (Psat2g013240-F115-R1060) and digested with *TaqI* restriction enzyme. M-molecular marker, A - variant polymorphism for dormant lines, B - variant polymorphism for non-dormant lines, A/B – heterozygous variant polymorphism, X – empty lane without variant polymorphism.

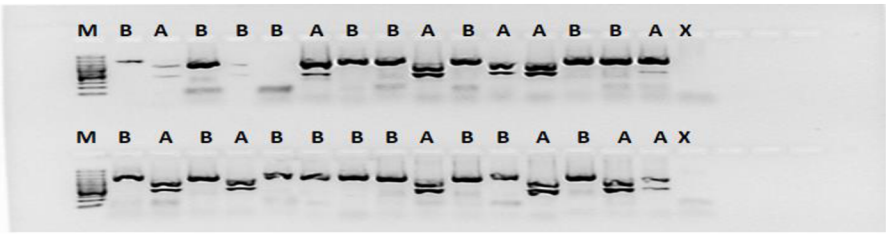


Figure 7: Sample of an electrophoretic gel showing segregation patterns of JI64 x JI92 RILs amplified by primer combination D (Psat2g024960-F961-R2157) and digested with *HaeIII* restriction enzyme. M- Molecular marker, A- variant polymorphism for dormant lines, B- variant Polymorphism for non-dormant lines, X- empty lane without variant polymorphism.

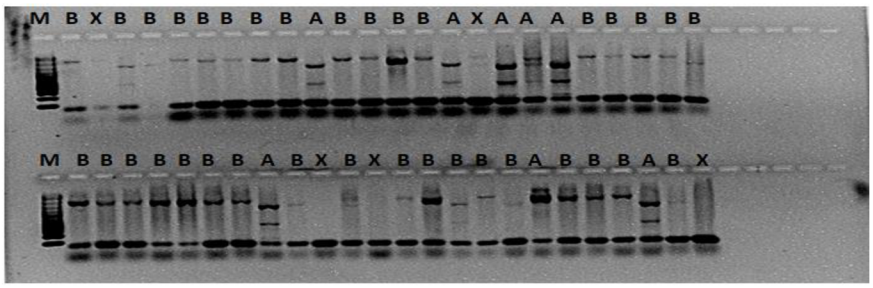


Figure 8: Sample of an electrophoretic gel showing segregation patterns of JI92 x JI64 RILs amplified by primer combination A (Psat2g013240-F115-R1060) and digested with *TaqI* restriction enzyme. M-molecular marker, A-variant polymorphism for dormant lines, B- variant polymorphism for non-dormant lines. X- empty lane with no variant polymorphism.

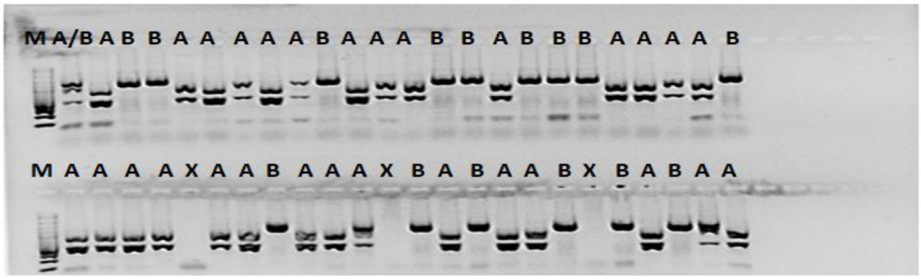


Figure 9: Sample of an electrophoretic gel showing segregation patterns of JI92 x JI64 RILs amplified by primer D (Psat2g024960-F961-R2157) and digested with *HaeIII* restriction enzyme. M-molecular marker, A- variant polymorphism for dormant lines, B- variant polymorphism for non-dormant lines. X- empty lane with no variant polymorphism.

5.3. EVALUATION OF SEED COAT PIGMENTATION

Seed coat pigmentation was evaluated visually with the differences between pigmented and non-pigmented ones. Wild pea (JI64) was characterized with small and uniformly grey pigmented (dark brown until black) seeds, while cultivated pea (JI92) were larger and dot pigmented (**Figure 10**). Pigmentation starts to develop first at the hilum and was present as dots or patches on the seed coat, seed color and seed size varied among lines. The color changes from light brown to brown and black (**Figure 11**).

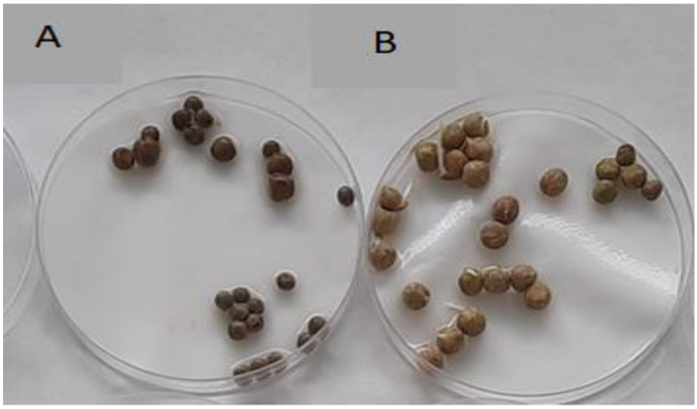


Figure: 10: Difference in seed pigmentation pattern of parental genotypes, JI64 (A) and JI92 (B). JI64 - (Wild pea-*Pisum elatius*), JI92 - (Domesticated pea- (*Pisum sativum*).

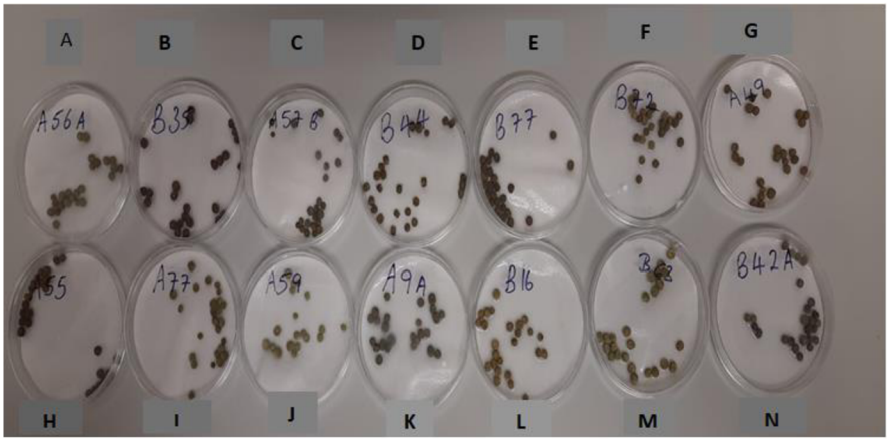


Figure 11: Showing sample of different colored seeds of selected RILs. A56A (A), B35 (B), A57B (C), B44 (D), B77 (E), B72 (F), A49 (G), A55 (H), A77 (I), A59 (J), A9A (K), B16 (L), B63 (M), B42A (N).

After 10 days of imbibition, there were considerable differences in pigmentation and texture surface of individual RIL lines. It was observed that JI64 was grey pigmented, gritty and imbibed slowly which is in contrast to JI92. Also RILs derived from crosses of JI92 and JI64 imbibed easily at a ratio of 60 – 100 percent while RILs from JI64 and JI92 crosses imbibed slowly at 0 – 40 percent ratio.

5.4. GENE POLYMORPHISM EVALUATION OF RECOMBINANT INBRED LINES

Assessment of variant polymorphism for each tested gene of JI64 x JI92 RILs and its reciprocals JI92 x JI64 and level of recombination between them, both genes are located at chromosome 2 at a distance of 17 Mbp apart. Out of the 71 lines tested for JI64 x JI92, 30% recombined and 3.9% lines were heterozygote. While for gene JI92 x JI64 RILs, 60% lines recombined out of 79 lines tested and 1.2% was heterozygote. This is double recombination frequency of J64 x JI92 compared to reciprocal cross. According to the result, JI92 x JI64 RILs were more able to recombine in comparison to JI64 x JI92 RILs (**Table 5 and 6**).

Table 5: Assessment of genes polymorphism of JI64 x JI92 (71 RILs) population

Number	RILs (JI64x 92) line F6	(Psat2g013240-F115-R1060)	(Psat2g024960-F961-R2157)	Recombination between A and D
1	1	B	X	
2	3	B	X	
3	5	B	X	
4	6	B	A	1
5	11	X	X	
6	12	A	X	
7	16	A	X	
8	19	A/B	A	
9	20	B	A	1
10	21	B	X	
11	23	B	B	
12	24	B	X	
13	25A	B	X	
14	25B	A	X	
15	26	A	X	
16	30	A	A	
17	32	X	A	
18	34A	A	X	
19	34B	A	X	
20	35	B	B	
21	38	A	A	
22	39	A	B	1
23	41	A	B	1
24	42A	B	A	1
25	42B	A	A	
26	43	B	B	
27	44	A	A	
28	45	B	B	
29	46	A	A	
30	47	B	B	
31	48	A	A	
32	50	B	B	
33	51	A	B	1
34	52	B	A	1
35	55	B	B	
36	56	X	B	
37	58	A	A	
38	59	B	B	
39	60	A	A	
40	61	B	B	
41	63	A	B	1

Number	RILs (JI64x 92) line F6	(Psat2g013240-F115-R1060)	(Psat2g024960-F961-R2157)	Recombination between A and D
42	64	X	A	
43	66	B	B	
44	69	X	X	
45	70	X	B	
46	72	B	B	
47	73	B	A	1
48	74A	B	A	1
49	74B	A	A	
50	75	B	B	
51	76	A	A	
52	77	B	A	1
53	78	B	A	1
54	82	A	B	1
55	83	A	A	1
56	84	A	A	
57	86	X	B	
58	87	B	A	1
59	88	B	B	
60	89	A	B	1
61	90	B	A	1
62	91	B	A	1
63	93	A	B	1
64	94	B	X	
65	95	B	A	1
66	96	A/B	B	
67	97	A/B	A	
68	98	A	B	1
69	99	B	A	1
70	100A	A	A	
71	100B	B	A	1

Legend: A – allele from JI64 (dormant), B – allele from JI92 (non- dormant), A/B – Heterozygote alleles, orange color row – number of recombination of tested genes, X – blank lane on electrophoretic gel.

Table 6: Evaluation of genes polymorphism of JI92 x JI64 (79 RILs) population

Number	RIL (JI92x64) line F6	(Psat2g013240-F115-R1060)	(Psat2g024960-F961-R2157)	Recombination Between A and D
1	1	B	A/B	
2	2		A	
3	9A	B	B	
4	9B	B	B	
5	10	B	A	1
6	11	B	A	1
7	12	B	A	1
8	13	B	A	1
9	15B	B	A	1
10	16	A	B	1
11	18	B	A	1
12	19	B	A	1
13	20	B	A	1
14	21	B	B	
15	22A	A	B	1
16	22B	X	A	
17	25	A	B	1
18	27	A	B	1
19	29	A	B	1
20	30A	B	A	1
21	30B	B	A	1
22	31	B	A	1
23	32	B	A	1
24	33	B	B	
25	34	B	A	1
26	35	B	A	1
27	36	B	A	1
28	37	B	A	1
29	38	B	X	
30	40	B	A	1
31	41	B	A	1
32	44	A	B	1
33	45	B	A	1
34	46A	X	A	
35	46B	B	A	1
36	47	X	X	
37	49	B	B	
38	51	B	A	1
39	84A	B	B	
40	54B	B	A	1
41	55	B	A	1

Number	RIL (JI92x64) line F6	(Psat2g013240-F115-R1060)	(Psat2g024960-F961-R2157)	Recombination Between A and D
42	56A	B	B	
43	56B	B	X	
44	57A	B	B	
45	57B	B	A	1
46	58	A	B	1
47	59	B	A	1
48	61	B	A	1
49	62	A	B	1
50	63	A	B	1
51	65	B	B	
52	66	B	A	1
53	67	A	A	
54	70A	B	A	1
55	70B	X	A	
56	73	B	B	
57	74	B	A	1
58	75	B	X	
59	77	B	B	
60	78	B	X	
61	83	B	A	1
62	84	B	A	1
63	86	A	B	1
64	88	X	B	
65	89	B	B	
66	90A	B	A	1
67	90B	X	A	
68	87	B	A	1
69	91A	X	A	
70	91B	X	A	
71	93	B	B	
72	96	B	B	
73	100	B	A	1
74	10	B	A	1
75	12	B	A	1
76	22B	B	A	1
77	31	A	A	
78	32	A	A	
79	55	B	A	1

Legend: A – allele from JI64 (dormant), B - allele from JI92 (non- dormant), A/B - Heterozygotes allele, orange color row – number of recombination of tested genes, X – blank lane on electrophoretic gel.

5.5. DIVERSITY ANALYSIS OF CALCINEURIN-LIKE GENES IN PEA GERMPLASM

In order to analyze the scenario of possible domestication bottleneck and diversity selection acting on studied (Psat2g013240 and Psat2g024960) genes the fragments were amplified and sequenced in the diversity panel consisted of *P. fulvum* (6 accessions), *P. elatius* (21 ac.), domesticated *P. abyssinicum* (7 acc.) and *P. sativum* geographically diverse (14) accessions (total of 48 samples). In the case of Psat2g013240, a fragment of 945 bp (in Cameor reference genome) was amplified using primer combination A (Psat2g013240-F115 and R1060). Upon editing and gaps removal (two 5 and 10 nt insertions/deletions) the total length of 835 nt was used for further analysis. There were 25 polymorphic SNP sites in final alignment used for Neighbor-joining tree analysis (**Figure 12**). These showed the monomorphic (single allele) status of P. abyssinicum, single cluster of P. fulvum accessions, while wild P. elatius and domesticated P. sativum samples formed several clusters and were intermixed. Interestingly, 6 *P. sativum* accessions shared single allele, while wild *P. elatius* were more diverse. Notably, studied JI64 and JI92 parental genotypes were positioned relatively close to each other. In case of causality of the given gene being responsible for seed dormancy and its respective removal in domesticated accessions, there is expected to be single of very few alleles in non-dormant material. Monomorphism observed in the case of Ethiopian *P. abyssinicum* is not surprising and corresponds to other genes, and seems to be related to extreme genetic bottleneck of this pea (Trněný *et al*. 2018).

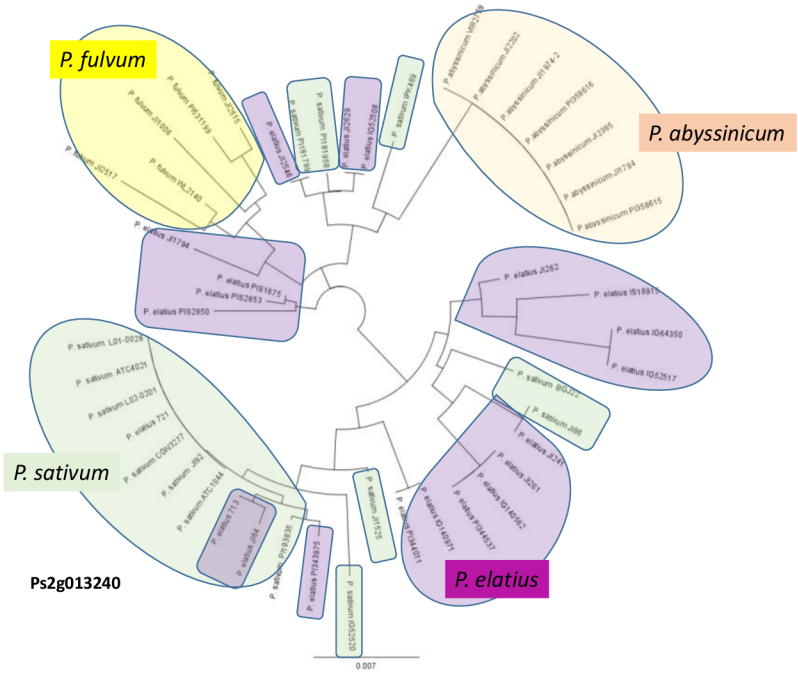


Figure 12: Neighbor-joining tree analysis of Psat2g013240 gene in panel of 48 diverse *Pisum sp.* samples. Cultivated *P. sativum* genotypes are encircled in green, wild *P. elatius* in violet*, P. fulvum* in yellow and *P. abyssinicum* in brown.

Identical analysis of Psat2g024960 gene was possible in 43 out of 48 samples, due to sequencing quality. The respective amplified region using primer combination D (Psat2g024960-F961-R2157) yielded of 960 bp (in Cameor reference genome) and was trimmed to 755 bp region consisting of 2 and 9 bp insertion/deletions which were also removed for the final analysis. There were 32 polymorphic SNP sites in the final alignment and Neighbor-joining tree (**Figure 13**). Similarly to Psat2g013240 gene, there was a nearly monomorphic status of *P. abyssinicum* except of JI2853 accession which had a different allele, single but yet diverse cluster of *P. fulvum* accessions, while wild *P. elatius* and domesticated *P. sativum* samples formed several clusters and were intermixed. In general, there was less similarity as compared to. Notably, domesticated *P. sativum* accessions were more variable without evidence of selection.

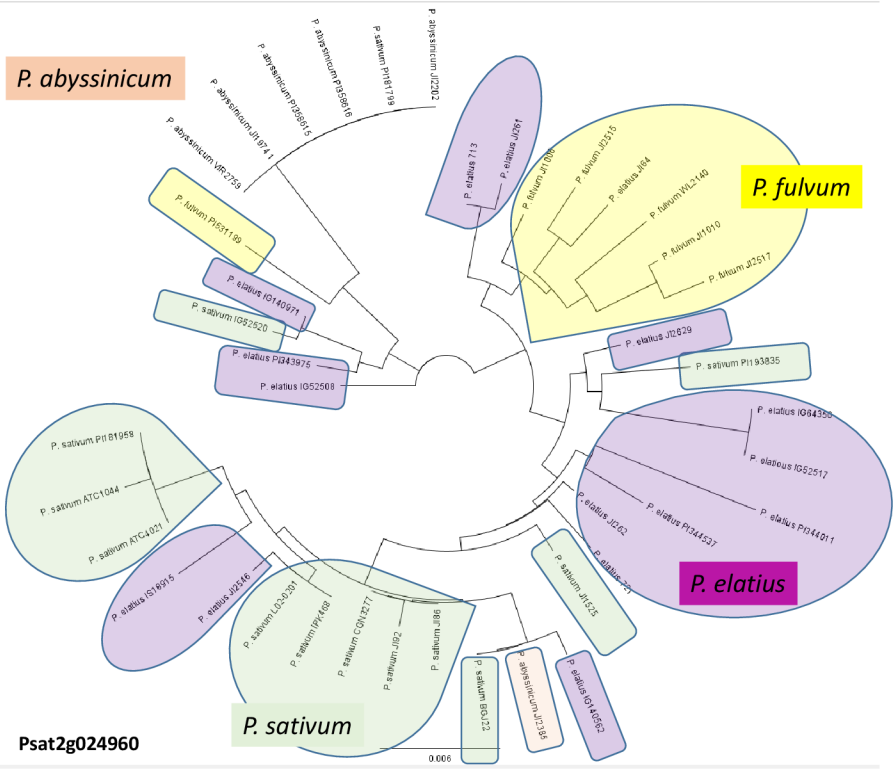


Figure 13: Neighbor-joining tree analysis of Psat2g024960 gene in panel of 48 diverse *Pisum sp.* samples. Cultivated *P. sativum* genotypes are encircled in green, wild *P. elatius* in violet*, P. fulvum* in yellow and *P. abyssinicum* in brown.

5.6. TESTING OF SEED DORMANCY

Using contrasting parental lines of cultivated pea, *Pisum sativum* (JI92) and wild pea, *Pisum elatius* (JI64), a mapping population of 130 recombinant inbred lines was derived from their crosses (RILs). From a total of 130 RILs tested at 10 days, 58 lines were evaluated as germinating (non-dormant), that is, with a germination rate of (60 - 100%), 61 lines as dormant (0 - 40%) non-germinating seeds and 20 lines were intermediate with level between 40 to 60%. As indicated in **Figure 14,** the category with the largest number of lines was the 0-20% category, followed by the 80-100% category.

(A). **(B).**

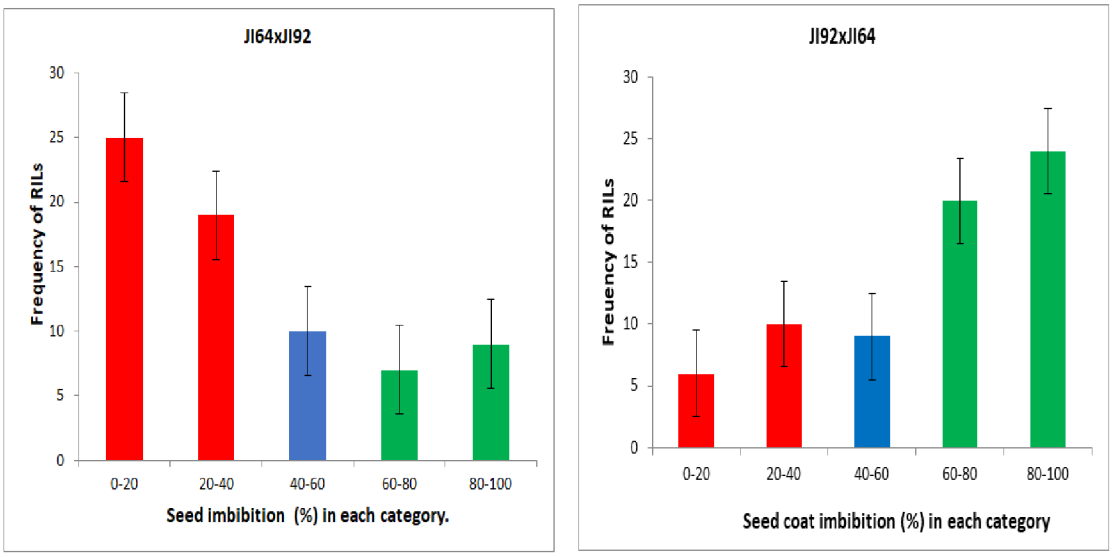


Figure 14: Distribution of seed dormancy among 130 RILs of JI64 x JI92 (A) and JI92 x JI64 (B) scored as seed imbibition at 10 days.

Dormant lines were denoted (with red color bars), non-dormant lines (with green color bars) and the intermediate lines (with blue color bars), with an average value of 38 and 68 and a standard error of 3.4 and 3.3 for **Fig. 14** (A and B) respectively. Considering the graph on **Figure 14 (A)**, 44% were dormant, 16% were non dormant and 10% were intermediate while in **Figure 14 (B)**, 17% were dormant, 42% were non dormant and 9% were intermediate. Analyzing the graphs, it indicate that JI64 x JI92 RILs is more dormant and possess lower rate of imbibition in comparison to its reciprocal JI92 x JI64 RILs which has more than double rate of imbibition.

5.7. SEED COAT THICKNESS

Seed coat thickness of selected recombinant inbred lines of parental genotypes (JI64) and (JI92) was also measured and compared as shown in (**Figure 15**).

(A). **(B).**

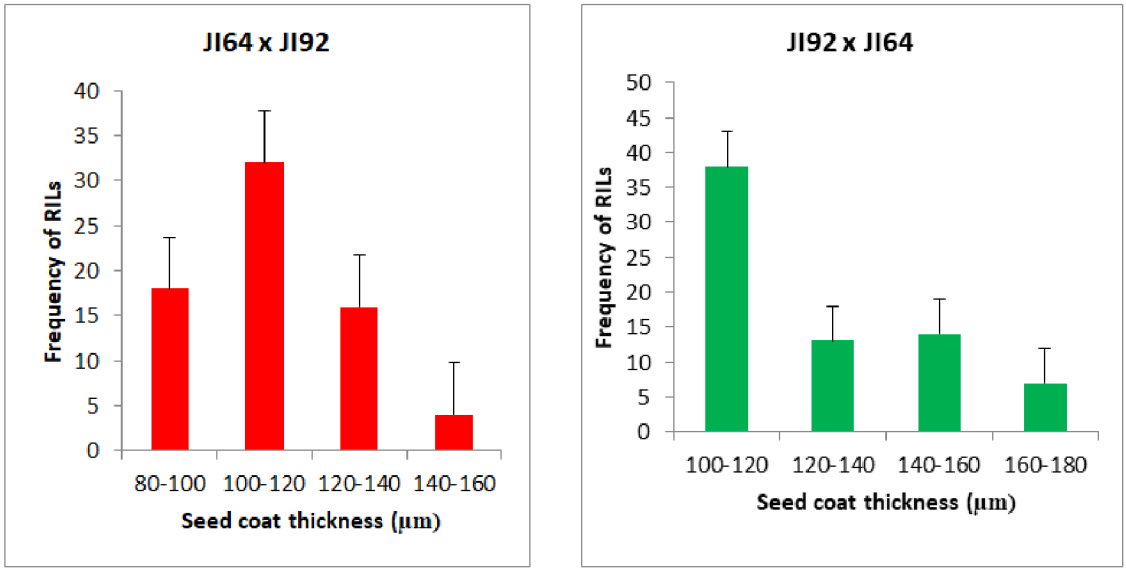


Figure 15: A graph comparing the measurements of seed coat thickness of RILs of JI64 x JI92 (A) and its reciprocal JI92 x JI64 (B) population. Dormant lines are represented with red color bars while non dormant lines are represented with green color bars.

The seed coat of 71 RILs of JI64 x JI92 genotype showed highest measurement between 100 – 120 µm and lowest between 140 – 160 µm, with a mean value (114) and standard error (2.1). While its reciprocal, JI92 x JI64 RILs had highest measurement between 100-120 µm and lowest between 160 – 180 µm, with a mean value (129) and standard error (2.7).

5.7.1. ANATOMICAL ANALYSIS OF SEED COAT THICKNESS

In order to assess the relationship between measured seed coat and behavior of RIL lines during dormancy testing, selected lines were subjected to microscopic analysis of mature seed coat. They were selected parental genotype JI64, JI92 and 4 non-dormant RIL lines A20, A30A, A45, A49, and contrasting 6 dormant lines B16, B19, B45, B48, B61, B82 (**Figure 16**).

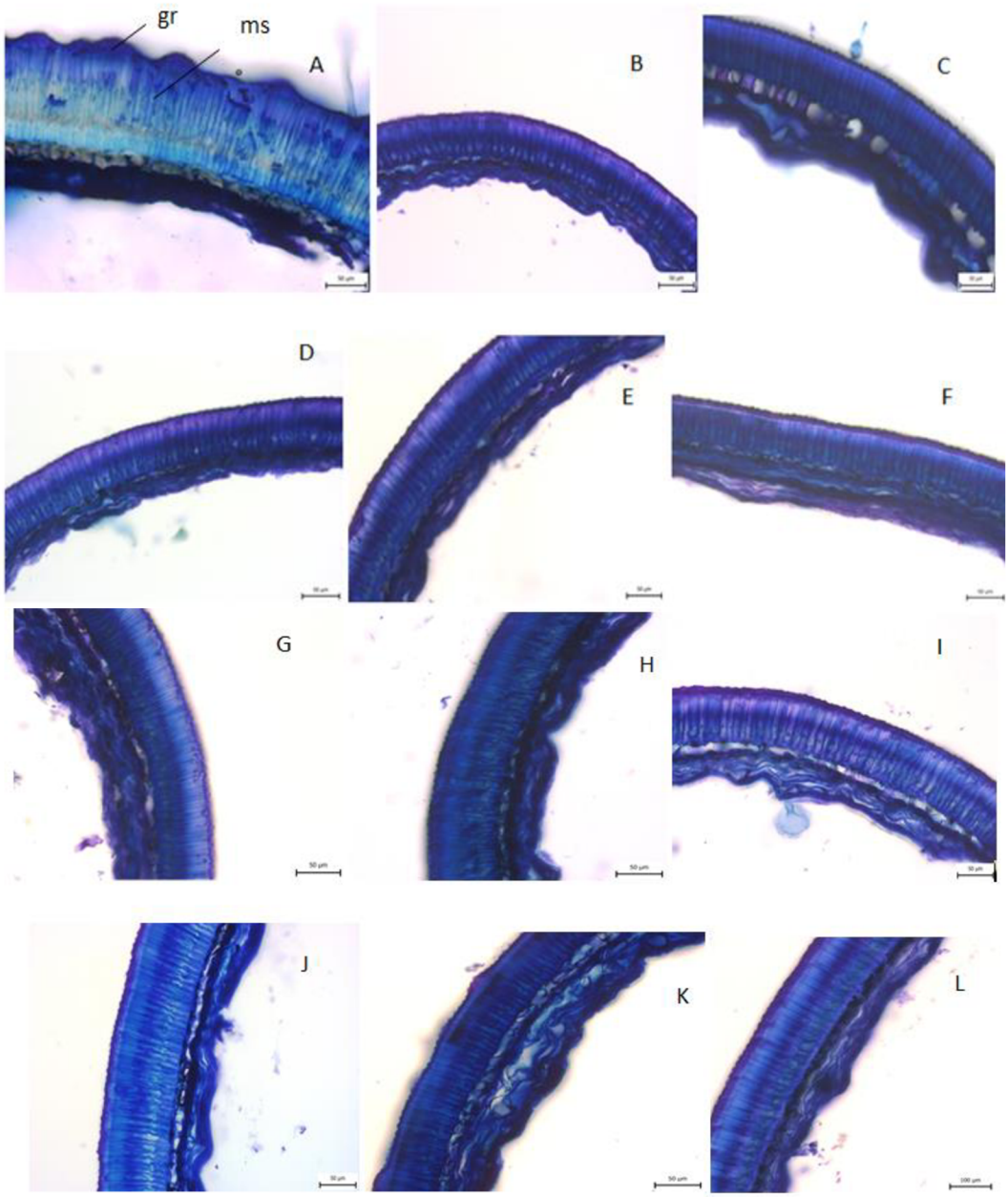


Figure 16: Microscopic analysis of seed coat sections of wild pea – JI64 (*Pisum sativum* subsp. *elatius*) (A), cultivated pea – JI92 (*Pisum sativum* subsp. *sativum*) (B), and their recombinant inbred lines A30A (C), A20 (D), A45 (E), A49 (F), B16 (G), B19 (H), B45 (I), B48 (J), B61 (K), B82 (L), stained with Toluidine blue. Scale bars 50 µm for (A - K), 100 µm for (L). gr (gritty), ms (macroscleried cells).

Based on the anatomic analysis, there was a presence of grittiness in JI64 (wild pea) and recombinant inbred line B61 coming from the wild parental genotype. Each of the structure possessed a macroscleried of different length.

5.8. STATISTICAL ANALYSIS OF SEED COAT RELATIONSHIP TO DORMANCY

Correlation between parental alleles in the recombinant inbred lines was analyzed to test the relationship between seed coat thickness and the level of imbibition after 10 days. Based on the plotted graphs, it indicated that thickness of seed coat influences its rate of seed imbibition (**Figure 17 and 18**).

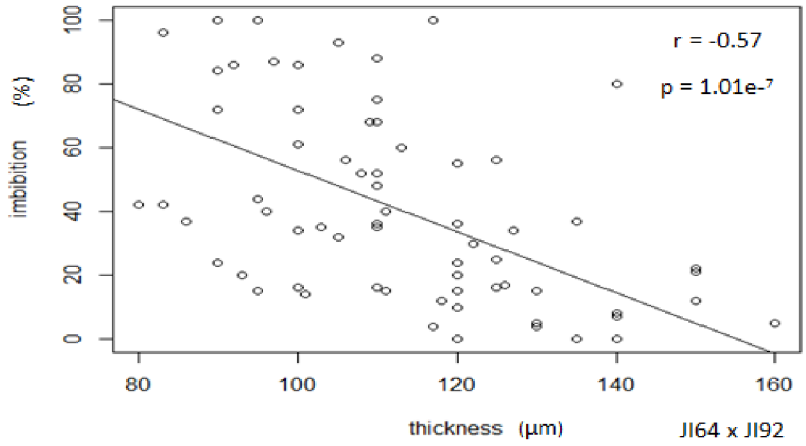


Figure 17: A scatter plot graph describing the relationship of seed coat thickness to level of imbibition of JI64 x JI92 (71 RILs). Vertical axis- percentage of seed imbibition of JI64 x JI92 RILs, horizontal axis- measurement of seed coat thickness of JI64 x JI92 RILs, r- Pearson correlation coefficient, p- "P value".

The graph shows a "P value" of $1.01e\text{-}^{7}$ and a correlation coefficient of -0.57 indicating a strong negative correlation between both variables. This clearly emphasized that as seed coat thickness increases, the rate of imbibition decreases drastically.

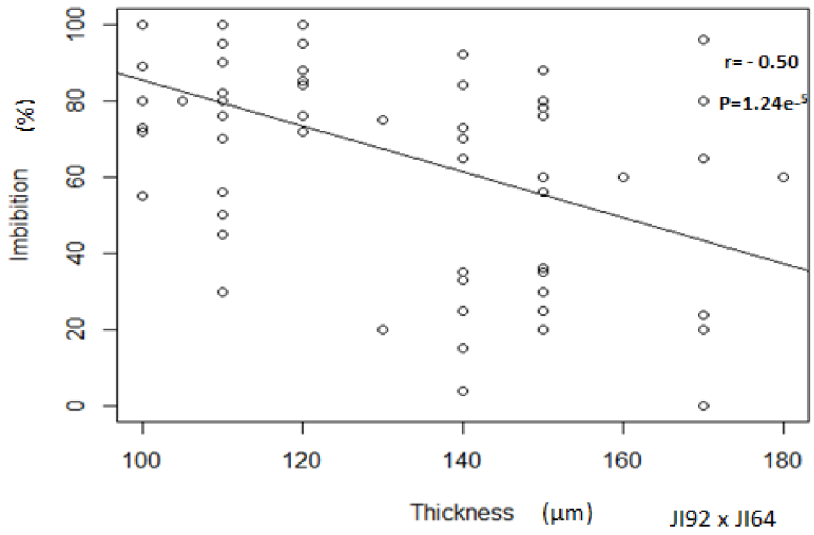


Figure 18: A scatter plot graph describing the relationship of seed coat thickness to level of imbibition of JI92 x JI64 (79 RILs). Vertical axis- percentage of seed imbibition of JI92 x JI64 RILs, horizontal axis- measurement of seed coat thickness of JI92 x JI64 RILs, r- Pearson correlation coefficient, p- "P value".

The graph shows a "P value" of $1.24x10^{-5}$ and a correlation of – 0.50, indicating a moderate negative correlation between both variables. This means that there is a noticeable but weak relationship between seed coat thickness of JI92 x JI64 RILs and its rate of imbibition. In other words, as seed coat gets thicker its rate of imbibition decrease slightly. Seed coat of JI92 x JI64 RILs have higher imbibition rate in comparison to JI64 x JI92 RILs.

Further statistical analysis was ascertained to check for the allelic relationship of tested calcineurin genes in set of 71 lines of JI64 x JI92 and its reciprocal of 79 lines of JI92 x JI64 to their level of imbibition (dormancy) with their respective primer combination A and D. Considering the graphs on **Figure 19 – 23**, it indicates that the rate of imbibition is different between Psat2g013240 and Psat2g024960 genes respectively.

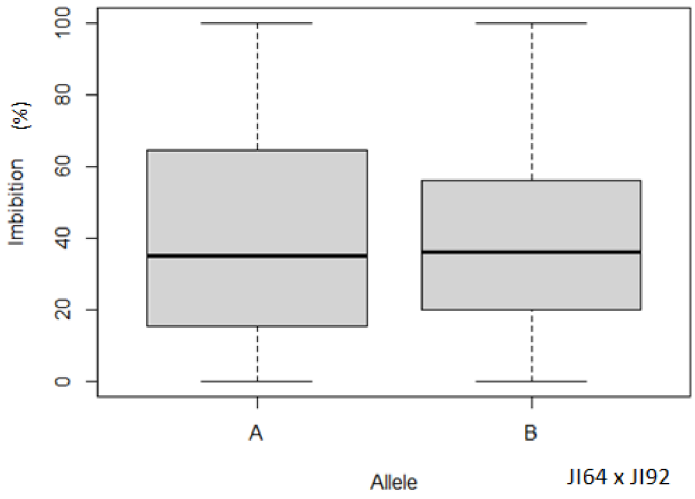


Figure 19: A box plot describing the allelic relationship of Psat2g024960 gene to level of imbibition in RIL population. A - allele from Psat2g013240 gene (JI64), B - allele from Psat2g04960 gene (JI92) amplified with primer combination D (Psat2g024960-F961-R2157). Vertical axis – percentage of imbibition of allele A and B, horizontal axis - allele A and B from JI64 x JI92 RILs. The bold line in the middle of box plot represent median.

According to the graph on **Figure 19,** the median of box plot for allele B is slightly higher than that of allele A with a mean value of 40 for allele B and 39 for allele A and a 95% confidence interval of -16 and 15 respectively, indicating that Psat2g024960 gene (JI92) had almost same rate of imbibition with Psat2g013240 gene (JI64) and also overlaps between each other, emphasizing that the difference in median is not statistically significant. Both alleles were negatively skewed with no observable outliers. There was a less association between Psat2g013240 gene (JI64) and Psat2g024960 gene (JI92) to the level of imbibition.

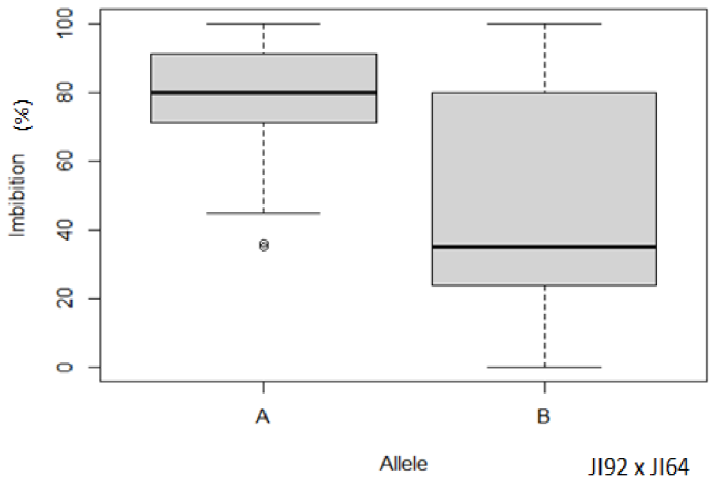


Figure 20: A box plot describing the allelic relationship of Psat2g024960 gene to level of imbibition. A - allele from Psat2g013240 gene (JI64), amplified with primer combination D (Psat2g024960-F961-R2157), B - allele from Psat2g04960 gene (JI92). Vertical axis – percentage of imbibition of allele A and B, horizontal axis - allele A and B from JI92 x JI64 RILs. The bold line in the middle of box plot represent median.

Based on the graph on **Figure 20**, the median of box plot for allele A is higher than that of allele B with a mean value of 78 for allele A and 49 for allele B and 95 confidence interval of 16.8 and 41.9 respectively, indicating that Psat2g013240 gene (JI64) had higher rate of imbibition in comparison to Psat2g024960 gene (JI92), emphasizing that the difference in median is statistically significant. Allele A is positively skewed with two outliers while allele B is negatively skewed with no observable outlier. However, there might be an association between Psat2g013240 and Psat2g024960 genes to the level of imbibition.

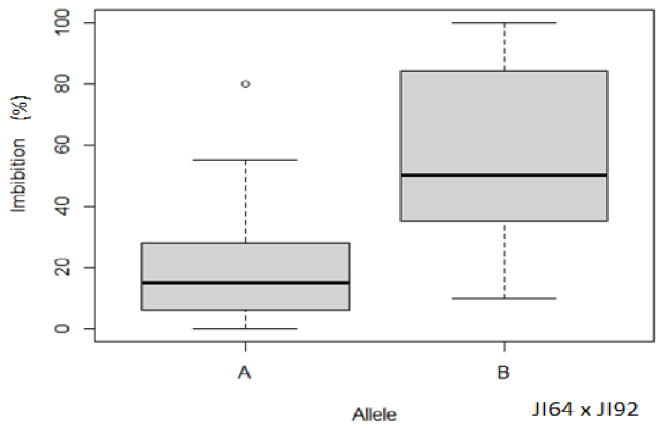


Figure 21: A box plot describing the allelic relationship of Psat2g013240 gene to level of imbibition. A - allele from Psat2g013240 gene (JI64), B - allele from Psat2g024960 gene (JI92) amplified with primer combination A (Psat2g013240-F115-R1060). Vertical axis – percentage of imbibition of allele A and B, horizontal axis - allele A and B from JI64 x JI92 RILs. The bold line in the middle of box plot represent median.

Based on the graph on **Figure 20**, the median of box plot for allele B is higher than that of allele A with a mean value of 20 for allele A and 55 for allele B with 95% confidence interval of -47.3 and -22.6 respectively, indicating that Psat2g024960 gene (JI92) had higher rate of imbibition in comparison to Psat2g013240 gene (JI64), also both do not overlap with each other, emphasizing that the difference in median is statistically significant. Both alleles were negatively skewed with one outlier in allele A, suggesting that, there was probably an association between Psat2g013240 and Psat2g024960 genes to the level of imbibition.

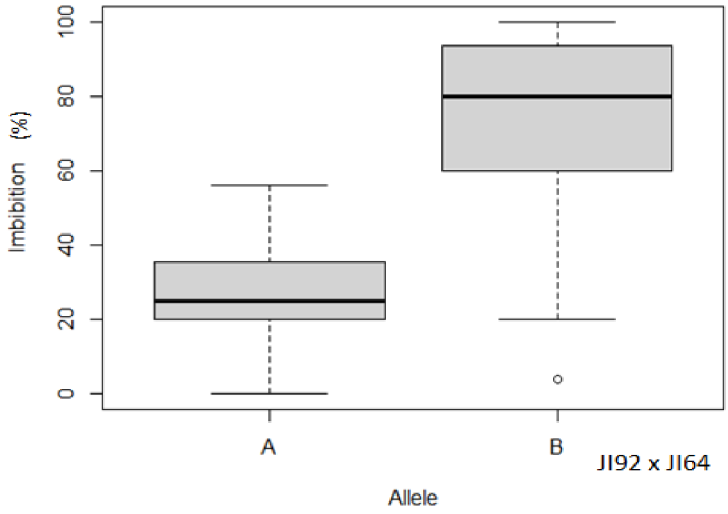


Figure 22: A box plot describing the allelic relationship of Psat2g013240 gene to level of imbibition, with primer combination A (Psat2g013240-F115-R1060). A- allele from Psat2g013240 gene (JI64) amplified with primer combination A (Psat2g013240-F115-R1060), B - allele from Psat2g024960 gene (JI92). Vertical axis - percentage of imbibition of allele A and B, horizontal axis - allele A and B from JI92 x JI64 RILs. The bold line in the middle of box plot represent median.

According to the graph on **Figure 22**, the median of box plot for allele B is higher than that of allele A with a mean value of 27 for allele A and 75 for allele B with a 95% confidence interval of -62.3 and -32 respectively, indicating that Psat2g04960 gene (JI92) had a higher rate of imbibition in comparison to Psat2g013240 gene (JI64), emphasizing that the difference in median is statistically significant. Allele A is negatively skewed while allele B is positively skewed with one outlier. Suggesting that, there was probably an association between Psat2g013240 and Psat2g024960 genes to level of imbibition.

5.8.1. SUMMARY PRESENTATION OF ALL BOX PLOTS

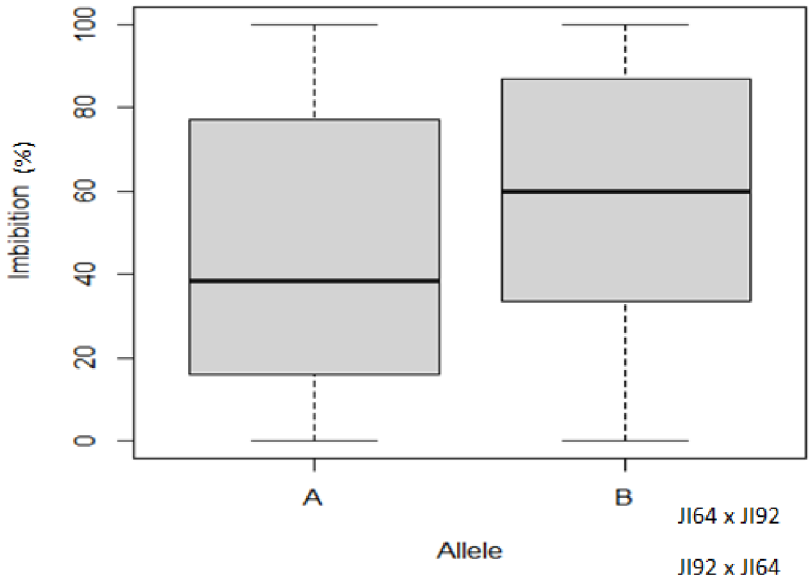


Figure 23: A box plot describing the allelic relationship of Psat2g024960 and Psat2g013240 genes to their level of imbibition, with their respective forward and reverse primer combinations A (Psat2g013240-F115-R1060) and D (Psat2g024960-F961-R2157). A - allele from Psat2g013240 gene (JI64), B - allele from Psat2g024960 gene (JI92). Vertical axis – percentage of imbibition of allele A and B, horizontal axis - allele A and B from JI64 x JI92 RILs and JI92 x JI64 RILs. The bold line in the middle of box plot represents median.

According to the graph, median of box plot for allele B is higher than that of allele A with a mean value of 47 for allele A and 58 for allele B and a 95% confidence interval of 19.1 and 3.3 respectively, indicating that Psat2g024960 gene (JI92) had a higher rate of imbibition in comparison to Psat2g013240 gene (JI64), emphasizing that the difference in median was statistically significant. Allele A is negatively skewed while allele B is positively skewed. Suggesting that, there was an association between Psat2g013240 and Psat2g024960 genes to level of imbibition, as one increase, the other decreases simultaneously.

6. DISCUSSION

This work focused on analysis of candidate gene(s) encoding calcinerium and testing its association with seed dormancy in pea, as one of the two key domesticated traits in leguminous crop. The removal of dormancy from cultural form, thus allows rapid germination after sowing and at the same time swelling of the seeds in connection with cooking and subsequent digestibility. To identify the gene locus conditioning seed dormancy in wild pea (*Pisum elatius* L.), two genetically divergent parents of wild (JI64) and cultivated (JI92) pea which show clear difference in the dormancy were selected for genetic mapping based on genome - wide association mapping on RIL population (Hradilová *et al*. 2017). This analysis resulted in identification of two loci, one of them placed at chromosome 2. Searched at current pea genome (Cameor version 1.0) indicated 49 gene of which two were homologues genes encoding calcineurin-like protein. This is in accordance with previous studies on mapping of quantitative trait locus (QTL) underlying hard seedness to an overlapping region on soybean position at chromosome 2 (Sun *et al*. 2015), this revealing a linkage between *GmHs1-1* and the markers defining the common QTL region also on chromosome 2 (Sun *et al*. 2015), ultimately leading to identification of respective gene encoding calcineurin-like protein (Sun *et al*. 2015). Notably, about the same time this quantitative trait locus was identified to encode also endo-1,4-β-glucanase (Jang *et al*. 2015), which seems to be involved in the accumulation of β-1,4-glucan derivatives that reinforce the impermeability of seed coats in soybean. This suggests that the hard seedness investigated in this pea analysis in comparison to previous studies might be controlled by the homologues genes. Since there were two homologues genes encoding calcineurin-like protein, this study has analyzed each of these two separately. These are positioned at 12,022,981 nt (Psat2g013240) and 28,971,472 nt (Psat2g024960) at reference Cameor v1.0 genome, e.g. with the physical distance of about 17 Mbp. The rate of recombination of these two genes was also compared between RILs of JI64 x JI92 and its reciprocal JI92 x JI64. Based on the PCR followed by restriction analysis, out of the 71 lines tested from JI64 x JI92, 30% recombined and 3.9% lines were heterozygues. In contrary to the reciprocal cross, JI92 x JI64

RILs, 60% lines recombined out of 79 lines tested and 1.2% was heterozygote. This is the double recombination frequency of J64 x JI92 compared to its reciprocal cross. According to the result, JI92 x JI64 RILs are more able to recombine in comparison to JI64 x JI92 RILs. However, there were some lines without results which were denoted as empty lanes. I assumed this could be an error either from sample pipetting or PCR amplification failure. Notably these two reciprocal RIL populations differ also in proportion of seed dormancy, with JI64 x JI92 being more dormant than the JI92 x JI64. The reason for this is unknown, and we cannot exclude the influence of maternal genetic components (chloroplast and mitochondria genomes) in pea typically inherited from mother.

The mechanism controlling seed dormancy in the leguminous crop is also mediated by the anatomic structure, morphology and chemical composition of the seeds, such as phenolic contents, cuticle composition (Smýkal *et al*. 2014). The use of cultivated and wild pea parental genotypes provides an effective means of identifying the mechanism underlying physical dormancy of legume crops. It has been indicated that reduction of seed coat thickness led to reduction of seed coat impermeability (Smýkal *et al*. 2014), which was demonstrated with wild (JI64) and cultivated (JI92) pea. Asides thickness, the structure of the palisade and cuticular layer (Vu *et al*. 2014) and presence or absence of cracks (Meyer *et al*. 2007; Koizumi *et al*. 2008) was proposed to be associated with seed hardness (Hradilová *et al*. 2017; Liu *et al*. 2014), this was also identified in peas, where the wild (JI64) pea had a thicker macrosclereid palisade layer compared to the domesticated type, which probably attributes to water impermeability in seed coats as suggested by Miao *et al*. 2001. In this study, only subset of phenotypically contrasting lines was analyzed, and the results support the findings in previous studies on dormant chickpea seed genotype possessing thicker macrosclereid palisade layer (Miao *et al*. 2001). However there was not complete agreement between the seed coat thickness and dormancy (assessed as imbibition of seeds) in case of RIL lines, indicating that, it is not just physical thickness of the seed coat that is responsible for dormancy. Previous analyses also suggest the existence of chemical substances providing this seed coat property (Hradilová *et al*. 2017, Krejčí *et al*. 2022).

Indeed parental genotype, JI64 and JI92 differ substantially in palisade cells length, which probably contributes to overall testa thickness. Dormant genotype JI64 has significantly thicker testa, which probably contribute to the water impermeability of seed coat of dormant pea genotypes. Also, there are considerable differences in the pigmentation and texture surface of individual RIL lines. As denoted from this study analysis, after 10 days of imbibition, JI64 seeds were grey pigmented, gritty and imbibed slowly and this is in contrast to JI92. Also higher proportion of RILs derived from crosses of JI92 and JI64 imbibed easily at a ratio of 60 – 100 percent while in RILs from reciprocal JI64 and JI92 crosses more lines imbibed slowly at 0 – 40 percent ratio. These findings are in agreement with previous studies conducted on pea by (Smýkal *et al*. 2014) where RILs from JI92 (or cv. Cameor) imbibe readily and germinate within 24 h and wild pea (JI64) seeds remain highly dormant, imbibe and germinate at 8 percent or 30 percent levels, respectively (for VIR320) after 7 days.

Based on previous findings on several legumes such as pea, common bean, chickpea, and faba bean, it was demonstrated that seed coat pigmentation was shown to correlate with imbibition ability. The presence of proanthocyanidins (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, Balarynová *et al.* 2022). In soybean, 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. On the other hand, similarly to seed coat thickness, its pigmentation is not directly involved in seed dormancy, as this trait can be decoupled by genetic crosses in respective RIL lines (Hradilová *et al*. 2017, Balarynová *et al*. 2022). However, I assumed that those seeds from RILs used in this analysis which did not imbibed even after 10 days, will definitely imbibe if these seeds are scarified and soak in water for a longer period of time under a right environmental condition.

In other to infer the association or discrepancies between JI64 and JI92 as well as their respective RILs to the thickness of seed coat and imbibition rate, a statistical analysis was done. In the statistical analysis between the alleles of RILs of JI64 x JI92 genotype, the result shows a

seed coat thickness between 160-180 µm, have a strong negative correlation with imbibition while the result from its contrasting RILs of JI92 x JI64 genotype shows a seed coat thickness between 140-160 µm, indicate a moderate negative correlation between both variables. Based on the result analysis, seed coat of JI92 x JI64 RILs have a lesser seed coat thickness and higher imbibition rate in comparison to JI64 x JI92 RILs. Inferring that seed coat thickness might influence the level of imbibition.

The genetic diversity analysis based on sequencing analysis of respective calcineurin genes, has shown monomorphic diversity of all tested *P. abyssinicum* accessions (7) which is in line with previous results of strong genetic bottleneck (Trněný *et al.* 2019). Notably, both tested genes showed sequence variability in domesticated, non-dormant pea genotypes, excluding domestication based selection as expected for key genes. Thus no single allele was detected in the panel of geographically structured cultivated pea genotypes. On the other hand, due to the length of the entire gene (3 kb) we have sequenced only an 850 bp fragment. Altogether, this suggests that calcineurin-like gene(s) might be associated with seed coat permeability and thus dormancy, but not being causative. Further analysis using genetic complementation is needed to exclude or support this hypothesis. Supporting evidence for the role of at least Psat2g013240 gene comes from RNAseq analysis of seed coat samples from several developmental stages of wild and cultivated genotypes, including JI92 and JI64, with significantly higher expression in wild pea sample (Balarynová, Smýkal, personal communication). Moreover, it has to be mentioned that genetic mapping was based on Afghan type landrace (JI92) and wild pea (JI64) while reference genome is from modern type pea cv. Cameor. It is known that there is chromosomal inversion between wild and cultivated pea, as well as we cannot rule out the possibility that the respective gene(s) is even not in modern pea genome. Thus wild pea genome would be highly desirable for this analysis.

7. CONCLUSION

This thesis tested the loci conditioning the dormancy of the wild ancestor of pea (*Pisum elatius* L.) using two contrasting parental genotypes (JI64 and JI92) and two homologues genes (Psat2g024960 and Psat2g013240) encoding calcineurin-like proteins. Genetic mapping based on genome - wide association mapping on RIL population identified the loci at chromosome 2, which is assumed to be associated with hardness in seed coat. This corresponds with previous studies on soybean mapping analyses (Sun *et al.* 2015). Anatomical analysis of the palisade cells length, revealed differences in parental genotypes which contribute to overall testa thickness. JI64 genotype has significant thicker testa, which contribute to the water impermeability in seed coat of dormant pea genotypes in comparison to JI92 genotype. However in the case of RIL lines, seed coat thickness did not completely agree with dormancy indicating that dormancy is not only mediated by seed coat thickness. Statistical analysis inferred that, there is a relationship between the seed coat thickness and seed dormancy. Dormant RILs had thicker testa (mean of 114 μm) than non-dormant RILs (mean of 129 μm). However for conclusive evidence of relationship, entire RIL population (130 lines) would need to be tested as shown by Hradilová *et al*. (2017). Candidate gene allelic analysis in panel of recombinant inbred lines indicated association between Psat2g013240 gene and the level of imbibition. These results suggest that Psat2g013240 gene of wild pea genotype (JI64) is related with dormancy. However sequence variability detected in set of domesticated, non-dormant pea genotypes excluded domestication based selection as expected for key genes. Thus respective gene might be associated but not causative with dormancy status in pea.

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