Fine-mapping of the major locus for vicine and convicine in faba bean (*Vicia faba* L.) and application of the findings for winter faba bean breeding

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Fine-mapping of the major locus for vicine and convicine in faba bean (*Vicia faba* L.) and application of the findings for winter faba bean breeding

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1.1 General introduction of *Vicia faba* (L.)

Faba bean (*Vicia faba* L.) is an annual legume crop with high agronomic value (Köpke and Nemecek 2010). Faba beans can fix high amounts of nitrogen compared to other legumes and therefore contribute to a positive N-balance in the soil (Schmidtke and Rauber 2000). In addition, for many rotations they have a phytosanitary effect on the soil (Duc et al. 2015). This makes faba bean a good choice for farmers, especially in organic farming (Köpke and Nemecek 2010). In addition, faba beans have a high seed protein content of 25-30% protein in dry matter (Link et al. 1994; Heuzé et al. 2016). Other agronomically beneficial characteristics of faba bean are its comparably high yield potential of about 70 dt/ha (Link et al. 1994; Jeroch et al. 2016), the high shattering resistance of its pods and its relatively low lodging tendency (Köpke and Nemecek, 2010). These features, among others, make faba beans a valuable source for food, especially in Mediterranean countries and China, and feed, especially for industrialized countries like Germany (Crépon et al. 2010).

Further, Europe is highly dependent upon protein sources from abroad (BMEL 2022). The increased cultivation of native crops with high protein contents such as faba bean has the potential to improve this situation. Consequently, the German Ministry for Nutrition and Agriculture (Bundesministerium für Ernährung und Landwirtschaft; BMEL) supports pertinent research and development in its "funding strategy for protein plants" ("Eiweißpflanzenstrategie") to thus increase the domestic production of protein from native and local crops (BMEL 2022).

Besides its many advantages, faba bean also have negative agronomic characteristics. Most prominent is its lacking yield stability, i.e. its sensitivity to biotic and abiotic stresses (Ghaouti et al. 2007). Faba bean seeds contain diverse anti-nutritive compounds, amongst others, tannins, vicine and convicine (VC), lectine, saponine, trypsine-inhibitores, and pythic acid (Römer, 1998). Most notable are tannins, which are solely found in the seed coat, and the pyrimidin derivates vicine and convicine (Ghaouti et al. 2007; Crépon et al. 2010).

It is necessary to improve faba beans by breeding efforts to enhance the faba bean cultivation in agriculture. To this aim, this work's focus is on the aspect of VC in faba bean, on the potential agronomic relevance of these compounds, on their genetic background and, consequently, on the possibility of breeding faba beans with markedly lowered VC amounts.

1.2 Botanic and breeding characteristics of *Vicia faba*

Faba beans, of genus *Vicia* and species *faba*, belong to the family of *Fabaceae* and are taxonomically grouped mainly according to their seed size into the sub-species *ssp. minor* (small seeded with 1000-seed weight less than about 500 g), *ssp. equinor (*1000-seed weight between 500g and 1000g*)*, and *ssp. major* (1000-seed weight more than 1000 g; Diepenbrock et al. 1999, Hebblethwaite 1983). The

small seeded faba beans are mainly used as high protein compound in feed, while medium sized seed and big seeded faba beans are mainly used a food (Duc et al., 2015; Link et al., 2008).

Faba beans are annual crops, which live in symbiosis with bacteria of the genus *Rhizobium* (Hebblethwaite 1983; Roskothen 1989). The plants form, in combination with the bacteria, nodules on the roots in which the bacteria can convert atmospheric nitrogen into ammonia, which is used by the plant as a nitrogen source (van Rhijn and Vanderleyden 1995). Faba beans can become as tall as two meters. They have pinnate leaves and inflorescences, which are set in racemes and consist of two to eight flowers (Hebblethwaite 1983). Flower color is variable; wild-type faba beans have white petals and a prominent black spot on the wing petals (Link et al. 2008). Faba bean pods can be up to 18cm in length and carry two to seven seeds (Hebblethwaite 1983; Link et al., 2008).

Faba beans are diploid, with 2n=12 chromosomes as opposed to other diploid *Vicia* species, which have 14 chromosomes (e.g *Vicia dumetorum, Vicia ervilia, Vicia sativa, Vicia narbonensis, Vicia michauxii, Vicia peregrina*; Heitz, 1931). The genome size of *Vicia faba* is large with 13,000 Mb (Satovic et al. 2013). Faba beans propagate by partial allogamy, a mixture of self- and cross-fertilization. The degree of cross-fertilization varies between faba bean genotypes, locations and cultivation years from about 30-60% (Link 1990; Suso and Moreno. 1999; Ghaouti 2007) and is facilitated by bumble-bees (*Bombus* spp.), honey bees (*Apis mellifera)* and solitary bees (Hebblethwaite 1983; Suso and Moreno 1999; Brünjes and Link 2021, Marzinzig et al. 2018). Plants with low auto-fertility require pollinators for self-fertilization, which can be spontaneous, or pollinators can facilitate it. The plants' ability to self-fertilize spontaneously (i.e., without stimulus via pollinators) is called auto-fertility (Link 1990). Faba bean genotypes exhibit a wide range of auto-fertility from 1% (near auto-sterility) to 98% (near complete auto-fertility; Link 1990). Auto-fertility and cross-fertilization have been shown to be negatively correlated and are additionally influenced by the heterozygosity status of the plant (Link 1990). This signifies that inbred plants tend to cross-fertilize while non-inbred plants tend to self-fertilize.

Breeding of faba beans employs methods of line breeding and population breeding (Adhikari et al. 2021; Duc 1997; Link et al. 2008; Becker 2011). Faba bean cultivars are frequently bred as synthetic cultivars (synthetics; Link 2009, Becker 2011), which are a specific form of population cultivars. They exploit heterosis as well as heterogeneity to produce high and relatively stable yield (Link 2009; Stelling et al. 1994). Synthetics are open pollinated populations created via selected parental components, for which the optimal number is about six or higher (Gasim et al. 2004; Link and Ederer 1993).

There have been multiple attempts to conduct hybrid breeding with faba beans and therefore to establish a working CMS system for this crop. However, due to a lack of stability of the pollen

sterility (Link 1998, 2009; Pfeiffer et al. 1993) this was not possible until today, even though a CMS system and hybrid breeding could unfold high potential for faba beans.

1.3   Winter and spring faba beans

In Europe, north of Pyrenees and Alpes, there are two seasonal types of faba beans – autumn-sown winter faba beans and spring-sown spring faba beans. The majority of faba beans varieties are spring sown faba beans (Bundessortenamt, 2022). Spring faba beans are sown between end of February until mid-April and are then harvested by August-September (Sauermann and Sass 2016). Winter beans are sown in autumn from the end of September to the end of October, preferably in regions with usually mild winters. The harvest of winter beans is then in August. As young plants, spring faba beans have a frost tolerance, which allows them to survive temperatures around -6°C while winter faba beans survive temperatures around -16°C (Herzog 1988; Link et al. 2009).

Winter beans comprise advantages over spring beans; their drought stress tolerance as well as their yield tend to be higher, due to head start in spring and their longer vegetation period (Flores et al. 2012). A negative aspect is the risk of winter kill (Link et al. 2010; Flores et al. 2012), since their ability to survive harsh winter conditions is not only dependent on their frost tolerance, but also on their resistance against further abiotic and against biotic stresses during winter and early spring time (Flores et al. 2012). In addition, the sowing before winter raises the likeliness of frost damage upon the roots of the plants and with that the possibility of infection of the roots by *Fusarium* (Link 2010). German winter faba beans tend to be taller than spring faba; this feature can be a disadvantage as it raises the likeliness of lodging (Link 2010).

Further breeding efforts on winter faba beans are therefore necessary. There are mainly four breeding companies in Europe currently working on winter faba bean breeding, namely NPZ Lembke in Germany, Gleisdorfer Saatzucht in Austria, Agri-Obtentions in France, and Senova in Great Britain.

1.4   Pests and diseases of *Vicia faba*

Occurance of certain weeds, pests and diseases usually complicate the cultivation of *Vicia faba*. Even though faba bean shows a superior ability to compete with weeds compared to other pulse crops such as chickpea, weeds can be a major limitation for faba bean growing and can reduce yield by up to 50% (Frenda et al. 2013; Karkanis et al. 2018). Therefore, to prevent faba beans to be overgrown by weeds especially in its juvenile stages, herbicides are applied on conventional faba bean growing (Ghaouti et al. 2015; Ghaouti et al. 2016; Karkanis et al. 2018), or extensive mechanical weeding is necessary. Additionally, in Mediterranean countries, faba bean is frequently parasitized by broomrape species such as *Orobanche spp*. and *Phelipanche spp.*, with *Orobanche crenata* Frosk (bean broomrape) being the main offending species (Karkanis et al. 2018; Pérez-de-Luque et al. 2010).

The broadbean seed beetle (*Bruchus rufimanus*) poses increasingly problems for seed production in faba bean (Pöhlitz & Reike 2019). The beetles deposit their eggs on the surface of the very juvenile pods. Here, the larvae find their way through the pod wall into the growing seed, where they grow until they hatch from the mature seeds as adult beetles, leaving round holes in the seeds that greatly limit the usability of the seeds for human consumption (Roubinet 2016).

Fungi can also lead to problems. Fungal infections on shoot, such as chocolate spot (caused by *Botrytis fabae*)*, Ascochyta* blight (caused by *Ascochyta fabae),* rust disease (caused by *Uromyces viciae-fabae*) and on root, such as *Fusarium* species (which can lead to wilting) can infect faba bean plants, especially in wet weather conditions (Karkanis et al. 2018). They can cause up to 60% yield loss, if counter measures, such as fungizides, are not applied in time (Karkanis et al. 2018).

An additional problem for faba bean cultivation is the so-called legume fatigue ("Legumi-nosenmüdigkeit"; bioaktuell.ch 2016). Legume fatigue describes yield losses of legumes on a field in which legumes have been grown for an extended period, and consequently, pests such as nematodes and fungi which specifically infect legumes have accumulated in the soil (Diepenbrock et al. 2016).

1.5   Anti-nutritive compounds of *Vicia faba*

1.5.1   Tannins

The seed coat of *Vicia faba* contains secondary metabolites called tannins (Crépon et al. 2010), which have anti-nutritive effects upon monogastric animals (Jeroch et al. 2008). Tannins reduce the digestibility of proteins as well as the activity of the enzymes α-amylase und cellulase (Duc 1997; Griffiths 1981).

The mean amount of tannin in faba beans seeds is 5-10g/kg dry matter; however, this degree can vary with the genotype (Duc et al. 2015). Tannins can be removed from faba bean seeds either by dehulling, since tannins are solely found in the seed coat, or by breeding. The two alleles of two independent genes zt1 and zt2 cause the zero-tannin phenotype, which is recessive (Duc et al. 2015, Link 2009). Tannins are a pleiotropic trait. Genotypes with tannins also show colorful flowers, brownish-buff seed coats and the presence of a black dot on the lower surface of the stipule (Link et al. 2008, Link 2009). Joint absence of these features has its cause in the same gene: the zero-tannin phenotype shows purely white flower color, a grey tinge of the seed coat and the absence of that black dot (Link et al. 2008). For further discussion, see Khazaei et al. (2018).

While zero-tannin faba beans can be beneficial for feed production, they can have agronomic disadvantages. Zero-tannin faba beans are during germination and emergence more susceptible against soil-borne pathogens than their tannin containing counterparts (Link and Hempel 1992).

1.5.2 Vicine and Convicine

The anti-nutritive pyrimidin derivates vicine and convicine (VC) can be found in the seeds as well as in in all other plant parts of *Vicia faba*. VC can only be found in *Vicia spp.* and in the bitter melon *Momordica charantia* (*M.c.*) specifically, no other legume species produces VC (Gauttam and Kalia 2013). However, convicine is next to non-existent in *M.c.,* with a ratio of vicine to convicine of approximately 20 to 0.005 (Khazaei et al. 2019). VC amount is highest in young faba bean seeds that are still green and vicine and convicine occur in a ratio of about 2:1, which stays stable during plant development (Crépon et al. 2010).

VC are problematic as they can cause oxidative stress upon ingestion. The enzyme β-glucosidase facilitates the transformation of VC to divicine and isouramile (Crépon et al., 2010). In humans with a genetic glucose-6-phosphat-dehydrogenase deficiency (G6PD), divicine and isouramil can cause hemolytic anemia (called favism; Crépon et al., 2010). Favism can most often be found in Mediterranean countries as well as the Middle East and the south of China (Crépon et al., 2010).

The enzyme β-glucosidase, which is responsible for the metabolic processing of VC, is present in pods and seeds of faba bean and can be inactivated by cooking or drying of the beans or through a treatment with acid (Crépon et al., 2010). As the human gene responsible for human G6PD deficiency lies on the X chromosome and the gastric acid is at a young age of humans often not strong enough to deactivate the enzyme, young boys suffer most often from this disease (Crépon et al., 2010). Interestingly, G6PD deficiency plus ingestion of VC can provide under specific conditions some protection against the tropic disease malaria (Link, 2009; Crépon et al., 2010; Arese et al. 2012).

1.6 Project Abo-Vici

The presented project and work was conducted within "Abo-Vici", a project founded by the German Ministry for Nutrition and Agriculture (BMEL) as part of its "funding strategy for protein plants" ("Eiweißpflanzenstrategie", BMEL 2022). The goal of the project was to gain more knowledge about the "breeding and agronomy of low VC faba beans for native protein feed" during the years 2017-2020 (Abo-Vici 2022).

The project aimed to improve the acceptance and usage of faba beans to produce protein-rich feed. To this end, the genetics behind VC as well as its potential agronomic implications had to be examined. Also, the project set the goal of breeding the first-ever low VC winter faba bean population, since winter faba beans promise higher yield than spring faba beans and could therefore, combined with a low VC feature, substantial raise the importance of faba bean (Abo-Vici 2022).

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Chapter 2: Zooming into the genomic vicinity of the major locus for vicine and convicine in faba bean (*Vicia faba* L.)

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2.1   Abstract

The versatility of faba bean (*Vicia faba* L.) seed as valuable protein feed is hampered by its relatively high level of the compounds vicine and convicine (VC), which are antinutritive factors in poultry and further non-ruminant feed. The objective here was to develop the first-ever genetically low-VC winter faba bean. Hence, the low VC allele vc-, that should be the basis of a known, major locus for VC, needed verification and molecular identification and be based on appropriately developed DNA markers; the low VC feature awaited its transfer into the high-performing winter faba bean germplams. Based on bi-parental F2-families and isogenic lines, we developed highly useful SNP markers exploiting transcriptomic data. Furthermore, we fine-mapped and, based on synteny to *Medicago truncatula* and *Cicer arietinum*, identified a candidate gene for the VC locus. A novel, genetically low VC winter faba bean population was bred. With these advances, the path was paved for further marker-based breeding progress.

2.2   Introduction

Faba bean (*Vicia faba* L.; *V.f.*) is a large-seeded, annual grain legume which is grown for food and feed. It is appreciated for its high protein content of approximately 30% (Crépon et al. 2010; Link et al. 1994). In Germany and Europe, it is one of the very sought-for sources of domestic, vegetal protein and it is an alternative crop to improve soil fertility (Köpke and Nemecek 2010; Kulak et al. 2013). Faba beans are cultivated in all non-tropical regions, the global area is about 2.4 million ha. In Germany, faba beans are grown on only approximately 50.000 ha (Statistisches Bundesamt 2020).

Genomic tools are still underdeveloped for faba bean (Annicchiarico et al. 2017); a well annotated sequence of its huge genome (13 Gb) is not yet available. Therefore, the high syntenic correspondence of *Vicia faba* to the sequenced genomes of *Medicago truncatula (M.t.), Cicer arietinum (C.a.)* and further legumes of the subfamily *Faboideaei* is of high importance for genomic analyses (Adhikari et al., 2021).

In addition to yield instability, the presence of the antinutritive compounds vicine and convicine (VC) limits the agronomic relevance and repute of faba bean (Adhikari et al., 2021). VC are two very similar pyrimidine glycosides. Where the pyrimidine of vicince holds an amino group, the pyrimidine of convicine holds a hydroxy group. Both compounds occur in faba bean seeds, their sum is approx. 0.3% up to 1.5% of the dry matter. This quantitative variation has a clear genetic component (Duc 1997; Frauen et al. 1984; Khamassi et al. 2013). Vicine and convicine occur in an approximate 2:1 ratio in faba bean seeds (Goyoaga et al. 2008). Faba beans contain VC in all parts of the plant (Goyoaga et al. 2008). These two compounds are nearly unique to the genus *Vicia*; *Momordica charantia* (*M.c.*) is the only species outside this genus containing vicine (Gauttam and Kalia 2013; Khazaei et al. 2019). Upon ingestion, vicine and convicine release divicine and isouramil (Hänsel and Sticher, 2007), which both cause oxidative stress for the red blood cells. Stress relaxation involves the activity of glucose-6-phosphate-dehygrogenase (G6PD).

More than 400 million people are, upon ingestion of vicine and convicine, at risk of suffering from hemolytic anemia, called favism, which is caused by a human X-chromosomal inherited genetic deficiency of G6PD. This human genetic condition has a delicate epidemiological connection to malaria (Arese 2006; Arese et al. 2012; Luzzatto and Arese 2018). Vicine and convicine can also have negative effects on monogastric livestock such as laying hens and broilers (Guillaume and Bellec 1977; Halle 2006; Larbier and Leclercq, 1994; Marquardt et al. 1981; Munduuli et al. 1981; Muduuli et al. 1982; Naber et al. 1988; Olaboro et al. 1981a; Olaboro et al. 1981b).

Duc et al. (1989) reported the presumably monogenetically inherited low vicine and convicine (LVC) content of the genebank accession 1268(4)(1) from Radzikov (Poland), which has since been used in breeding and research. This accession showed a seed content of 0.046% of VC, this is about 1/10 to 1/20 of the wild type seed content. That reduction is controlled by one locus, designated as VC locus, with alleles VC+ (wild type) and vc- (from genebank accession 1268(4)(1)). The low VC level in homozygous vc-/vc- faba bean genotypes prevents favism in G6PD-deficient humans (Gallo et al. 2018) and prevents dietary disadvantages if such faba beans are used as compounds of feed (Crépon et al. 2010). Heterozygosity at the VC locus causes approximately intermediate VC values.

Based on scattered hints in earlier literature, vicine and convicine are assumed to be formed in the faba bean seed coat and transported into the embryo (Duc et al. 1989; Brown and Roberts 1972).

The biosynthetic pathway to vicine and convicine was unknown until recently, when Björnsdotter et al. (2020) presented new, pioneering findings, which provided convincing experimental evidence that a bi-functional RIBA1 protein, which catalyzes the first step of the riboflavin biosynthetic pathway, also catalyzes the key step of the so far unresolved vicine and convicine pathway. Starting with the GTP cyclohydrolase II function of this protein, the two pyrimidine glucosides are synthesized from GTP as demonstrated by feeding isotopically labelled GTP precursor into roots of *Vicia faba*. However, the additional enzymatic steps, which finally lead to the compounds vicine and convicine, remain to be elucidated, although the authors propose that VC are synthesized in three additional steps from the first two intermediates in the riboflavin pathway, respectively, the first of which is probably catalyzed by an N-glycosidase described by Frelin et al. (2015). Björnsdotter et al. (2020) could also show that the allele vc- of the gene encoding the RIBA1 protein at the VC locus has its GTP cyclohydrolase II function destroyed by a frameshift mutation. This gene was consequently named VC1.

A major objective of research of VC characteristics is to facilitate breeding of faba beans which are genetically low in vicine and convicine seed content. The black and white hilum colors of faba bean seeds, a monogenic trait (Sirks 1931), can be used as a morphological marker to select for LVC status. The accession 1268(4)(1), source of the LVC feature, displays grains with white hilum. The linkage between the VC locus and the hilum color locus is relatively tight (5-10cM; Duc et al. 2004; Khazaei et al. 2017). Yet, white versus black hilum is only visible late in the life of plants, when their seeds are mature. In an early attempt to overcome this limitation, Gutierrez et al. (2006) developed two CAPS markers by bulked segregant analysis, which were linked to the VC locus. Later, Khazaei et al. (2015) were able to map the VC locus as a major QTL on the large first chromosome of faba bean. The QTL mapped in an interval of 3.6 cM, flanked by two pairs of co-segregating SNP markers. Unfortunately, the two markers closest to this mapped QTL (0.8 cM) proved to be not diagnostic for the trait in a larger set of diverse genotypes.

The genetic map used for the mapping of the VC locus had been constructed with SNP markers (Khazaei et al. 2014) that were a subset of a larger set of 687 SNP markers which were used to construct a comprehensive consensus map for faba bean (Webb et al. 2016). Most of these SNP markers had been developed based on selected mRNA contigs of faba bean that could be unambiguously assigned to a corresponding gene of *M.t.* by sequence comparisons. These markers therefore allow comparative mapping between faba bean and *M. truncatula.* Khazaei et al. (2015) used this approach

to identify a region on chromosome 2 of *M.t.* with strong collinearity to the region in faba bean carrying the VC locus. A total of 340 genes were found in *M.t.* in the interval delimited by the markers flanking the VC locus. However, due to lacking knowledge about the biosynthetic pathway for vicine and convicine, it was then not possible to identify a candidate gene for the VC locus. In an early approach to identify candidate genes for the VC locus, Ray et al. (2015) identified six contigs that were differentially expressed in immature seed coats of high and low VC genotypes. One of these contigs, contig 4518, was later located by Khazaei et al. (2017) in *M.t.* in the target region for the VC gene on chromosome 2. The contig 4518 corresponds to the *M.t.* gene Medtr2g009270, which is annotated as 3,4-dihydroxy-2-butanone 4-phosphate synthase. In soybean and chickpea, the same gene is annotated as RIBA 1, a bifunctional riboflavin biosynthesis protein (Khazaei et al. 2017) that has two catalytic domains, one with a 3,4-dihydroxy-2-butanone 4-phosphate synthase activity and a second one with a GTP cyclohydrolase activity. Based on contig 4518, Khazaei et al. (2017) developed an SNP marker whose segregation completely matched the segregation of VC content in a RIL population from a cross between a high and low VC genotype and in a set of 51 diverse genotypes. A comprehensive and detailed review on the current classical and molecular genetic background of the VC feature of faba bean was very recently presented by Khazaei et al. (2019).

Here, the main objectives were to develop additional, new SNP markers with close linkage to the VC locus via fine-mapping and to locate and preferably to identify the VC gene in our genetic material. Moreover, we aimed to employ all findings to develop a novel, LVC winter faba bean germplasm for breeding and research purpose.

2.3   Material and Methods

2.3.1   Plant material

Two pairs of near-isogenic lines (NILs) with contrasting VC status (high VC versus low VC) were utilized for mRNA analysis. Based on pedigree and phenotype, their contrasting VC status was caused by homozygosity for VC+ and vc-. Additionally, both pairs were used as crossing parents to develop two F2 mapping populations (Cross1, Cross2). These two NIL pairs were themselves generated from two crosses of a high VC (HVC) and a LVC parental line, respectively. The first NIL pair originated from the cross between the inbred line Mélodie/2 (LVC) and ILB938/2 (HVC). Mélodie/2 was bred via single-seed descent (SSD) originating from the French LVC cultivar Mélodie, whereas ILB938/2 was bred via SSD from the HVC gene bank accession ILB938 (Khamassi et al. 2013; Khazaei et al. 2015). The second NIL pair was accordingly generated from a cross facilitated by NPZ (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG), with the cultivar Fabelle as LVC parent. From both crosses, recombinant inbred lines (RILs) were developed until generation F5 (Khazaei et al. 2015). Here, one rare F5-individual per cross was identified which, upon selfing, still segregated for VC content and included homozygous HVC and LVC individuals. Two convenient, VC contrasting individuals were maintained by self-fertilization and became one pair of NILs. One such pair of NILs was derived from each of the two initial crosses. According to Mendel's rules, a degree of isogeneity of 15/16 is expected for each NIL pair.

Cross 1:

● "Mél/2*ILB938/2-139-1 (LVC)"

● "Mél/2*ILB938/2-139-2 (HVC)"

Cross 2:

● "NPZ-848-3 (LVC)"

● "NPZ-848-4 (HVC)"

2.3.2   Creation of mapping populations

To create the two F2- mapping populations, the F_1 of Cross 1 and of Cross 2 were self-fertilized (under pollinator-proof conditions). These two mapping populations contained N=751 (Cross 1) and N=899 (Cross 2) F_2- individuals. The two F_2 populations where subsequently used to generate linkage map fragments and to conduct fine-mapping analyses. Seed coat tissue from the two parental NIL pairs was collected and used for mRNA analyses.

2.3.3 Plant material for breeding novel, winterhardy, LVC faba bean lines

This breeding program started in 2006 by crossing Hiverna/2 and Mélodie/2. Hiverna/2 had been bred via SSD from Hiverna, an old, very winterhardy German winter faba bean cultivar with a HVC phenotype (Bundessortenamt 2019).

Until 2015 and generation BC_2F_2, the selection was carried out via hilum color among F_2, BC_1F_2 and BC_2F_2 individuals. Therefore, for actual backcrossing, corresponding F_3, BC_1F_3 and BC_2F_3 individuals had to be used, meaning an additional generation had to be grown until further backcrossing was possible. Starting in BC_3F_2, selection was based on VC assessment in mature seed via high liquid pressure chromatography (HPLC) analyses (see below). Further on, we employed marker assisted selection for the subsequent next backcrossing program, hence speeding up the procedure.

2.3.4 mRNA sampling, RNA-Isolation, transcriptome, and SNP analysis

For mRNA analyses, seed coat material of immature seeds was collected from the isogenic of Crosses 1 and 2 (Figure 1), in their developmental stages four, five and six (Borisjuk et al. 1995).
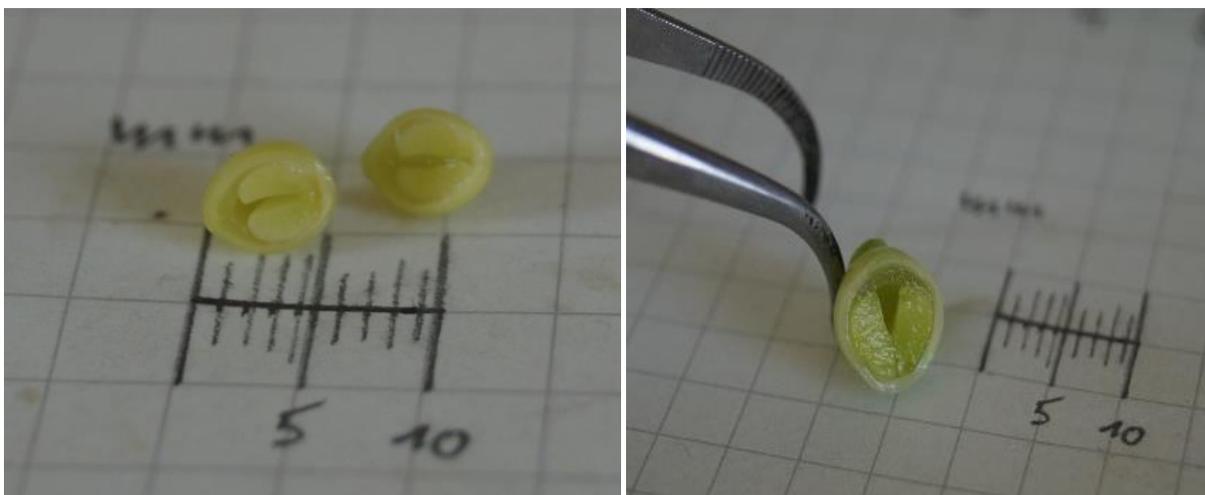
Figure 1. Cross-section of a *Vicia faba* seed in developmental stage 4 (left) and stage 5 (right).

The frozen tissue was ground to a fine powder under liquid nitrogen conditions using a mixer mill (MM200, Retsch, Haan, settings 30 Hz for 2 min). Total RNA was isolated with the InviTrap® Spin Plant RNA Mini Kit (STRATEC SE, Birkenfeld). Aliquots of this RNA were treated with Baseline Zero™ DNase (Biozyme Scientific GmbH, Hessisch Oldendorf) according to the manufacturer's instructions to eliminate contaminating gDNA, purified using the MinElute Cleanup Kit (Qiagen, Hilden) and finally photometrically measured (ND-1000, NanoDrop Technologies, Wilmington, USA). The RNA integrity number values of all RNA samples to be sequenced were determined using a 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany). Individual RNA samples for transcriptome analysis were converted into 3´-prime specific MACE libraries (Bojahr et al. 2016) and sequenced with 1x 75 b reads on a HiSeq 2000 machine (Illumina) resulting in 3,1 - 5 million quality filtered reads. For each line of the NIL pairs, RNA samples which derived from seeds of the developmental stages 4 and 5 were pooled on a ratio of 1:1 and subsequently converted into RNASeq libraries and paired-end sequenced at 2x75 b on a Hiseq2000 device yielding 64-77 million quality filtered reads per sample. Combined high-quality reads from all samples were used for a *de novo* assembly and annotation of a duplicate

free testa-specific reference transcriptome as described in Santos et al. (2018). The software package JointSNVMix was used to detect single nucleotide variants (Roth et al. 2012). For SNP candidates, a locus must have had at least five reads in both samples where one of two possible bases might occur (either allele A or B). A maximum of one percent false allele reads was tolerated. NGS template preparation, sequencing and bioinformatics took place at GenXPro GmbH (Frankfurt am Main). Transcriptome data evaluation for the identification of SNPs that correlated with the VC phenotype was performed stepwise: for the RNASeq and MACE libraries separately and for each of the two NIL pairs (parents of Cross 1 and Cross 2). For each genotype, MACE-data sets derived from developmental stages 4, 5, and 6 were pooled to increase sensitivity for SNP-detection. Subsequently, those SNP candidates which were common to both NIL pairs were identified.

2.3.5 SNP marker development

KASP (Kompetitive allele specific PCR) marker development, DNA extraction and KASP analysis were conducted at TraitGenetics (Gatersleben, Germany). KASP assay designs were based on app. 50 bps to the left and right side of SNPs found via the mRNA analysis.

2.3.6 Marker analysis and map construction

Throughout, the genetic linkage maps were constructed using R (R Core Team 2019) and the R package ASMap (Taylor and Butler 2017). For all linkage maps, the Kosambi function was used to calculate the map distances in cM (Kosambi 1943). The functions of the package were used as described by Taylor and Butler (2017). Missing marker data and marker segregation ratios were identified with the R Package ASMap. Markers that were monomorphic in a cross and markers showing strongly skewed segregations (-log(P) of 2.0 or higher) were excluded from further analyses. The selected SNP markers were used for the linkage map fragment construction.

2.3.7 Determination of VC content

Determination of VC values was conducted via high-liquid chromatography analysis (HPLC; Khamassi et al. 2013) by the National Institute of Agricultural Botany in England, NIAB institute (Cambridge, UK; NIAB 2021).

2.3.8 Fine mapping

For the manual fine-mapping of the sought-for VC-locus, HPLC VC results were displayed along with the SNP genotypes of the F_2 individuals of Cross 1 and Cross 2. The SNP markers were ordered according to their linkage map positions. The correspondences between genotype and phenotype were thoroughly studied.

2.3.9 Blast search of SNPs from fine-mapping

To determine candidate genes, *V.f.* specific KASP marker sequences were BLASTed against the genomes of *M.t.* (LIS - Legume Information System; https://legumeinfo.org/) and of *C.a.* to define their physical positions.

2.3.10 Analysis of synteny between *Vicia faba* and *Cicer arietinum*

To determine the synteny between *V.f.* and *C.a.* in the chromosomal interval of the putative VC locus, the syntenic positions of the markers in the final linkage map fragments on the *C.a.* genome were established. For this, the contig cDNA sequences from which the new markers were derived and the flanking cDNA sequences of the markers from the initial marker set (Webb et al. 2016) were BLASTed against the proteins of the reference sequence of *C. arietinum* (Varshney et al. 2013; NCBI: RefSeq assembly accession no. GCF_000331145.1). BLAST analyses were conducted using the blastx application from the NCBI BLAST+ package (v. 2.7.1). The positions of the transcripts encoding the respective proteins on the reference sequence were extracted from the annotation gff3 file of the sequence in R and assigned to the markers as syntenic positions. To extract all transcripts and the corresponding genes and proteins encoded in the region of the *C.a.* genome corresponding to the core region from the annotation file of the reference sequence, the intersect function of bedtools (v2.27.1) was used.

2.4 Results

2.4.1 SNP marker selection

For the marker-assisted selection, mapping and fine-mapping, markers within the region around the putative VC locus (Khazaei et al. 2015) were chosen and tested. The markers Vf_Mt2g005900_001 and Vf_Mt2g015010_001 from Khazaei et al. (2015) were used to serve as initial boundary markers to define the initial interval-of-interest and encompass all other chosen markers. In total, six markers from Khazaei et al. (2015), including the initial boundary markers, were employed. Ten markers from Webb et al. (2016) were added since they were determined to be in the interval-of-interest according to the physical placement of the initial boundary markers in the *M.t.* genome and the genetic map of Webb et al. (2016). Additionally, the marker SNP384 from Song (2017) was included. These 17 markers made up our initial marker set (Table 1) which was used for our marker assisted selection in the backcross and the construction of draft maps for Cross 1 and Cross 2.

Only subsets of the 17 markers (initial marker set) were polymorphic in the respective genetic backgrounds (Table 1). Most notably, the marker Vf_Mt2g015010_001, which served as initial boundary marker, was monomorphic. The polymorphic marker Vf_Mt2g013690_001 was hence chosen as substitute (see Table 1). So, markers Vf_Mt2g005900_001 and Vf_Mt2g013690_001 were subsequently used to define a search frame for the mRNA analyses (Table 2). All markers polymorphic in Cross 1 and Cross 2 were used for the construction of the draft maps. Only three markers were polymorphic

in all genetic backgrounds (Table 1, markers SNP384, Vf_Mt2g009320_001 and Vf_Mt2g010740_001) and their chromosomal vicinity was deemed as the most likely region to contain the VC gene, based on the data of Song (2017) and Khazaei et al. (2017), and was therefore the focus for our search for new SNP markers and the VC gene in the transcription analysis (Table 2). In addition, the 7 markers polymorphic in the backcross were used for marker assisted selection in the backcrossing program.

Table 1. Seventeen SNP markers from Webb et al. (2016), Song (2017) and from D. O'Sullivan (personal communication; initial marker set). Their function in Cross 1 and 2 and for the backcross is noted. Markers in yellow are the initial boundary markers and encompass the chromosomal interval-of-interest. Blue markers denote the region we suspected the VC gene to be located in. Markers are sorted after Webb et al. (2016).

SNP-Marker	Usability in families		
	Cross 1	Cross 2	Backcross
Vf_Mt2g005900_001*	X	-	X
Vf_Mt1g083330_001*	X	-	-
Vf_Mt2g007220_001*	X	-	-
Vf_Mt2g007390_001	-	X	X
Vf_Mt2g008150_001	-	X	-
Vf_Mt2g008180_001	X	-	-
Vf_Mt2g008226_001	X	-	-
Vf_Mt2g009230_001	-	-	-
SNP384	X	X	X
Vf_Mt2g009320_001	X	#	X
Vf_Mt2g010740_001*	X	#	X
Vf_Mt2g010880_001*	X	-	-
Vf_Mt2g010970_001	-	#	-
Vf_Mt2g011080_001	-	-	X
Vf_Mt2g013690_001	-	X	-
Vf_Mt2g013900_001	-	-	X
Vf_Mt2g015010_001 *	-	-	-

"*": Marker from Khazaei et al. 2015; "-" : not polymorphic; "#": removed from the analysis due to skewed segregation patterns; "X" used in draft maps and final genetic maps and for marker assisted selection

Joint analyses of RNAseq and MACE data of Cross 1 and Cross 2 sets revealed a total of 448 unique SNP candidates within 123 contigs (Table 2) between markers Vf_Mt2g005900_001 and Vf_Mt2g013690_001. These contigs could be assigned to chromosome 2 of *M.truncatula*. Of these, 135 unique SNPs from 35 contigs were found within the closer search frame between markers SNP384 and Vf_Mt2g010740_001. A total of 73 SNPs (range within markers Vf_Mt2g005900_001 and Vf_Mt2g013690_001) and 22 SNPs (range within markers SNP384 and Vf_Mt2g010740_001), respectively, were found within both data sets, RNAseq and MACE.

From this initial data set of putative SNPs, new markers were developed (Table 3). A number of 33 SNPs were taken from those 73 SNP candidates which mapped to the range between markers Vf_Mt2g005900_001 and Vf_Mt2g013690_001 onto the genome of *M.t.* (*M.t.* chr. 2; 390,001-

3,620,000 bp, see Table 2) and which were detected with both sequencing techniques, RNAseq and MACE. A further subset of 19 SNPs was selected from the 320 SNPs which were detected by means of RNAseq only (393 SNPs – 73 SNPs = 320 SNPs, see Table 2). Moreover, six SNP candidates whose corresponding contig sequences could not be mapped to the *M.t.* genome were included, chosen from the 57 SNPs mentioned in Table 2.

Table 2. Joint comparison of LVC vs. HVC sequence data from transcriptome analyses of Cross 1 and Cross 2. Given are the numbers of detected SNPs and corresponding contigs which were found in the specified selection ranges, with different sequencing methods and search frames.

	Range within markers					
Data source	Vf_Mt2g005900_001 and Vf_Mt2g013690_001 (*M.t.* chr. 2; 390,001-3,620,000 bp)		SNP384 and Vf_Mt2g010740_001 (*M.t.* chr. 2; 1,851,023-2,500,001 bp)		SNPs & contigs with base count/expression differences between LVC and HVC but not mappable to *M.t.*	
	SNPs	Contigs	SNPs	Contigs	SNPs	Contigs
RNAseq	393	112	117	30	55	21
MACE	128	59	40	17	4	4
Both, RNAseq & MACE	73	48	22	12	2	2
RNAseq & MACE, non-re-dundant	448	123	135	35	57	23

Of the 58 newly developed markers, 42 markers were used for the construction of the final linkage map fragment and fine-mapping of Cross 1 while 38 markers were used for Cross 2 (see Table 3), since these marker subsets were polymorphic and did not show skewed segregation patterns in the corresponding genetic background. For the backcross, 34 markers were polymorphic and therefore had the potential to be used in marker-assisted selection. Those markers of the initial marker set which were polymorphic for the genetic backgrounds of Cross 1 and 2 plus the above described newly developed markers formed the new marker set.

Table 3. Fifty-eight SNP markers derived from mRNA analyses (RNAseq and MACE). Their origin as well as their applicability in the three families (Cross 1 and 2 and backcross) is noted.

Marker	Filtering process	Usability in families		
		Cross 1	Cross 2	Back-cross
VFS18002.A03	From the 73 common SNP markers identified with both techniques, RNAseq and MACE (Table 3)	X	X	-
VFS18002.A04		X	X	X
VFS18002.A05		X	X	-
VFS18002.A06		#	X	X
VFS18002.A07		X	X	X
VFS18002.A08		X	X	X
VFS18002.A09		#	#	X
VFS18002.A10		#	#	-
VFS18002.A11		-	-	-
VFS18002.A12		X	X	X
VFS18002.A13		X	X	X
VFS18002.A14		X	X	X
VFS18002.A15		X	X	-
VFS18002.A16		X	X	X
VFS18002.A17		X	X	X
VFS18002.A18		#	#	-
VFS18002.A19		X	X	X
VFS18002.A20		-	-	-
VFS18002.A21		X	#	X
VFS18002.A22		-	-	-
VFS18002.A23		-	-	-
VFS18002.A24		X	X	-
VFS18002.A25		-	-	-
VFS18002.A26		X	-	-
VFS18002.A27		#	#	-
VFS18002.A28		X	-	X
VFS18002.A29		X	#	X
VFS18002.A30		X	X	X
VFS18002.A31		X	X	-
VFS18002.A32		X	X	-
VFS18002.A33		X	#	-
VFS18002.A34		X	X	-
VFS18002.A35		X	X	X
VFS18002.A36	From MACE & RNAseq-data, not mappable to *M.t.*	#	-	-
VFS18002.A37		X	-	X
VFS18002.A38		X	X	X
VFS18002.A39		X	X	X
VFS18002.A40		X	X	X
VFS18002.A41		X	X	X
VFS18002.A42	From RNAseq only, mapped to *M.t.*	X	X	X
VFS18002.A43		X	X	X
VFS18002.A44		-	#	X
VFS18002.A45		-	-	-
VFS18002.A46		-	X	-
VFS18002.A47		#	X	X
VFS18002.A48		X	X	X

Table 3 continued. Fifty-eight SNP markers derived from mRNA analyses (RNAseq and MACE). Their origin as well as their applicability in the three families (Cross 1 and 2 and backcross) is noted.

Marker	Filtering process	Usability in families		
		Cross 1	Cross 2	Back-cross
VFS18002.A49		X	X	X
VFS18002.A50		#	-	X
VFS18002.A51		X	X	X
VFS18002.A52		X	X	X
VFS18002.A53		X	X	-
VFS18002.A54	From RNAseq only, mapped to *M.t.*	X	X	X
VFS18002.A55		X	X	X
VFS18002.A56		X	#	-
VFS18002.A57		X	X	X
VFS18002.A58		X	X	-
VFS18002.A59		X	X	-
VFS18002.A60		X	X	X

"- ": not polymorphic; "#": removed from the analysis due to skewed segregation patterns; "X" used in final genetic maps and for marker assisted selection

2.4.2 Linkage maps

For the construction of the draft linkage maps, all F_2 genotypes present in the F_2 families were used, i.e., 751 individuals for Cross 1, and 899 individuals for Cross 2. The initial marker set that was used to create the draft maps is depicted in Table 1. The marker order in the draft maps followed nearly always the order of their physical placement in the genome sequence of *M. truncatula.* The new marker set gave the final linkage map fragments for Cross 1 and Cross 2.

Only informative F_2 genotypes were genotyped with the new marker set. Such informative F_2 genotypes had one or more putative recombination events as determined by their marker genotypes in the draft mapping. A total of 70 individuals for Cross 1 and 74 individuals for Cross 2 were thus re-analyzed with the new marker set. In addition, and as controls, the parental genotypes (10 plants each) of the respective cross were genotyped with the new marker set. The marker data of the remaining F_2 individuals were inferred based on results from the draft maps.

SNP results of the informative and control individuals were checked for missing data points and for plausibility of their numbers of single and double crossovers. Five F_2-individuals of Cross 1 and nine of Cross 2 were excluded based on more than 10 missing marker data per individual and on extremely unlikely recombination patterns, such as the apparent occurrence of two or more crossovers in both gametes within 3 cM. This cleansing combined with exclusion of markers with strongly skewed segregations (see "SNP marker selection", Table 3) reduced the length of the map fragments from app. 20 cM in a first, uncleansed approach to between 3 and 6 cM for the final linkage map fragments.

2.4.3   Fine mapping of the VC locus

For manual fine-mapping of the sought-for VC-locus, the informative F_2 plants of Cross 1 and Cross 2 were phenotyped (HPLC; via NIAB 2021) for their VC-content. As checks and benchmarks for the HPLC results, additional F_2 individuals, with marker-deduced homozygous and heterozygous VC genotypes, were identified and tested for their HPLC values (see Table 4).

Table 4. VC contents of marker-deduced homozygous and heterozygous F_2 individuals of Cross 1 and Cross 2.

Genotype of F_2 individuals		No. of individuals	VC range of HPLC values	Mean	Standard error	VC content category
Cross 1	Homozygous HVC	6	$0.54 - 0.89$	0.6428	0.0502	HVC
	Heterozygous	8	$0.47 - 0.49$	0.4798	0.0088	intermediate
	Homozygous LVC	5	$0.04 - 0.05$	0.0421	0.0015	LVC
Cross 2	Homozygous HVC	4	$0.51 - 0.61$	0.5520	0.0243	HVC
	Heterozygous	7	$0.32 - 0.34$	0.3311	0.0080	intermediate
	Homozygous LVC	5	$0.02 - 0.03$	0.0270	0.0020	LVC

The hypothetical VC genotypes of the informative F_2 plants were subsequently deduced from their VC phenotypes based on an established reference (Table 4) and matched with their respective SNP marker genotypes. For this purpose, SNP markers were arranged according to the final linkage map fragments (Figure 2). The F_2 individuals were grouped correspondingly as homozygous HVC, heterozygous and homozygous LVC. Crossovers were thus made visible (see Tables 5A and 5B).

It was therefore determined which crossovers were closest to the VC locus (see Tables 5A and 5B). The smallest possible SNP interval bearing the sought-for VC locus was determined for both crosses. This interval is referred to as the core region (depicted by orange background of marker names in Tables 5A and 5B).

Table 5A. Fine-mapping of the vicinity of the VC locus for Cross 1. F_2 genotypes of the respective individuals are sorted according to their haplotypes, with homozygous for SNP-allele (green, A) as detected in LVC parent on the left to heterozygous (yellow, H) in the middle to homozygous for SNP-alleles (red, B) as detected in HVC parent on the right. The respective HPLC analysed VC contents are given above with the individual numbers of genotypes. Marker names as well as their positions on the genetic linkage map fragments (see Figure 2) are displayed, thus crossovers are visualized. Orange colored marker names define outer boundaries of the interval which, according to this fine-mapping, contains the VC locus. The light green colored marker name depicts the SNP384 which was defined by Khazaei et al. (2017) as a diagnostic marker for VC content.

Position in genetic map (cM)	Marker	F_2 individuals					
		587	273	440	765	404	313
		VC content (HPLC; Total % Vicine/Convicine (dry weight))					
		0.046	0.044	0.483	0.468	0.582	0.561
		Marker genotypes					
2.28	VFS18002.A39	A	H	A	H	B	H
2.28	VFS18002.A49	A	H	A	H	B	H
2.48	VFS18002.A51	A	A	A	H	B	B
2.55	VFS18002.A12	A	A	H	H	B	B
2.55	VFS18002.A41	A	A	H	H	B	B
2.55	VFS18002.A52	A	A	H	H	B	B
2.55	SNP384	A	A	H	H	B	B
2.61	VFS18002.A54	A	A	H	H	H	B
2.61	VFS18002.A13	A	A	H	H	H	B
2.61	VFS18002.A53	A	A	H	H	H	B
2.68	Vf.Mt2g009320	A	A	H	H	H	B
2.75	VFS18002.A14	A	A	H	A	H	B
2.75	VFS18002.A55	A	A	H	A	H	B

Table 5B. Fine-mapping of the vicinity of the VC locus for Cross 2.

Position in genetic map (cM)	Marker	F_2 individuals					
		223	589	11	129	267	885
		VC content (HPLC; Total % Vicine/convicine (dry weight))					
		0.031	0.031	0.320	0.342	0.510	0.536
		Marker genotypes					
0.98	VFS18002.A39	A	A	H	H	H	B
1.10	VFS18002.A49	A	A	H	H	H	B
1.26	VFS18002.A51	A	A	H	H	B	B
1.26	VFS18002.A12	A	A	H	H	B	B
1.26	VFS18002.A41	A	A	H	H	B	B
1.26	VFS18002.A52	A	A	H	H	B	B
1.26	SNP384	A	A	H	H	B	B
1.26	VFS18002.A54	A	A	H	H	B	B
1.26	VFS18002.A13	A	A	H	H	B	B
1.37	VFS18002.A53	A	H	H	H	B	B
1.49	VFS18002.A14	A	H	H	B	B	B
1.49	VFS18002.A55	A	H	H	B	B	B

The region between markers VFS18002.A51 and VFS18002.A54 (Cross 1) was 0.14cM smaller than the region between markers VFS18002.A49 and VFS18002.A53 (Cross 2; Table 5A, 5B). All markers in this Cross 1 region were included in the region of Cross 2, and in the same order. VFS18002.A51 and VFS18002.A54 of Cross 1 (0.13cM) are consequently defined as the final boundary markers which define the core region where the VC locus must be located.
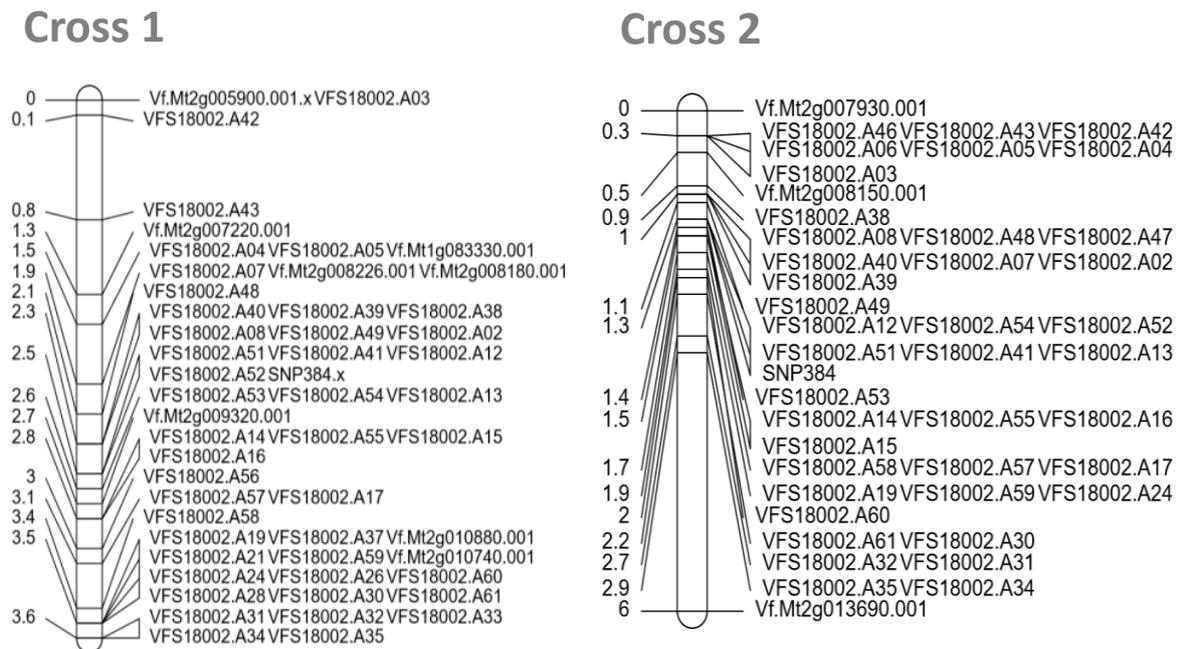
Figure 2. Final linkage map fragments for Cross 1 and Cross 2. Depicted are fragments of chromosome 1 of *V.f.* showing the putative region of the VC locus. Positions of markers are given in cM on the left, marker names for the respective positions are given on the right.

2.4.4 SNP marker validation for breeding novel, winterhardy, vc- faba bean lines

Out of the 7 markers in the initial marker set and the 34 SNP markers of the new marker set which proofed polymorphic for the backcross material, nine SNP markers were located inside the outer boundaries of the intervals in Cross 1 and Cross 2 (see Tables 5A and 5B). They were tested to identify the most suitable candidates for a marker assisted selection for LVC. The usability of these markers for the backcross material and the accuracy of the respective marker prediction for the VC content was verified with BC_3F_2 genotypes and their respective HPLC results (via NIAB 2021; Khamassi et al. 2013).

Eight out of these promising nine SNP markers proved to be precise selection tools for our backcrossing program, namely markers VFS18002.A49 to VFS18002.A13 (see Table 6) while one marker was not polymorphic (VFS18002.A53). Two markers did not match perfectly; they were indeed not included in this group of promising markers (markers VFS18002.A14 and VFS18002.A55; see Table 6).

Table 6. SNP Markers tested for marker-assisted selection potential in Hiverna/2 * Mélodie/2 back-cross material. Markers were selected from the fine-mapping of Cross 1 and Cross 2 (Tables 5A and 5B). They are ordered according to the fine-mapping of Cross 1 (Table 5A).

Marker	F_2 individuals of Hiverna/2 * Mélodie/2 backcross generation BC_3											
	VC content (HPLC; Total % vicine/convicine (dry weight))											
	0.031	0.037	0.040	0.035	0.406	0.408	0.415	0.548	0.554	0.596	0.603	0.629
	Marker genotypes											
VFS18002.A49	A	A	A	A	H	H	H	B	B	B	B	B
VFS18002.A51	A	A	A	A	H	H	H	B	B	B	B	B
VFS18002.A12	A	A	A	A	H	H	H	B	B	B	B	B
VFS18002.A41	A	A	A	A	H	H	H	B	B	B	B	B
VFS18002.A52	A	A	A	A	H	H	H	B	B	B	B	B
SNP384	A	A	A	A	H	H	H	B	B	B	B	B
VFS18002.A54	A	A	A	A	H	H	H	B	B	B	B	B
VFS18002.A13	A	A	A	A	H	H	H	B	B	B	B	B
VFS18002.A53	Monomorphic in the backcross material											
VFS18002.A14	A	A	A	H	H	H	H	B	B	B	B	B
VFS18002.A55	A	A	A	H	H	H	H	B	B	B	B	B

2.4.5 Putative candidate genes from synteny studies and from transcriptome analyses

The core region which was identified via fine-mapping of Cross 1 and Cross 2 (Tables 5A and 5B) was inspected for possible candidate genes. The sequences of the markers covering the core region were compared by BLAST algorithms to the genomes of *M.t.* and *C.a.* (see Table 7) and genes within the core region were noted (see Table 8).

Table 7. Markers of core-region (comp. Table 5A) and their physical position in the *M.t.* genome.

Position in *V.f.* (cM)	Marker name	Position in *M.t.* (bp)
2.48	VFS18002.A51	1 764 412
2.55	VFS18002.A12	1 828 300
2.55	VFS18002.A41	no match on Chr. 2*
2.55	SNP384	1 851 023
2.55	VFS18002.A52	1 852 191
2.61	VFS18002.A54	1 974 254

* Contig TRINITY_DN49974_c1_g1_i2 (319 bp), from which SNP VFS18002.A41 was deduced, overlaps with 117 bases (93% identity) with contig Contig14304 (SNP VFS18002.A52). Even though SNP 18002.A41 does not fall into the overlapping region, it is likely that this SNP also maps into this same region on Chr. 2.

A total of 30 genes was thus found in *M.t.*, while a subset of 17 of these could also be found in the corresponding region of *C.a.* (Table 8).

2.4.6 Breeding progress of novel, winter hardy, LVC faba bean lines

The backcrossing program, with the selection based on hilum color and on HPLC results to breed a winter hardy, LVC faba bean line, was carried out until 2017 and up to generation BC_3F_2. Thereafter, KASP markers were employed (Khazaei et al. 2015; Webb et al. 2015). Starting in 2018, we employed

KASP markers derived from our own transcription analyses. KASP marker predictions were verified by HPLC results (compare Table 4). Our markers allowed identifying VC+ /vc- heterozygous BC4F1 plants and directly employing them for further backcrossing (instead of using BC_4F_3), thus we could very markedly speed up the breeding process.

Table 8. Genes located in the region of *M.t.* and *C.a.* syntenic to the core region in *Vicia faba*.

Gene name	Start position *M.t.* genome (bp)	Product	Assessable in *C.a.* core region equivalent
MTR_2g009090	1 771 882	Splicing factor 3B subunit, putative	-
MTR_2g009110	1 780 752	Splicing factor 3B subunit 1	Yes
MTR_2g009130	1 796 938	Hypothetical protein	-
MTR_2g009140	1 801 324	DNA (cytosine-5)-methyltransferase	Yes
MTR_2g009150	1 807 363	Transmembrane protein, putative	-
MTR_2g009190	1 810 285	Minichromosome maintenance (MCM2/3/5) family protein	Yes
MTR_2g009200	1 822 351	Serine carboxypeptidase-like protein	-
MTR_2g009220	1 828 042	2-oxoglutarate/malate translocator	Yes
MTR_2g00923	1 833 036	L-ascorbate oxidase	-
MTR_2g009270	1.848.020	3,4-dihydroxy-2-butanone 4-phosphate synthase	Yes
MTR_2g009275	1 854 017	Transmembrane protein, putative	Yes
MTR_2g009290	1 860 925	Transmembrane protein, putative	-
MTR_2g009310	1 866 319	RING-H2 zinc finger protein	Yes
MTR_2g009320	1 869 086	Ubiquinol-cytochrome C chaperone protein	Yes
MTR_2g009330	1 877 274	Pyruvate decarboxylase	Yes
MTR_2g009340	1 883 129	Phototropic-responsive NPH3 family protein	Yes
MTR_2g009360	1 890 536	Transcription factor	Yes
MTR_2g009390	1 899 088	FKBP-type peptidyl-prolyl cis-trans isomerase	Yes
MTR_2g009410	1 904 618	Oligosaccaryltransferase	-
MTR_2g009415	1 905 205	tRNA-His	Yes
MTR_2g009430	1 911 762	Hypothetical protein	-
MTR_2g009450	1 915 096	Leguminosin group485 secreted peptide	Yes
MTR_2g009480	1 929 386	Leguminosin group485 secreted peptide	Yes
MTR_2g009500	1 937 541	Carbonic anhydrase family protein	Yes
MTR_2g009520	1 948 713	Chaperone DnaJ-domain protein	-
MTR_2g009530	1 952 263	Hypothetical protein	-
MTR_2g009550	1 957 898	ATP synthase subunit beta, putative	-
MTR_2g009560	1 958 801	F0F1 ATP synthase subunit beta	-
MTR_2g009580	1 964 581	Ulp1 protease family, carboxy-terminal domain protein	Yes
MTR_2g009590	1 975 139	Hydroxyproline-rich glycoprotein family protein	-

In 2018, the genetic diversity of the backcrossing program was widened. Selected BC_3F_2 individuals (Tacke and Link, 2018) were crossed with non-Hiverna/2 winter lines: S_062, S_300, S_306 and S_340 (Gasim and Link, 2007). These lines were chosen for their superior winter hardiness and agronomic performance at Göttingen.

In 2020, we arrived at generation BC_4F_4. From this material, four LVC components (see Table 9) were sown in October 2019 as generation Syn 0 and in October 2020 as Syn 1, to initiate a novel LVC synthetic variety of winter faba beans as experimental cultivar and as breeding germplasm pool.

Table 9. Four LVC components were used to initiate Syn0 of a winter hardy, LVC synthetic population.

Field book numbers	Backcross generation	Detailed pedigree	Component in Syn 0
F19_[622-631]	BC_4F_4	BC_4F_4 (**S_062** * BC_3F_2(Hiv/2 * Mél/2))	1
F19_[633-638]	BC_4F_4	BC_4F_4 (**S_300** * BC_3F_2 (Hiv/2 * Mél/2))	2
F19_[675-698]	BC_4F_4	BC_4F_4 (**S_306** * BC_3F_2 (Hiv/2 * Mél/2))	3
F19_[702-720]	$BC_3 F_4$	BC_3F_4 (**S_340** * BC_2F_2 (Hiv/2 * Mél/2))	4

2.5   Discussion

In 2015, the group of Khazaei (Khazaei et al., 2015) presented a genetic map of the fragment of faba bean chromosome 1 which most likely contains the VC locus. This was the first mapping attempt of this locus and the VC gene. It reduced the search interval for the causative gene down to approximately 4cM. For this, the authors employed a set of 210 F_5 recombinant inbred lines (RILs) of a cross between Mélodie/2, a LVC inbred line, and ILB938/2, a HVC inbred line. Genotyping was done with a set of 188 polymorphic SNPs (Khazaei et al. 2015).

Gutiérrez et al. (2016) reported corresponding findings from their cross Vf6 * 1268. The putative region of the respective locus in their approach amounts also to app. 4cM.

Here, two crosses with two different genetic backgrounds were employed, containing 751 and 899 individuals, respectively. Seventeen markers from Webb et al. (2016) and Song (2017) allowed the draft mapping. By means of two different transcriptome sequencing techniques, 58 new markers were developed. Due to the different sequencing approaches used (RNASeq usually covers the whole mRNA sequence while MACE focusses on the 3´-region of the mRNA templates only) these 58 markers consisted of 2 groups according to their contigs ability to match the *M. t.* genome: 33 SNPs which mapped to the range between markers Vf_Mt2g005900_001 and Vf_Mt2g013690_001 onto the genome of *M.t.* (*M.t.* chr. 2; 390,001-3,620,000 bp, see Table 2) and were detected with RNAseq and MACE, 19 SNPs which were solely detected by RNAseq. The second group consisted of 6 SNPs which could not be mapped to the *M.t* genome (see Table 3). *M.t.* lacks the ability to synthesize VC (Gauttam and Kalia 2013; Khazaei et al. 2019). As the VC gene did not necessarily have a counterpart in *M.*

t., these 6 SNPs were included (Table 3). They were chosen from the 57 SNPs mentioned in Table 2, based on maximum base counts and on strictest and most symmetric distribution of SNP alleles across the two pairs of VC-contrasting, near-isogenic lines.

Of the new 58 SNP markers (Table 3), 42 and 38 were polymorphic for the two genetic backgrounds and were thus used for the creation of final genetic linkage map fragments. The two resulting maps show, for those markers which were also used by Khazaei et al. (2017), almost the same order. The previous empty gaps between those markers are now enriched with 42 and 38 new markers, respectively. The subsequent fine mapping resulted in a core region for the VC gene of approximately only 0.13 cM.

This relatively small interval (core region) yielded a set of putative candidate genes (Table 8). Additionally, expression data derived from our transcriptomics analyses (Abo-Vici 2021, data not shown) revealed that only two contigs in this narrow search area were significantly differentially expressed, namely TRINITY_DN44878_c1_g2_i2 and Contig14304. One of these contigs (Contig 14304) included a SNP used in this study, namely VFS18002.A52. Both contigs could be matched successfully to the *M.t.* genome, revealing the gene "3,4-dihydroxy-2-butanone 4-phosphate synthase" (or in *C.a.* "bifunctional riboflavin biosynthesis protein RIBA 1") as a possible candidate gene (Table 8). A third contig (TRINITY_DN49974_c1_g1_i2), which could not be mapped to the *M.t.* genome, nonetheless showed significant sequence overlap with at the very 3´-end of Contig14304 (see subtitle Table7) and yielded the SNP VFS18002.A41, which was also used in this study. Whether the corresponding sequence is a 3´prime variant of RIBA1 mRNA could not be clarified due to the lack of genomic data from *V. faba*.

A fourth contig (Contig4411) showed significant differential expression levels as well (Abo-Vici 2021), it was though located slightly outside our core region, as the BLAST revealed. This contig could be BLASTed to the gene "Glycosyltransferase" of *C.arietinum* (Abo-Vici 2021)*.*

Recently, Björnsdotter et al. (2020) named the gene RIBA 1 as the putative candidate gene for the LVC phenotype. Their findings strongly suggest that vicine and convicine are side products of the riboflavin biosynthesis from the purine nucleoside triphosphate GTP. Their hypothesis for the cause of the LVC phenotype is a frame shift insertion in the RIBA1 enzyme. They accordingly named the gene for the RIBA1 enzyme VC1. Our findings support the conclusion of Björnsdotter et al. (2020).

Additionally, the mRNA analyses lead to a set of new markers in the VC core region, ready for use for marker assisted selection. We employed them and bred new, LVC winter faba bean lines, aiming at highly versatile feed stuff from highly productive winter beans. Syn 1 of the first, novel LVC winter faba bean variety is currently (2021 season) in the field.

Although there are hints that vicine and convicine may confer antifungal effects (Bjerg et al. 1984; Pavlik et al. 2002), there were no agronomic disadvantages of LVC material detected in accompanying field trials (Abo-Vici, 2021); moreover, data is lacking on the actual VC content in roots and shoots of HVC and LVC types, be it spring or winter types of faba bean.

In conclusion, the current genetic investigation of the chromosomal vicinity of the VC locus supports the findings of Björnsdotter et al. (2020); the gene MTR_2g009270 starting in the *M.t.* genome at 1.848.020bp, a 3,4-dihydroxy-2-butanone 4-phosphate synthase, should be considered as genetic cause for the HVC and LVC status of faba bean. This finding was based on the combination of fine-mapping and mRNA expression data. Furthermore, eight markers were presented, they are ideally suited for breeding LVC winter faba beans, as demonstrated here. This introduction of the LVC feature in winter faba beans will make them more attractive for farmers and feed producers.

2.6   Acknowledgements

2.7   Literature

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Chapter 3: The impact of low vicine and convicine (VC) levels on field performance of faba bean (*Vicia faba* L.), the distribution of VC in shoot and root, and genetic analyses of VC levels across parents and offspring.

This chapter is published solely here; being part of this Docotral Dissertation.

3.1   Introduction

Faba bean (*Vicia faba* L.) is an annual legume with high potential as regional vegetal protein source, especially for organic farming (Köpke and Nemece 2010). It has a yield potential of about 70 dt/ha (Link et al. 1994) and is a valuable source of protein (25-30% protein in seed dry matter) with a high content of several essential amino acids (Link et al. 1994; Heuzé et al. 2016), making faba bean a good source for food and feed. Breeders were able to improve many traits during the recent decades, such as lodging and shattering (Link 2009). Additionally, faba bean lives in symbiosis with bacteria (genus *Rhizobium*, Hebblethwaite 1983) and have been shown to fix a large amount of nitrogen and accordingly to contribute to a positive N-balance in the soil (Schmidtke and Rauber 2000). Therefore, faba bean is valued for its characteristic of improving soil fertility and breaking rotation circles of pests and diseases (Köpke and Nemecek 2010; Kulak et al. 2013).

Even though faba bean cultivation has some major advantages, the area it is grown on in Germany is still rather small, with 59.500 ha (Statistisches Bundesamt 2020). Reasons for this lie, among others, in its sensitivity to biotic and abiotic stresses (Ghaouti 2007), as for example lacking tolerance to water deficiency (Cernay et al. 2015; Mwanamwenge et al. 1999). Inadequate yield stability and the presence of anti-nutritive seed compounds such as tannins and vicine and convicine (Köpke and Nemecek, 2010; Römer, 1998; Crépon et al. 2010) also pose problems for faba bean use and marketing.

The pyrimidine derivates vicine and convicine (VC) have been described to occur in an approximate ratio of 2:1 in faba bean seeds, with 0.3% up to 1.5% of seed dry matter. They have been reported to be found in all plant parts (Goyoaga et al. 2008). Scattered hints in literture imply that VC is formed in the faba bean seed coat and subsequently this compound is transported into the embryo. Since seed coat is maternal tissue, this notion makes seed VC levels potentially maternally dependent (Griffiths and Ramsay 1992; Duc et al. 1989; Brown and Roberts 1972). VC is found in all plant parts; however, little is known about the exact nature of VC inheritance and distributrion patterns so far.

Vicine and Convicine can, after consumption, cause hemolytic anemia (favism) in humans with a genetic deficiency of the enzyme Glucose-6-Phosphat-Dehydrogenase (G6PD; Crépon et al., 2010);

moreover, VC have been described to have negative health and productivity consequences for animals such as laying hens and broilers (Guillaume and Bellec 1977; Halle 2006; Larbier and Leclercq, 1994). Therefore, faba beans without or with very low VC content are highly attractive.

In 1989, the group of Duc et al. (1989) found that genebank accession 1268(4)(1) from Radzikov (Poland) contained only about 1/10 to 1/20 of the so-called wild type seed content; more specifically a seed content of 0.046% of VC, here termed as low VC content (LVC). Much later it was shown that faba beans with that LVC level prevent favism in humans and do not cause dietary disadvantages for laying hens and broilers as compared to faba beans wild wild type or high VC (HVC) levels (Gallo et al. 2018; Crépon et al. 2010; Abo-Vici 2022). Based on Mendelian genetics and QTL-mapping, it was shown that this large variation of the trait is controlled by one locus (VC locus), with alleles VC+ (wild type) and vc- (name for allele which causes LVC phenotype; Duc et al. 1989; Tacke et al. 2022).

Recently, the group of Björnsdotter et al. (2021) uncovered in a pioneering finding the bi-functional RIBA1 protein as key catalyst of the VC pathway. It catalyzes as one of its activities the firth step of the riboflavin biosynthetic pathway. An allel at the locus for RIBA1 was found to cause the LVC phenotype and is therefore the above-mentioned vc- allel. Consequently, the locus, i.e. the gene encoding RIBA1 was named VC1.

Further breeding efforts towards improved faba bean cultivars are necessary to enhance the acceptance and cultivation of faba beans in agriculture (Brünjes 2014, Link 2009). A major breeding objective for faba beans is a LVC content. There already exist certain LVC spring-sown faba beans, for example the cultivars Mélodie and Tiffany.

However, winter beans have been known to have a high seed- and protein yield and a high agronomic value in rotation circles and are therefore even more suitable to improve acceptance of faba bean (Link 2009). Therefore, it seems advantageous to breed for winter faba beans with the LVC feature.

In terms of these breeding objectives, it is important to answer questions of whether a LVC content might have indirect, auxiliary effects on the plants (especially in case of negative side effects). Seed-borne vicine and convicine are most probably formed in the faba bean seed coat and transported into the embryo (Duc et al. 1989; Brown and Roberts 1972), yet, VC can be found in the whole plant, from leaves and shoots to roots (Goyoaga et al. 2008; Ramsay and Griffith 1996). Since the seed VC amount is supposedly maternally inherited (Griffiths and Ramsay 1992; Duc et al. 1989; Brown and Roberts 1972), it can be assumed juvenile plants or seedlings emerging from LVC seed still exhibit such LVC status. There is scattered speculation that the HVC or LVC status of a plant might influence

its resistance to fungi and insects. Vicine and convicine have proven fungistatic effects (against *Botrytis*, *Ascochyta* and *Pyrenophora*; Bjerg et al. 1984; Pavlik et al. 2002) and it has been reported that LVC content was difficult to genetically combine with high *Ascochyta* resistance in spring sown faba beans (Jellis and Vassie, 1995). These findings suggest that VC might have beneficial effects against pests for the faba beans seeds and a LVC faba bean cultivar might therefore have agronomic disadvantages.

Here, our main objectives were therefore to search for differences between LVC faba beans and HVC faba beans in agronomic traits other than seed quality, hence, to search for potential agronomic disadvantages of the LVC type.

Additionally, we strove to uncover more about the inheritance patterns of the VC content in seed and further plant organs of faba beans to gain a more thorough understanding of this feature and to facilitate the breeding of LVC faba bean cultivars in the future.

3.2   Material and Methods

3.2.1   Project parts

The project is divided into 5 main parts (Tab. 1), two of which are related to the features of VC in the plant, its distribution patterns as well as inheritance (VC distribution study, VC inheritance study). Three further parts of the project mainly address the agronomic behaviour of the HVC and LVC faba bean in the field; mainly their resistance against soil-borne fungi. Here, the Legume fatigue trial 1 is a large field study with 14 faba bean entries while the Legume fatigue trial 2 is, in a similar way, focusing on isogenic LVC and HVC lines and their potentially differential reaction to soil-borne fungi. The Pathogen study, finally, was carried out in the laboratory with a focus on pathogenic fungi occurring on roots that were harvested from the mentioned Legume fatigue trial 1.

Table 1. Overview and subdivision of workflow.

Project part	General description	Corresponding tables	Experimental year(s)/season	Experimental condition
VC distribution study	Where and when can VC be found in faba bean?	Table 3	2019	Greenhouse experiment
VC inheritance study	How is VC inherited?	Table 4	2018-2019	Greenhouse experiment
Legume fatigue trial 1	Do VC play a role for resistance to soil-borne fungi?	Table 5	2017-2019	Field trial
Legume fatigue trial 2	Resistance study with isogenic LVC/HVC entries	(explained in 3.2.5.3)	2019	Field trial
Pathogen study	Do VC play a role for resistance to specific soil-borne fungi?	(explained in 3.2.9)	2017-2018 (within legume fatigue trial 1)	Lab analyses

3.2.2 Isogenic families

To allow strong inference of results while comparing HVC and LVC entries, we used so-called isogenic families (IFs). These were entries which were genetically highly similar and mainly differed for their VC values. Several of these entries were even available as pairs of lines with one HVC line and one LVC line, which were genetically comparable to each other, i.e. isogenic lines. All isogenic families are mentioned in Table 2. Isogenic families are used in each part of the project; further genotypes will be introduced below when describing the corresponding chapters, i.e. project parts.

Table 2. Isogenic families of faba beans with HVC and LVC seed content. Given are the designations, the specific genotypes, the VC type of seed, seasonal types of faba bean, tannin type.

Desig-nation	Genotype	Description	VC type of seed	Tan-nin type	Winter-/Spring-Type	Descendent from plant with inbreed-ing degree
IF1	Hiverna/2	Line bred from winter faba bean culti-var Hiverna	HVC	+	Winter	F>8
IF2-4	BC1F4 ((Hiverna/2*Mé-lodie/2)* Hiverna/2)	Mixture of 9 lines from backcrosses be-tween Hiverna/2 and Mélodie/2	LVC	+	Winter	BC1F4
IF5	NPZ 14.8099 HIGH	Both: mixture of 10 lines with HVC/LVC content from a cross between a LVC and a HVC spring cultivar, conducted by NPZ Lembke KG	HVC	+	Spring	F5; same ancestors
IF6	NPZ 14.8099 LOW		LVC	+	Spring	
IF7	Isogenic, NPZ 14.8099 LVC	Lines developed from the same F5 indi-vidual from a cross between a LVC and a HVC spring cultivar, conducted by NPZ Lembke KG (part of IF5 and IF6)	LVC	+	Spring	F5
IF8	Isogenic, NPZ 14.8099 HVC		HVC	+	Spring	F5
IF9	Isogenic, Mél/2* ILB938/2 LVC	Lines developed from the same F5 indi-vidual from cross between lines Mé-lodie/2 (LVC) and ILB938/2 (HVC), de-veloped from spring bean cultivar Mé-lodie and genebank accession ILB938	LVC	+	Spring	F5
IF10	Isogenic, Mél/2* ILB938/2 HVC		HVC	+	Spring	F5
IF11	Highly isogenic, NPZ 14.8099 LVC	Lines developed from same F7 individ-ual from a cross between a LVC and a HVC spring cultivar, conducted by NPZ Lembke KG	LVC	+	Spring	F7
IF12	Highly isogenic, NPZ 14.8099 HVC		HVC	+	Spring	F7
IF13	Isogenic, Hiv/2*Mél/2 LVC	Lines from the same BC2F3 individual from backcrosses between Hiverna/2 and Mélodie/2 towards Hiverna/2 (based on IF2-4)	LVC	+	Winter	BC3F3
IF14	Isogenic, Hiv/2*Mél/2 HVC		HVC	+	Winter	BC3F3

3.2.3 Vicine and convicine distribution study

3.2.3.1 Plant material for VC distribution study

The VC distribution study focused on the content of VC in plant tissues beyond the seed content. It was conducted with four faba bean entries (Table 3). These were spring faba beans and are isogenic pairs (Table 2). Three to four biological repetitions per genotype were evaluated.

Figure 1. Harvested plants at eight leave stadium.

Table 3. Genotypes used in VC distribution study. Given are the designations, the specific geno-types, the VC type of seed, tannin type, and types of faba bean.

Designation	Genotype	VC type of seed	Tannin type	Winter-/Spring-Type
IF7	Isogenic, NPZ 14.8099 LVC	LVC	+	
IF8	Isogenic, NPZ 14.8099 HVC	HVC	+	
IF9	Isogenic, Mél/2*ILB938/2 LVC	LVC	+	Spring
IF10	Isogenic, Mél/2*ILB938/2 HVC	HVC	+	

3.2.3.2 Execution of VC distribution study

The four entries were grown in a greenhouse in the winter season of 2018/2019. Twelve plants per entry were planted. Six plants per entry were harvested at 8 to 13 leave stage ("harvesting time point before flowering") and the other six when two to six inflorescences were flowering ("harvesting timepoint at flowering").Plants were analyzed individually, as biological replicates. Homogeneity of plant growth stage and number of harvested plants was not always ensured.

At harvest, each plant was divided into roots, shoots and leaves plus flowers. These plant tissues were frozen in liquid nitrogen. Subsequently, the samples were freeze-dried, ground and evaluated via HPLC analysis (see "3.2.7 HPLC analysis").

3.2.4 VC inheritance study

3.2.4.1 Plant material for VC inheritance study

For the VC inheritance study, the three different plant tissues (roots, shoots, leaves plus flowers) were used to analyze the VC values of parents, F1 and F2 offspring to gain more knowledge about gene action of VC+ vs. vc- alleles (dominance vs. partial dominance in gene action). For this, spring bean entries, namely isogenic pairs IF7 and IF8 and IF9 and IF10, the F1 offspring of their reciprocal crosses and their F2 offspring (from self-fertilization of the F1) were used (see Table 4). These samples were analyzed with the HPLC method (see "3.2.7 HPLC analysis"). Also, backcrosses between Mélodie/2 (SB3, see Table 5) and Hiverna/2 (IF1), which had been produced in the breeding of LVC winter faba bean lines (Tacke et al. 2022), were used in their generation BC3F3 (i.e. seeds harvestes from BC3F2 plants which had been marker-selected for being heterozygous in their VC status). The seeds harvested from these parents and offspring of these crosses were tested for their VC values via the Sixdenier method (absorbances are measured; values below an absorbance of 0.15 are low, values between 0.15 and 0.30 are medium and values above 0.3 are high; "Photometric method (Sixdenier method)"; Sixdenier et al. 1996).

Table 4. Genotypes of seeds used for VC inheritance studies.

Designation	Genotype	Expected VC values
Spring beans; plant tissues: Roots, shoots, leaves plus flowers (6 biological repetitions), seeds (2 biological repetitions); VC analysis conducted by HPLC		
IF7	Isogenic, NPZ 14.8099 LVC	LVC
IF8	Isogenic, NPZ 14.8099 HVC	HVC
F1(IF7*IF8)	F1 (NPZ 14.8099 LVC * NPZ 14.8099 HVC)	?[1]
F2(IF7*IF8)	F2 (NPZ 14.8099 LVC * NPZ 14.8099 HVC)	?
F1(IF8*IF7)	F1 (NPZ 14.8099 HVC * NPZ 14.8099 LVC)	?
F2(IF8*IF7)	F1 (NPZ 14.8099 HVC * NPZ 14.8099 LVC)	?
IF9	Isogenic, Mél/2*ILB938/2 LVC	LVC
IF10	Isogenic, Mél/2*ILB938/2 HVC	HVC
F1(IF9*IF10)	F1 (Mél/2*ILB938/2 LVC * Mél/2*ILB938/2 HVC)	?
F2(IF9*IF10)	F2 (Mél/2*ILB938/2 LVC * Mél/2*ILB938/2 HVC)	?
F1(IF10*IF9)	F1 (Mél/2*ILB938/2 HVC * Mél/2*ILB938/2 LVC)	?
F2(IF10*IF9)	F2 (Mél/2*ILB938/2 HVC * Mél/2*ILB938/2 LVC)	?
Winter beans; Plant material: seeds (8, 7 and 19 biological repetitions)		
IF1	Hiverna/2	HVC
IF13 (BC2F3)	BC2F3(Hiv/2*Mél/2)*Hiv/2 LVC	LVC
BC3F3	BC3F3(Hiv/2*Mél/2)*Hiv/2	?

[1] It was, prior to the study, still unclear which VC values the F1 and F2 entries would have. Therefore, the table shows "?" for their expected values.

3.2.5   Legume fatigue trials

3.2.5.1 Location of legume fatigue trials

The legume fatigue trial 1 was conducted at Reinshof (Agricultural Education Station Göttingen). The soil type is gleyic fluvisol and the medium yearly precipitation amounts to 645mm with a medium yearly temperature of 8.7 °C (Augustin and Müller 2022).

Figure 2. Legume fatigue trial 1 in 2019 at Reinshof, Göttingen. Fence and nets protected the faba beans from birds and from lodging.

The trial was implemented on a field site upon which only faba beans were grown in the last three years before the onset of the current experiment (Martsch 2017, personal communication) in 2017. By this measure, the legume fatigue of the soil, i.e., the enrichment of faba bean specific soil pathogens was induced. The legume fatigue trial 1 was conducted as monoculture with faba beans in the seasons 2017, 2018, and 2019.

Sowing was in October for winter beans and in March or April for spring beans. The only exception were the winter beans for the first experimental season, 2017, which were sown at the same time as spring beans.

To keep herbivorous predators away from the trial, a fence was erected, and a supportive net was placed horizontally (see Figure 2). The net served the dual purpose of protecting the juvenile plants against birds and later providing support for the tall plants, to protect them against potential lodging.

To not impact the soil microbes and fauna, no herbicides or other pesticides were used, weeding was done by hand.

3.2.5.2 Plant material for legume fatigue trial 1

In legume fatigue trial 1, 14 entries were used in all three years with few exceptions. Those 14 entries addressed pertinent, agronomically interesting traits of faba beans (see Table 5). Six winter-type faba bean entries and seven spring-type faba bean entries were used. The winter-types entries include

- the line Hiverna/2 (here coded as "IF1"), which is a homozygous line bred from the Hiverna winter bean cultivar; it is a HVC type.
- BC1F4 (termed "IF2-4"), a mixture of seven highly related LVC lines from backcrosses be-tween Hiverna/2 and Mélodie/2; the latter is homozygous and descended from Mélodie, a French spring faba bean cultivar with LVC content (homozygous for vc- allele; BSA - EU 2023).
- the four lines Wab98-98-3 ("WB1"), S_016 ("WB2"), S_054 ("WB3") and S_175 ("WB4"), which are descendants from the "Göttinger Winterbohnen Population („GWP"). The GWP is a winter faba bean breeding population, founded in 1989 by cross-breeding 11 diverse in-bred winter-type HVC lines, and which is characterized by its superior winter-hardiness (Gasim & Link, 2007).

The seven spring-type faba bean entries used in this study are the following:

- the near-isogenic pair NPZ 14.8099 HIGH ("IF5", mixture of lines) and NPZ 14.8099 LOW ("IF6", mixture of lines), which stems from the same ancestral F5 individual (from a cross between a LVC and a HVC spring cultivar, conducted by NPZ Lembke KG)
- the HVC faba bean cultivar Fuego ("SB1", BSA 2023)
- the spring cultivar Tiffany ("SB2"), which in 2017 was in Germany the only released LVC cultivar (BSA 2023)
- the homozygous line Mélodie/2 already mentioned above (BSA – EU 2023)
- the two HVC cultivars Taifun (BSA 2023) and Tattoo (BSA 2016), which have been chosen because of their zero-tannin feature. Taifun entered the experiments only in 2018 (was absent in the first season, 2017).

Additionally, a spring pea was added as negative control for an absolutely zero VC value; like almost all legumes, peas do not produce VC (see General Introduction). Four of the faba bean entries which were used in this trial form two isogenic families, namely IF1 plus IF2-4 and IF5 plus IF6 (Table 2). These two pairs are genetically isogenic and mainly differ for their VC values, as per pair always the one member has HVC content (IF1; IF5) and the other has LVC content (IF2-4; IF6, see Table 2).

Table 5. Faba bean lines and varieties employed for the legume fatigue field trial 1 of 2017 – 2019. Given are the designations, the specific genotypes, the VC value, types of faba bean, and tannin status. Red, yellow, and green denote high, medium, and low VC status, respectively.

Designation	Genotype	VC (% in seed DM, HPLC; means over three years, 2017-2019)	SD of VC values	Winter-/Spring-Type	Tannin	Analyzed for soil-borne pathogens
IF1	Hiverna/2	0.57	0.129	Winter	+	+
IF2-4	BC1F4	0.06	0.022	Winter	+	
IF5	NPZ 14.8099 HIGH	0.56	0.155	Spring	+	+
IF6	NPZ 14.8099 LOW	0.03	0.006	Spring	+	+
SB1	Fuego	0.56	0.134	Spring	+	+
SB2	Tiffany	0.05	0.023	Spring	+	+
SB3	Mélodie/2	0.02	0.003	Spring	+	-
WB1	Wab98-98-3	0.68	0.115	Winter	+	
WB2	S_016	0.58	0.086	Winter	+	+
WB3	S_054	0.62	0.121	Winter	+	-
WB4	S_175	0.54	0.129	Winter	+	-
SE	Navarro	0.00	0.001	Spring (pea)	+	+
Tf1	Taifun	0.67[1]	-	Spring	-	-
Tf2	Tattoo	0.53	0.112	Spring	-	+

[1] Only results from 2019 available

3.2.5.3 Plant material for legume fatigue trial 2

In the legume fatigue trial 2, which was conducted in 2019, the focus was on pairs of highly isogenic lines (see table 2). Two of these pairs (IF7 and IF8; IF11 and IF12) stem from the same genetic background as IF5 and IF6, while one pair (IF9 and IF10) stems from a cross between Mélodie/2 and ILB938/2. The latter is a descendent from the exotic HVC gene bank accession ILB938 (Khamassi et al. 2013; Khazaei et al. 2015). IF7 and IF8 as well as IF9 and IF10 share the fact that they descended from an F5 individual and are isogenic while IF11 and IF12 are higher isogenic by descending from an F7 individual (see Chapter 4). The pair IF13 and IF14 stems from the same BC2F3 individual of the back-

Figure 3. Half-harvested faba bean field with equipment for harvest-by-hand.

crossing program of the lines Hiverna/2 and Mélodie/2 and is also considered isogenic – though less so than the other pairs (see Chapter 4).

3.2.5.4 Structure of field trial

Legume fatigue trial 1 was conducted over three years (2017-2019) with five replicates throughout. Plot size and correspondingly size of the trial increased year by year. In 2017, 10 plants were grown per plot, while 20 were grown in 2018 and 40 in 2019. An exception was the pea Navarro, it was sown with 12, 24, 48 seeds per plot, respectively. Plots were arranged with one row, two rows, and four rows with 10 plants per row, in the three seasons, respectively.

For sowing, spacing between the faba bean seeds within rows was 10 cm; rows were 30 cm apart from each other. Per replicate, winter faba bean entries were sub-blocked, and spring bean were sub-blocked too, to avoid competition between the different seasonal types. The winter bean sub-blocks and the spring-sown bean sub-blocks were separated from each other by buffers rows of the winter bean line S_062 and of the the spring-sown cultivar Fuego. Sub-blocks and entries within sub-blocks were randomized within each replicate.

Figure 4. Harvested seeds in paper bags in 2019.

Legume fatigue trial 2 was carried out in 2019 and was embedded within legume fatigue trial 1 of 2019. Only one replicate per entry was grown and the members of one isogenic pair were always grown next to one another. The rows of the four isogenic pairs of legume fatigue trial 2 were separated from the rows of legume fatigue trial 1 by buffers.

3.2.5.7 Scoring of traits

Within the legume fatigue trials 1 and 2, the focus was on yield per plant. Field plots were harvested by hand (see Figure 3 and 4). After harvest, seed were dried in ambient temperature for more than six weeks. Yield was assessed as seed weight per plant (g/plant).

3.2.6 Pathogen study

Over the course of the two years 2017 and 2018, the roots and app. 1cm of the shoot directly above the root – called 'root crown' from here on – of seven entries of legume fatigue trial 1 were analyzed

regarding their colonizing pathogens. The entries chosen for this experiment were selected due to their diversity regarding VC status, their type (i.e. winter-/spring-type) and their tannin content. Namely, entries chosen were IF1, IF5, IF6, SB1, SB2, Tf2 and WB2 (see table 5). Each year, one plant was extracted per entry from legume fatigue trial 1. Dr. A. Mavridis of the Department of Plant Pathology and Plant Protection (Dept. Plant Patholgiy and Plant Protection 2023) then evaluated the roots and root crowns of these plant parts regarding the pathogens found on them. For this, roots of each plant were cut into 20, root crowns of each plant into 10 pieces. Each piece was then placed in a quarter of a petri dish upon culture medium (i.e. four pieces per petir dish). For each piece, it was estimated which pathogens could be detected around it. It was then recorded on how many of the root and root crown pieces a certain pathogen could be found, the highest possible count being 20 for roots, 10 for root crowns, the lowest possible count being 0 for both.

3.2.7 Measuring VC content

Measuring VC contents was necessary for many parts of this project. Generally, plant tissues that where evaluated where seeds, roots, shoots and leaves plus flowers of faba beans. Different plant tissues where evaluated in different parts of the project. For this, two different methods were used, which will be described in the following.

3.2.7.1 HPLC analysis

HPLC analysis was conducted at NIAB institute in UK by Helen Appleyard's team (NIAB 2023). The HPLC method followed Khamassi et al. (2013) with little adaptions. Duplicate samples of 0.5g flour of faba bean or other faba bean tissue were water-extracted and sonicated for 10 minutes and allowed to settle overnight at 4°C. Aliquots of 1 ml were centrifuged at 14000 rpm for 15 minutes and filtered (0.2micron) to prepare for analyses. Dionex Ultimate 3000 HPLC was used with a Kinetex 5μm C18 100A column (Phenomenex); 100% mobile phase, phosphate buffered water pH 7 with flow rate of 1.5ml/min and with 10-minute runs. Vicine and convicine peaks were observed at 280nm (Appleyard 2021, personal communication). The grouping of HPLC values into categories "low", "medium" and "high" follows the method described in Tacke et al. (2022). The categories of the given VC contents are marked throughout with the colors red (high VC), yellow (medium VC) and green (low VC).

3.2.7.2 Photometric method (Sixdenier method)

This photometric method of detecting the VC content in seeds of faba bean was conducted as described in Sixdenier et al. (1996), in the laboratory and with support of NPZ Lembke GmbH (NPZ 2023). VC was extracted from a sample of two faba bean seeds and the absorbance at 274 nm was read on a spectrophotometer (Sixdenier et al. 1996). Values below an absorbance of 0.15 were read as low, values between 0.15 and 0.30 were read as medium and values above 0.3 were read as high VC content. The defining of absorbances below 0.15 as low VC contents results from experience and

knowledge available at NPZ Lembke GmbH (personal comm. with G. Welna and O. Sass); the categories of medium and high were assigned after observations in the work of Allemann (2019). Allemann (2019) compared the Sixdenier method to the HPLC method with different faba bean lines, among which were also entries of Legume fatigue trial 1. The categories of the VC contents as received from the Sixdenier method are marked in the same way as the HPLC results (see above).

3.2.8 Data evaluation

The statistical analyses were conducted with the statistical software program R (R Core Team 2019), employing standard package and the 'ggplot2' package (Wickham 2009).

Boxplots displayed in this work represent the distribution of the respective samples. They are composed of the median as the central bar, the 25 and 75% quartiles as the top and bottom bars of the boxes as well as the whiskers, which represent minimum and maximum at their end bars. Outliers of the respective boxes are represented with circles above or below the respective boxes.

The analyses of variance (ANOVAs) as well as Tukey and Student's T-Tests were conducted with the standard package of R (Hoff 2008). Normal distribution of residues and homogeneity of variance was checked visually (Hoff 2008). Potential deviations of the distribution of the residuals from normality was additionally inspected via QQ-plots (Chambers 1983).

3.3 Results

3.3.1 Vicine and convicine distribution study

The means of VC values for the tested plant tissues from the isogenic pairs given in Table 3 were calculated and selected pairs of values were statistically tested for their significance (t-test, p<0.05). It could be observed that for each plant tissue (namely roots, shoots, and leaves plus flowers), the VC values decreased significantly from the harvesting time point before flowering to the harvesting timepoint at flowering (Table 6). Also, it could be shown that the VC values were significantly higher for roots than for the other plant tissues, even in LVC plants.

Table 6. Results of VC distribution study. Given are the VC HPLC values of genotypes given in Table 3 of roots, shoots and leaves and flowers divided for two harvesting times. The results are grouped into LVC and HVC types and means of these groups (with three to four replications per genotype) are given. HVC and LVC values are indicated with green and red, respectively. Values are given in total VC % in dry matter.

VC state in seed	Root		Shoot		Leaves and flowers	
	B.f. [1]	f. [2]	B.f.	f.	B.f.	f.
LVC	0.095	0.073	0.030	0.024	0.000	0.000
HVC	0.479	0.224	0.076	0.029	0.039	0.012

[1] Before flowering; [2] flowering

3.3.2 Vicine and convicine inheritance study

The means of VC values for seeds and other plant tissues were calculated and selected pairs of values were statistically tested for their significance (t-test, p<0.05). As expected, F1 seeds (i.e. hybrid seeds from manual crossing) harvested from LVC mothers crossed with HVC fathers showed LVC pheno-types, while F1 seeds of HVC mothers showed HVC phenotypes (Table 7). F2 seeds harvested from F1 plants showed intermediate VC phenotypes. The seeds harvested from BC3F2 plants showed an in-termediate VC phenotype. The absorbance at 274nm (Sixdenier method) lay roughly in the middle between the values of the two parents IF13 (BC2F3) and IF1. The actual mean between the original crossing parents would have been 0.2883 as compared to the actual BC3F3 seed VC content of 0.2931. It has to be stated that the BC3F2 plants the BC3F3 seeds were harvested from had previ-ously been marker-selected for being heterozygous for their VC status. Therefore, this value is not directly comparable to the values for the F1 seeds from the spring faba bean crossings and can merely give an estimate of what the VC value of true F1 seeds from this winter faba bean crossing might have been.

Table 7. Results of VC inheritance study. Given are the HPLC VC results for seeds and roots of different generations (parents, F1 offspring, F2 offspring) of reciprocal crosses between one HVC and one LVC spring bean parent. Evaluated seeds are sister seeds to those seeds leading to plants which donated roots for the analyses. Values are given in total VC % in dry matter. Also given are the results of the Sixdenier method of absorbance at 274nm, which have been ascertained for winter bean backcrosses.

Designation	Genotype	Seeds
Spring beans		HPLC method; Total VC % in dry matter
IF7	Isogenic, NPZ 14.8099 LVC	0.0206
IF8	Isogenic, NPZ 14.8099 HVC	0.4485
F1(IF7*IF8)	F1 (NPZ 14.8099 LVC * NPZ 14.8099 HVC)	0.0312
F2(IF7*IF8)	F2 (NPZ 14.8099 LVC * NPZ 14.8099 HVC)	0.2939
F1(IF8*IF7)	F1 (NPZ 14.8099 HVC * NPZ 14.8099 LVC)	0.3874
F2(IF8*IF7)	F1 (NPZ 14.8099 HVC * NPZ 14.8099 LVC)	0.2499
IF9	Isogenic, Mél/2*ILB938/2 LVC	0.0177
IF10	Isogenic, Mél/2*ILB938/2 HVC	0.4927
F1(IF9*IF10)	F1 (Mél/2*ILB938/2 LVC * Mél/2*ILB938/2 HVC)	0.0355
F2(IF9*IF10)	F2 (Mél/2*ILB938/2 LVC * Mél/2*ILB938/2 HVC)	0.3413
F1(IF10*IF9)	F1 (Mél/2*ILB938/2 HVC * Mél/2*ILB938/2 LVC)	0.4362
F2(IF10*IF9)	F2 (Mél/2*ILB938/2 HVC * Mél/2*ILB938/2 LVC)	0.3444
Winter bean		Sixdenier method; ab-sorbance at 274nm
IF1	Hiverna/2	0.4524
IF13 (BC2F3)	BC2F3(Hiv/2*Mél/2)*Hiv/2 LVC	0.1241
BC3F3	BC3F3(Hiv/2*Mél/2)*Hiv/2	0.2931

3.3.3 Legume fatigue trial 1

3.3.3.1 Yield for winter faba beans

Grain yield for the six winter bean entries of the legume fatigue trial revealed no disadvantages for LVC winter beans (i.e. IF2-4) as compared to HVC winter beans (Figure 5A). Over the three years, differences between the winter bean entries were clearly visible. The years 2017 and 2018 showed performances that were significantly lower than the year 2019, with $p < 0.05$ (ANOVA; Figure 5B).
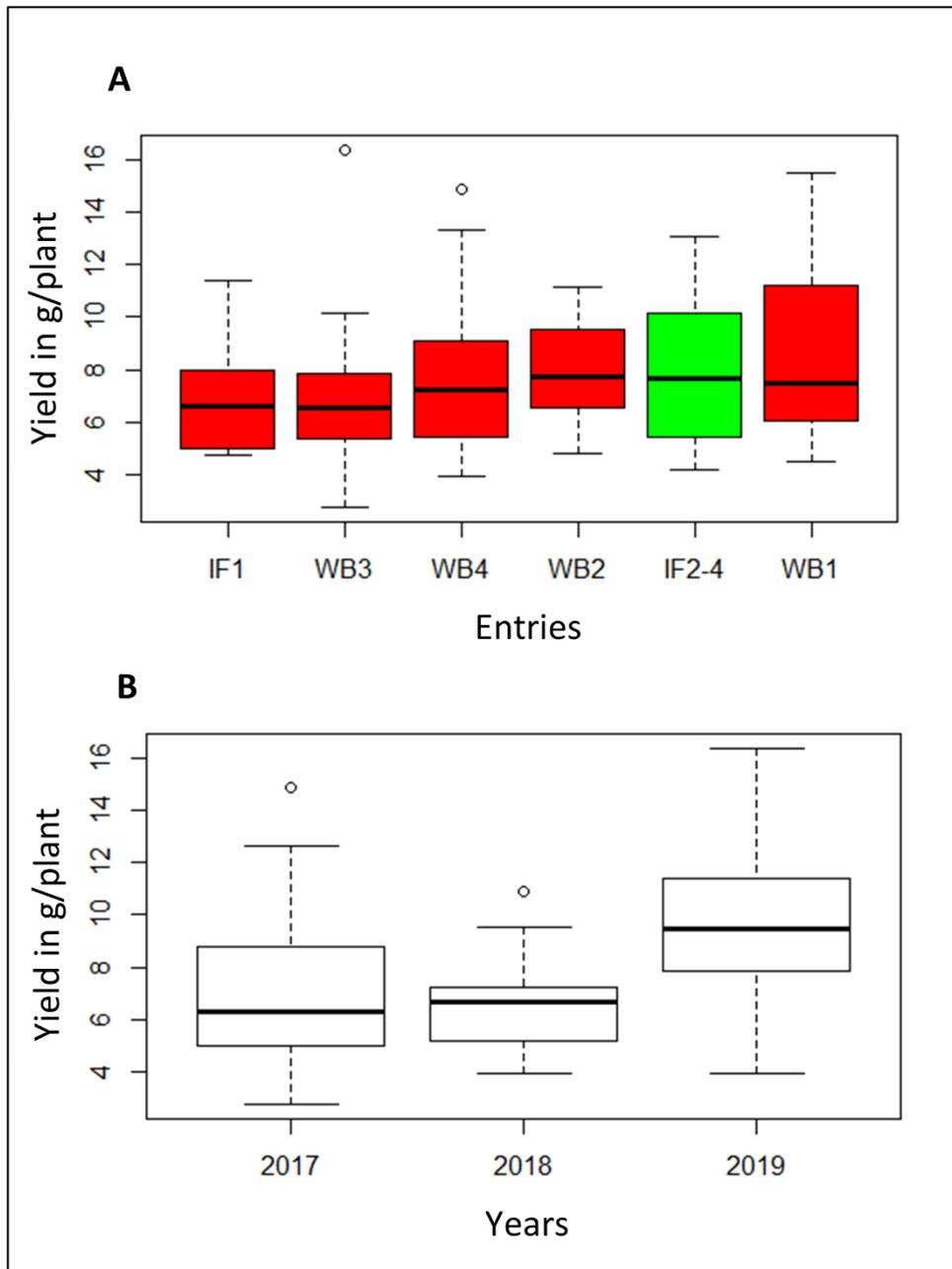
Figure 5. Yield of six winter beans in legume fatigue trial 1. Figure 5A gives the mean yield of the six winter bean entries over the years 2017-2019. Red color denotes HVC entries; green color shows the LVC winter bean entry (IF2-4). Figure 5B shows the mean across all winter bean entries per year. Yields are given in g/plant. Circles above/below the boxplots mark outliers.

The ANOVA revealed that genotypes were not significantly different for yield, as opposed to the significant year and the significant genotype * year interactions (Table 8).
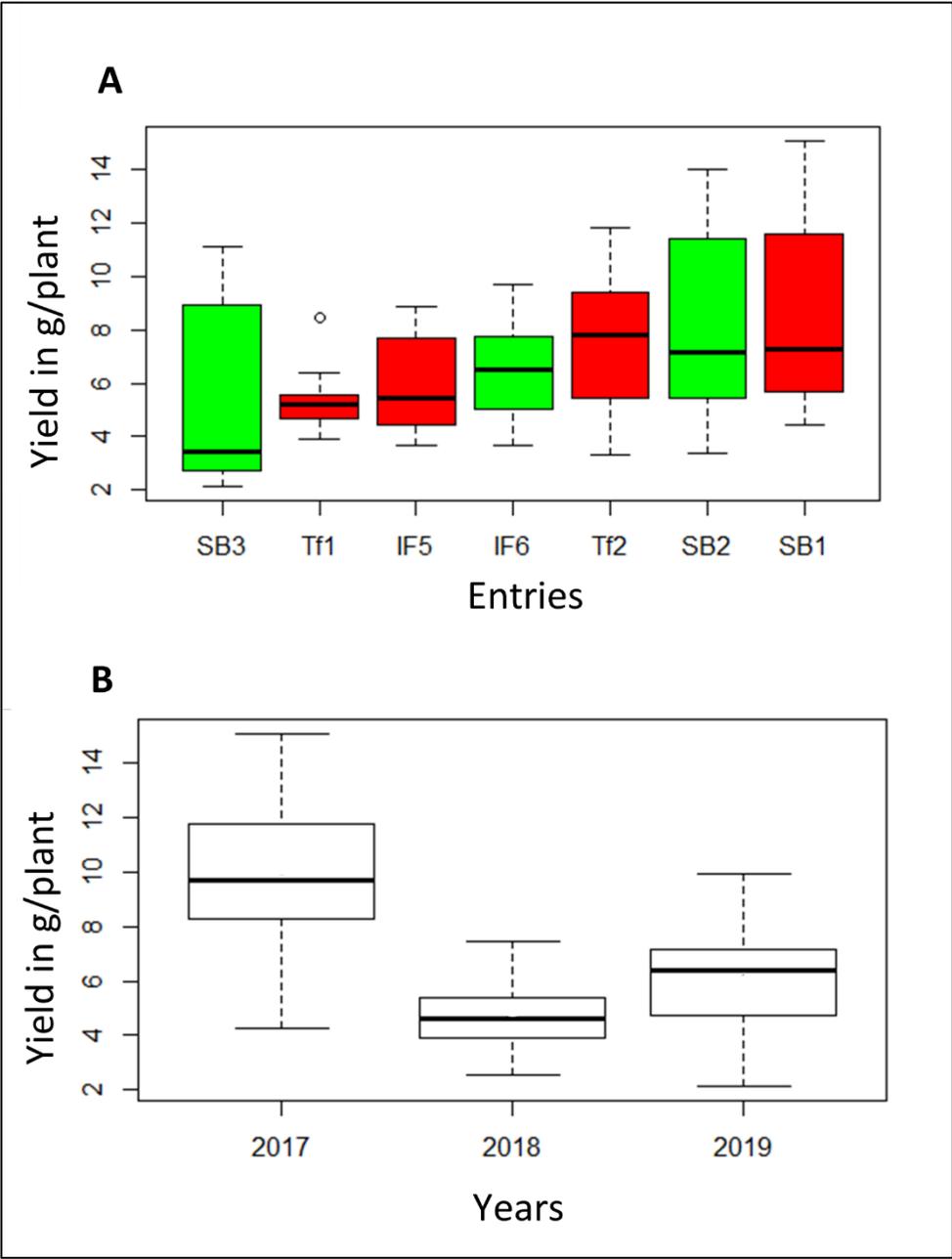

Figure 6. Yield of spring beans in legume fatigue trial 1. Figure 6A gives the mean yield of the seven winter bean entries over the years 2017-2019. Red color denotes HVC entries, green color shows the LVC spring bean entries. Figure 6B shows the mean across all spring bean entries per year of the trial. Yields are given in g/plant. Circles above/below the boxplots mark outliers.

3.3.3.2 Yield for spring-sown faba beans

Determination of yield for the seven spring bean entries of the legume fatigue trial 1 revealed no disadvantages for spring beans with LVC content as compared to the spring beans with HVC content (Figure 6A). Even though SB3 (LVC) showed very low yield, SB2 (LVC) had the second highest yield and was close to SB1 (HVC). The isogenic pair IF5 (HVC) and IF6 (LVC) showed no significant yield differences (t-test, $p<0.05$), with IF6 having the slightly higher yield (average across the years). The two faba bean entries with zero tannin content, Tf1 (HVC) and Tf2 (HVC), did not show notable differences in yield when compared to the other entries with tannin (Figure 6A). The three seasons caused clear, marked differences for their average spring bean yields (Figure 6B); grain yield was significantly lower in the years 2018 and 2019 than in 2017 (comp. Tab. 9, Tukey Test, $p<0.05$). The ANOVA of the spring beans (Table 9) revealed that the genotypes, years, and their interactions were significant sources of variation for yield per plant. Figure 6A clearly shows that spring bean entries showed markedly different yields, up to threefold differences occurred. SB3 was shown to be significantly lower in yield than all other entries (Tukey Test, $p<0.05$).

Table 8. ANOVA of yield of winter beans in legume fatigue trial 1 of the years 2017 – 2019.

Effects	Degrees of Freedom	Mean of Squares	F value	p-value	Significant (p<0.05)
Genotypes	5	5.97	1.42	0.2130	No
Year	2	91.90	21.83	$4.75*10^{-8}$	Yes
Genotype * Year	10	19.16	4.55	$6.19*10^{-5}$	Yes
Repetitions within Year	4	7.11	1.69	0.1625	No
Residues	68	4.21			

Table 9. ANOVA of yield of seven spring beans in legume fatigue trial 1 of the years 2017 – 2019.

Effects	Degrees of Freedom	Mean of Squares	F value	p-value	Significant (p<0.05)
Genotypes	6	22.81	12.93	$5.12*10^{-10}$	Yes
Year	2	234.93	133.21	$2.20*10^{-16}$	Yes
Genotype * Year	11	10.07	5.71	$1.258*10^{-6}$	Yes
Repetitions within Year	4	1.77	1.00	0.4113	No
Residues	76	1.76			

3.3.4 Legume fatigue trial 2

In legume fatigue trial 2, isogenic pairs of LVC and HVC lines were grown next to each other to directly compare the performance of lines that only differed in in the VC feature. Three of these pairs were spring beans while one pair was winter beans (IF13/IF14). Two pairs had the same genetic background but stemmed from plants of different inbreeding degrees and therefore potentially had different degrees of isogeneity (IF7/IF8 from a plant in F5 and IF11/IF12 from a plant in F7). Two pairs both originated from a plant with inbred generation F5 but came from different genetic backgrounds (IF7/IF8 and IF9/IF10; see Table 2). When comparing the LVC lines to the HVC lines within one pair, it is notable that the LVC lines yielded more than the HVC lines, even though these differences are not significant (Tukey Test, p<0.05). The winter bean pair yielded significantly less than the spring bean pairs (Tukey Test, p<0.05).
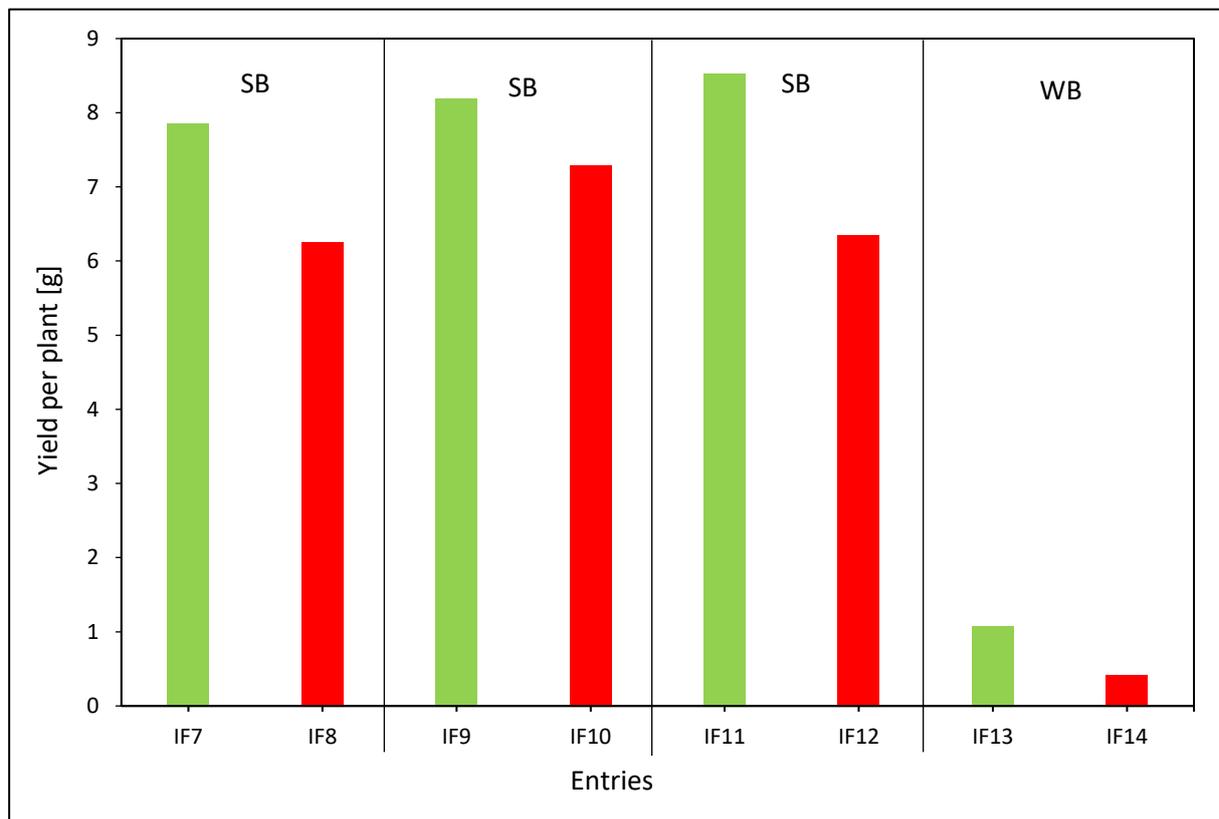
Figure 7. Yield of isogenic pairs of legume fatigue trial 2. Red color denotes HVC entries, green color shows the LVC spring bean entries. Isogenic pairs are implicated by black dividing lines. Spring bean isogenic pairs are marked by "SB" while the winter bean isogenic pair is marked by "WB".

3.3.5 Pathogen study

The pathogen study was conducted to evaluate whether VC had negative effects upon certain soile-borne pathogens and therefore positive effects upon the plants. In the years 2017 and 2018, one plant per entry of the seven entries IF1, IF5, IF6, SB1, SB2, Tf2 and WB2 (see table 5 and Chapter

3.2.6) was analyzed regarding the soile-borne pathogens found on its roots and root crown. Only the three pathogens *Fusarium spp., Phoma lingam* and *Pythium spp.* could be found in significant amounts (Figure 8). No significant differences could be found for the results of infected root crown pieces for any of the three pathogens (Tukey Test, $p<0.05$). Also, for root pieces infected with *Fusaium spp.*, no significant differences could be detected between the entries (Tukey Test, $p<0.05$). For *Phoma lingam*, significant differences could be found between Tf2 and the two entries WB2 and IF1, while for *Pythium spp.*, significant differences were found between IF5 and all other entries (Tukey Test, $p<0.05$). LVC status did not prove to increase the number of infected root or root crown pieces, as can be observed in Figure 8.
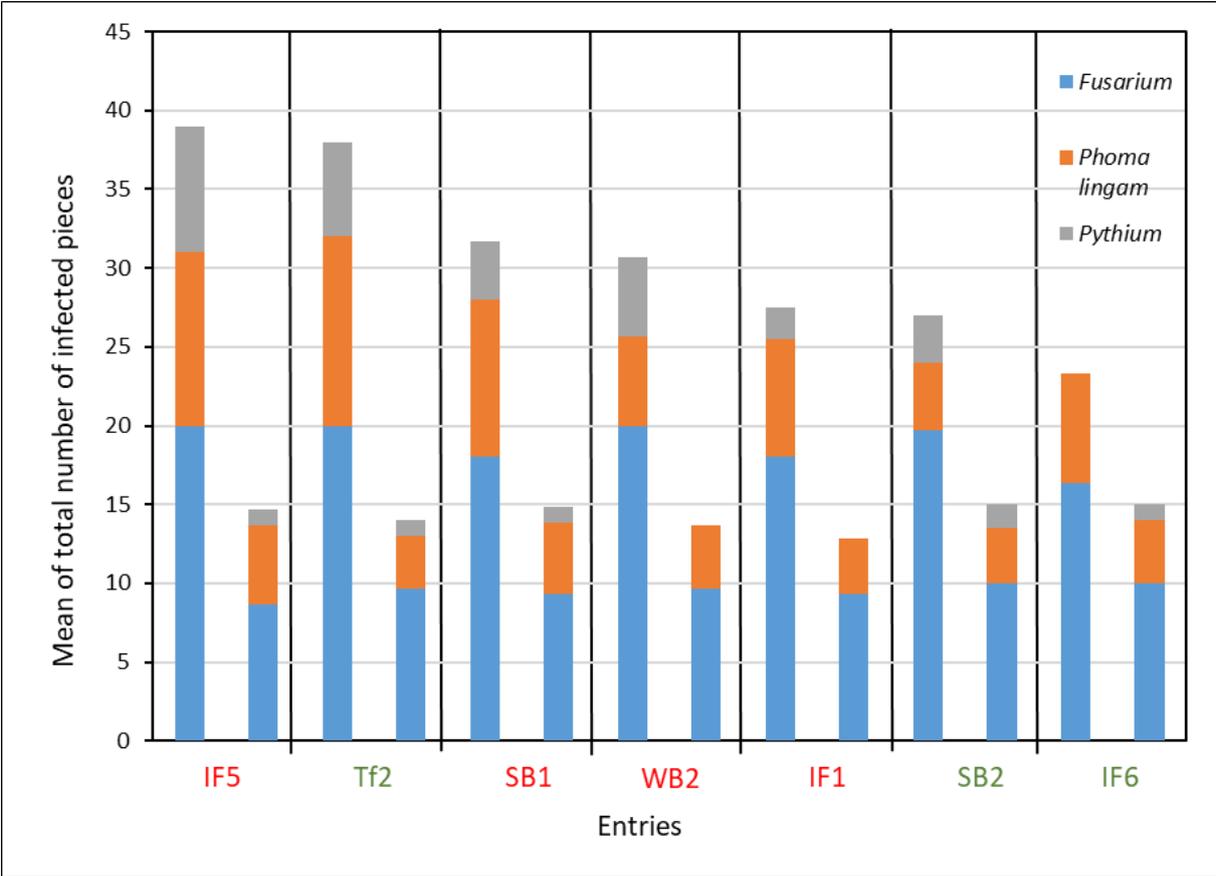
Figure 8. Results of infected root and root crown pieces from pathogen study. Given are the means of total sums of infected root and root crown pieces from seven entries of Legume fatigue trial 1 of years 2017 and 2018. Left column of each entry shows results for the root pieces (total number per entry per pathogen is 20, as 20 root pieces were evaluated), right column shows results for root crown pieces (total number per entry per pathogen is 10, as 10 root crown pieces were evaluated). Blue colour in columns denotes results for *Fusarium spp.,* orange colour results for *Phoma lingam* and grey colur for *Pythium spp.* LVC entries are marked by green writing, HVC entries by red writing.

3.4   Discussion

The VC distribution studies showed that, judged as percent of dry matter, the highest VC content is found in the roots of the faba bean plants (Table 6) as compared to shoots and leaves plus flowers. This, if not being just by chance but if having a reason, might point towards fungistatic effects of VC against soil-borne fungi. This was tested in our legume fatigue trials 1 and 2 on a part of a field on which legumes and faba beans had been grown for at least three years prior to the trials. The faba bean stands in this part of the field had been observed to be visibly shorter and weaker than the faba beans grown adjoining (Martsch, pers. communication). Therefore, the assumption can be made that on this part of the field, legume-specific pathogens had accumulated and created legume fatigue conditions. The legume fatigue trial 1 and 2 (Figures 5 and 6) did not reveal any disadvantages of LVC plants against HVC plants in this field setting with its presumably high pathogen pressure of soil-borne pathogens. The legume fatigue trial 1 was conducted with various lines and varieties of faba beans in pure stands. As such, the possibility remained that differences in the field or lack thereof might be grounded in other genetic characteristics of the entries than LVC and HVC. Therefore, legume fatigue trial 2 was conducted in addition to legume fatigue trial 1. In this trial, pairs if isogenic lines were used which mainly differed in their VC levels (see also Chapter 4). This trial revealed similar findings to legume fatigue trial 1 – i.e. no marked and no significant difference in field performance between plants with HVC and LVC. Additionally, a pathogen study was conducted to evaluate which soile-borne pathogens could be found on roots and root crowns of seven differing genotypes (LVC/HVC, tannin/zero tannin, winter-/spring-type) of legume fatigue trial 1 and if LVC or HVC status of the plants had any impact on the amount of pathogens found. The study, however, also reveald no significant differences for amounts of soile-borne pathogens on HVC and LVC plants. It can therefore be concluded that indeed VC status and with this, the VC value in its roots (cf. Table 6) does probably not have any important effects against soil-borne pathogens.

We could also show that VC can be found in differing amounts in all plant parts that were analyszed (not only in seed, but as well in root, shoot, leaves and flowers), with very low amounts in shoot, stem plus leaves and higher amounts in roots. These findings are similar to ones made by Ramsay et al. (1996). Additionally, the amount in the plant (in root, shoot, leaves plus flowers) could be shown to decrease over time (see Table 6), which Ramsay at al. (1996) also observed for shoots of faba bean.

The inheritance studies showed that seed carry the same VC phenotype as their mother plant (Table 7). Indeed, the VC phenotype of the harvested seed is equivalent with the phenotype value of the plant it was harvested from instead of being the VC phenotype of the plant that will grow from the

harvested seed itself. Seed harvested from LVC or from HVC mother plants show LVC or HVC pheno-types, respectively, even if the seed were hybrid seed and genetically heterozygous for the VC status. This confirms the notion that VC amount is maternally inherited (Griffiths and Ramsay 1992; Duc et al. 1989; Brown and Roberts 1972). One consequence is that the VC phenotype of F_2 plants from a VC heterozygous F1 plant can be assessed from few harvested seed per plant without worrying about segregation of the F_3 seed growing on F_2 plants.

Additionally, the inheritance study shows definite intermediate VC amounts for seeds harvested from F1 plants (Table 7). This confirms that VC gene action is most likely approximately intermediate (Ramsay et al. 1991).

The findings generated in these projects suggest that no great disadvantages arise from breeding LVC winter faba beans. The road to a LVC winter faba bean cultivar therefore has been cleared one step further.

3.5 Literature

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Chapter 4: Analyses of degree of isogeneity of important pairs of in-bred lines.

This chapter is published solely here; being part of this Docotral Dissertation.

4.1 Introduction

Faba bean (*Vicia faba* L.), an annual grain legume is a traditional and promising regional vegetal protein source (Link et al. 1994; Köpke and Nemece 2010; Sass, 2021, personal communication). Recently, genetic experiments and corresponding breeding research was conducted by our group to study the presence of the anti-nutritive seed compounds vicine and convicine (VC) (Köpke and Nemecek, 2010; Römer, 1998; Crépon et al. 2010). These compounds limit the usability of faba bean as they are problematic for the value chain. Vicine and convicine can cause favism (Arese et al. 2012) in humans and reportedly have negative consequences for laying hens and broilers (Crépon et al., 2010; Guillaume and Bellec 1977; Halle 2006; Larbier and Leclercq, 1994). Therefore, in our projects we concentrated on breeding faba beans with low vicine and convicine (LVC) amounts. To facilitate this venture, we aimed for the detection of the major genetic locus for VC and, consequently, also questioned any direct or indirect impact of the genetic VC status on field performances. For the detection of the major locus for VC and, especially, for the analysis of the potential impact of VC amount upon field performances of faba beans, we employed so-called near-isogenic lines. Near-isogenic lines (NILs) are often used for detection of quantitative trait loci (QTLs; Falke and Frisch 2011) and can be described as lines with a highly similar genotype, ideally only differing in one locus (see Figure 1). With these pairs of such near-isogenic lines (abbreviated isogenic lines hereafter) it
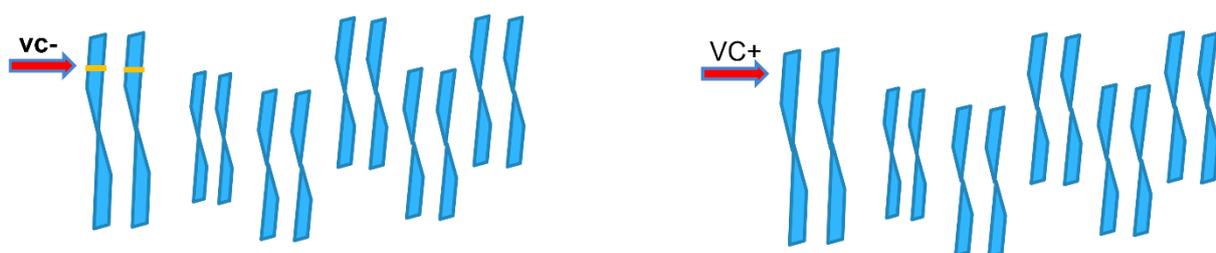
Figure 1. One pair of isogenic lines, each line with six chromosomes. Orange color denotes regions of the genome for which the two lines still differ, i.e. the locus for vicine and convicine content (VC; indicated by red arrow).

was possible for us to track the main VC locus as well as directly study potential effects of the genetic VC status on agronomic performance in our field experiments. However, the actual degree of isogeneity is important to meaningfully judge. This degree of isogeneity cannot be perfectly predicted based on pedigree alone, because meiosis adds randomness (Hillel et al. 1990) . Yet, the higher the actual degree of isogeneity, the stricter and the more stringent the conclusions from the

experiments are. In this study, we accordingly analyzed the extent and degree of isogeneity of our chosen pairs of isogenic lines (for pedigrees and discriptons of lines, see Chapters 2 and 3).

4.2 Material and Methods

4.2.1 Plant Material

For our analyses regarding the importance of VC in faba bean, we bred and used pairs of isogenic inbred lines. Here, we focused on obtaining four isogenic pairs with one high vicine and convicine (HVC) and one LVC line per pair. Each pair of lines was derived from one ancestral individual. This ancestral individual was, seeing its inbred status, a rare member in the family and generations that segregated from its initial cross; rare, because it was still heterozygous for the HVC/LVC status and hence was still able to segregate the sought-for homozygous offspring for both features. Specifically, this ancestral individual was either an F5 individual (Table 1, ancestral individual of isogenic pairs of lines IF7/ IF8; IF9/ IF10) or an F7 individual (Table 1, ancestral individual of isogenic pairs of lines IF11/ IF12) or an BC2F3 individual (Table 1, ancestral individual of isogenic pairs of lines IF13/14). This respective ancestral individual was self-fertilized once to release and create one pair of homozygous, isogenic lines. Each of the three initial crosses was a cross between a HVC and a LVC cultivar or line (Table 1): For the isogenic pairs of lines IF7/IF8 and IF11/IF12, it was a cross between Fabelle (LVC) and an NPZ-owned HVC-elite type, for the isogenic pair of lines IF9/IF10 it was a cross between Mélodie/2 (LVC) and ILB938/2 (HVC); or, in a backcross, Mélodie/2 (LVC) and Hiverna/2 (HVC) were used to create the ancestral individual for isogenic pair of lines IF13/IF14. For a closer description of the isogenic lines and their usage, see Material and Methods of Chapter 2 of this thesis.

Table 1. Four isogenic pairs of faba bean lines, each with a HVC line and LVC line. Given are the entry name, further entry details including the VC type, tannin type, types of faba bean. Genotype is given in column 2. Isogeneity degrees reflect the Mendelian generation of self-fertilization of the respective ancestral individual (column 3 "Description").

Desig-nation	Genotype/ Pedigree	Description	VC status	Tannin status	Winter-/ Spring-Type
IF7	Moderately isogenic, NPZ 14.8099 LVC	Developed from the same F5 individual from a cross between a LVC and a HVC spring cultivar, conducted by NPZ Lembke KG	LVC	+	Spring
IF8	Moderately isogenic, NPZ 14.8099 HVC		HVC		
IF9	Moderately isogenic, Mél/2* ILB938/2 LVC	Developed from the same F5 individual from a cross between the lines Mélodie/2 (LVC) and ILB938/2 (HVC), which were developed from the respective spring bean cultivars Mélodie and ILB938	LVC	+	Spring
IF10	Moderately isogenic, Mél/2* ILB938/2 HVC		HVC		
IF11	Highly isogenic, NPZ 14.8099 LVC	Developed from the same F7 individual from a cross between a LVC and a HVC spring cultivar, conducted by NPZ Lembke KG	LVC	+	Spring
IF12	Highly isogenic, NPZ 14.8099 HVC		HVC		
IF13	Isogenic, Hiv/2*Mél/2 LVC	Lines from the same BC2F3 individual from backcrosses between Hiverna/2 and Mélodie/2 towards Hiverna/2 (based on IF2-4)	LVC	+	Winter
IF14	Isogenic, Hiv/2*Mél/2 HVC		HVC		

4.2.2 50k SNP chip for genotyping of isogenic lines

To analyze the degree of isogeneity among the two lines of each isogenic pairs, all lines were genotyped. We thus genotyped the isogenic pairs (Table 1) as well as the parental lines used in the initial crosses (see Table 1; Mélodie/2, ILB938/2, Hiverna/2), except for the parental lines of the initial cross NPZ 14.8099, Fabelle (LVC) and the NPZ-owned HVC-elite type, because the employed NPZ parental lines were not available to us. Genotyping was conducted with the 50k Affymetrix chip for faba bean, in cooperation with O´Sullivan at Reading (UK; Angra and O´Sullivan, 2021; personal comm.). In all, 34320 SNPs were analyzed within the four isogenic pairs and the parental lines of their initial crosses (see Table 1, column "Genotype") and could be used for the assessment of isogeneity.

4.2.3 Calculation of isogeneity of isogenic pairs

The degree of isogeneity of an isogenic pair was calculated as ratio (expressed in percent) of SNPs that were identical between the two lines as well as in different states between "completely identical" to "completely different" (also considering heterozygotic states of both lines; see below) relative to the total number of SNPs . Each SNP locus received a score to weigh its contribution to the

overall degree of isogeneity. This score of isogeneity is based on the assessment of the inbreeding coefficient F that would occur in the hypothetical offspring from mating the respective pair of isogenic lines (see Figure 2). The genetic similarity of parents (coancestry) is then directly translated into the inbreeding coefficient of offspring (offspring from unrelated parents is non-inbred; Weir et al. 2006). A contribution of one locus, i.e. one SNP (linkage was not considered, see discussion), to the inbreeding of such hypothetical offspring varies between zero and one. The possible situations and concurring scores are given below:

- The two genotypes share same homozygous SNP (e.g., A/A vs. A/A): score = 1.00
- The situation is like C/C vs. T/C or like A/T versus A/A: score = 0.50
- The two genotypes share same heterozygous SNP (e.g., T/C vs. T/C): score = 0.50
- The two genotypes do not share any SNP allele (e.g., C/C vs. A/T): score = 0.00

AA // AA Score: 1.00	A	A
A	AA = „+"	AA = „+"
A	AA = „+"	AA = „+"

AT // AT Score: 0.50	A	T
A	AA = „+"	AT = „-"
T	AT= „-"	TT = „+"

AA // CC Score: 0.00	C	C
A	AC = „-"	AC = „-"
A	AC = „-"	AC = „-"

AT // AA Score: 0.50	A	A
A	AA = „+"	AA = „+"
T	AT= „-"	AT = „-"

Figure 2. Scoring system based on coefficient of similarity for every single marker. Shown in each 2x2 table is the situation that would occur if two lines with the respective genotypes given as row and column denominators (i.e. isogenic lines) were crossed. The resulting genotypes of the hypothetical offspring are given in the body of the table. The maximum of different genotypes is four (for a diploid organism and looking at one locus, i.e. SNP) and our score varies between zero and one. Hence, each cell of the table is given a value of 0.25, which is added to the inbreeding status (score) of the hypothetical genotype of the cell which is reporting the degree of isogeneity between the actual two parents of that SNP.

To determine the degree of isogeneity between the isogenic pairs, the following analysis steps were taken:

Firstly, where parental lines of the initial cross were available (i.e. isogenic pairs IF9/ IF10 and IF13/ IF14), these parental lines were analysed and compared for monogenic SNPs (i.e. all SNPs which were monogenic among either Mélodie/2 and ILB938/2 or between Mélodie/2 and Hiverna/2; Table 1).

Then, these monogenic SNPs were filtered out for that isogenic pair and the analysis was continued only with polygenic SNPs. For the pairs IF7 and IF8 and for IF11 and IF12, no such filtering could be applied.

Secondly, pairwise comparisons were made between the 4-5 biological repetitions (individual plants) per line of each isogenic pair – the purpose was to check whether the biological repetitions were 100% identical as expected or rather not (i.e. IF7 had 4 biological replications, hence, six pairwise comparisons were carried out between the four individuals within IF7). These comparisons should hence address the purity and genetic identity of each of the lines used, with a small margin of error remaining. If any of the biological replications showed a failed result at a SNP, this SNP was removed from all pairwise comparisons among the biological replications. The remaining SNPs were then used to calculate the score of isogeneity for each pairwise comparison between all biological replications (within a given entry). The mean of these comparisons was then calculated and represents the relative score of isogeneity within this respective line (i.e. within line IF7, assessing in asmuch these lines were "purely" bred).

Subsequently, isogeneity between the two lines of an isogenic pair was adressed. Thus, pairwise comparisons between all biological repetitions of an isogenic pair (i.e. IF7/ IF8) were conducted. For this, all SNPs which had failed results in any biological replications of either of the lines of the isogenic pair were excluded from the analysis. Then, all available plants of one line (i.e. IF7, 4 plants) were compared against all plants of the other line (i.e. IF8, 2 plants) of the isogenic pair. In this example, with IF7 having 4 plants and IF8 with 2 plants, in all, eight pairwise comparisons were carried out. The mean of the total score of isogeneity of these eight comparisons was calculated, to report the relative score of isogeneity within the two lines and between the two lines analysis of lines of the respective isogenic pair (Table 2).

4.3 Results

The results of the pairwise comparisons between individual plants within lines as well as between the two lines within one isogenic pair are given in Tables 2 to 4. It is notable that the values calculated for isogenic pairs IF7 and IF8 as well IF11 and IF12 lack the purging of monomorphic SNPs as for this cross, parental lines of the initial cross were not available.

In Table 2, the isogenic pair IF9/IF10 was evaluated for isogeneity, once including monomorphic markers, once excluding them. This was performed to later be able to adequately compare isogenic pairs IF7/IF8 and IF9/IF10 (on the common ground of including monomorphic markers), since both pairs originate from an individual in selfing generation F5 after the initial cross and thus deserve a direct comparison.

As can be seen in Table 2, the score of isogeneity differs between the two analyzation methods: With monomorphic markers included in the analysis, the relative score of isogeneity is quantified as 96.45%, while without these markers, it is 90.65%. The relative scores of isogeneity if calculated without monomorphic markers, between the pairs IF7/IF8 (Table 3) and IF9/IF10 (Table 2), i.e. those pairs originating segregating from an individual in selfing generation F5 of their respective initial crosses, are 95.23 % and 96.45 %, respectively. Isogenic pair IF11/IF12 shows a relative score of isogeneity of 99.09 %. The total numbers of markers used for the respective comparisons varies as different numbers of SNPs were purged due to failed results or monogeneity.

Table 2. Isogeneity of isogenic pair derived from initial cross between Mélodie/2 and ILB938/2. Total number of markers tested: 34321. Given are the means of the comparisons between the lines. Shown here is an analysis including and an analysis excluding the monomorphic markers.

Designation	Genotype	Genera-tion	Biological replica-tions	Total score of iso-genic loci	Relative score of isogeneity
Cross between lines ILB938/2 * Mélodie/2 – 23294 markers found to be monomorphic; marker number representing 100% in analyses: 34321 (analysis WITH monomorphic markers)					
Within IF9	Isogenic, Mél/2*ILB938/2 LVC	F5	4	34382.60	99.88
Within IF10	Isogenic, Mél/2*ILB938/2 HVC	F5	4	34234.78	99.79
Comparison between IF9 – IF10				32573.22	96.45
Cross between lines ILB938/2 * Mélodie/2 – 23294 markers found to be monomorphic; marker number representing 100% in analyses: 11027 (analysis WITHOUT monomorphic markers)					
Within IF9	Isogenic, Mél/2*ILB938/2 LVC	F5	4	10932.54	99.14
Within IF10	Isogenic, Mél/2*ILB938/2 HVC	F5	4	10987.12	99.63
Comparison between IF9 – IF10				9974.39	90.65

Table 3. Isogeneity of isogenic pairs derived from initial cross NPZ 14.8099. Total number of markers tested: 34320. The two isogenic pairs IF7/IF8 and IF11/IF12 are analyzed, originating from an individual in selfing generation F5 and F7 of the initial cross, respectively.

Designation	Genotype	Generation	Biological replications	Total score of isogenic loci	Relative score of isogeneity
Cross NPZ 14.8099 – no parental lines available, therefore analysis with all markers, including monomorphic markers; mean of markers defining 100% in analyses: 34310					
Within IF7	Isogenic, NPZ 14.8099 LVC	F5	4	34272.50	99.86
Within IF8	Isogenic, NPZ 14.8099 HVC	F5	2	34223.96	99.72
Comparison between IF7 – IF8				32674.10	95.23
Within IF11	Highly isogenic, NPZ 14.8099 LVC	F7	4	34299.68	99.94
Within IF12	Highly isogenic, NPZ 14.8099 HVC	F7	4	34139.41	99.47
Comparison between IF11 – IF12				33998.49	99.09

4.4 Discussion

Our results clearly demonstrate a rather high degree of isogeneity for all isogenic pairs (see Table 2 and 3) which are, literally defined, near-isogenic pairs.

The analysis of isogeneity between the biological repetitions of each singular line yielded very high scores. This confirms that the starting material for each line was very highly homozygous, the individual from which the seeds stemmed to sow the biological replications was very highly homozygous and that the technology yielded meaningful data. The fact that values for all biological replications stay beneath 100% is not unexpected: Errors in the KASP analysis or unclear results of the assays are part of the reasons for falling short to 100%. Also, it is possible that the original plant that yielded the seed for the near-isogenic pairs was not 100% homozygous itself. This would mean that other heterozygous loci – apart from the VC locus – were passed on. Since we know these margins of errors through the analysis of the biological replications, we can factor them into the comparison of the lines of an isogenic pair.

The degrees of isogeneity between the lines of an isogenic pairs range close to the expected degrees (see Table 4): Two individuals segregating from an individual in selfing generation F5 after the initial cross would be expected to have a relative score of isogeneity of 96.88%. The two isogenic pairs which each stem from an individual in selfing generation after the initial cross F5 are IF7/IF8 and IF9/IF10 and have a relative score of isogeneity of 95.23 % and 96.45 %, respectively. These values, however, have been measured without taking the monomorphic loci between the parental lines of the initial cross into account. In the case of isogenic pair IF9/IF10, where parental lines were available to us, the analysis excluding the monomorphic loci from the analysis gave a significantly lower score of 90.65%.

These unexpectedly lower degrees of isogeneity might stem from linkage in general, which was not considered in this analysis for reasons of feasibility, or specifically linkage around the selected-for VC locus: The expectation value given in Table 4 only considers a random individual in generation F5 after the initial cross and two random lines segregating from it. In our case, however, we have been selecting the propagated individuals across the selfing generations specifically for being heterozygous at one specific locus. To shed light on this problem, the distribution of the heterozygous loci and those loci differing between the two lines of the isogenic pairs should be determined: If many of those loci cluster around the VC locus, the reason for our lower-than-expected isogeneity might lie here.

The score of isogeneity for pair IF11/IF12, which stem from an individual in selfing generation F7 of the same initial cross as IF7/IF8, have a score of isogeneity of 99.09%. This is rather close to the expected value of 99.61%. The draw-back is, again, the missing filtering step of monomorphic SNPs before the analysis, which might lead to an overestimation of the isogeneity.

Table 4. Expected percentages of homozygous and heterozygous loci of one line in different generations of self-fertilization after a cross (covered here are only loci which are polymorphic between parents)

	F1	F2	F3	F4	F5	F6	F7	F8
Expected % homozygous loci	0	50	75	87.500	93.750	96.875	98.438	99.219
Expected % heterozygous loci	100	50	25	12.500	6.250	3.125	1.563	0.781
Exptected isogeneity of two random lines segregating from individual in given generation	-	75	87.500	93.750	96.875	98.438	99.219	99.610

Even though the calculated scores of isogeneity are all above 90%, they stay behind the expected scores. The scores of isogeneity for those pairs originating from an individual in selfing generation F5 (IF7/IF8 and IF9/IF10; in case if IF7/IF8 this is an approximation based on scores seen in IF9/IF10) are even below the score of isogeneity expected if lines were segregating from an individual in selfing generation F4. For the pair IF11/IF12, the score of isogeneity comes close to that of lines segregating from an individual in F7, as would be expected. Still, even considering these drawbacks of our isogenic pairs, the presented degrees of isogeneity are high and the lines of the pairs can be described in good conscience as near-isogenic.

The goal of this analysis was to answer the question whether differences in performance in the field of the lines of an isogenic pair can be attributed solely to the differing locus of VC or if the lines still generally differed greatly and there might therefore be different reasons for their differences in performance. Since the scores of isogeneity were satisfactory, it can be concluded that the field analyses were sound, and their results can be attributed mainly to the presence or absence of VC.

4.5   Literature

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Chapter 5: Comprising Discussion

This chapter is published solely here; being part of this Docotral Dissertation.

Faba bean (*Vicia faba* L.) is a high-yielding grain legume valued for its protein-rich seeds (Crépon et al. 2010; Link et al. 1994), and for its ability to improve soil fertility (Köpke and Nemecek 2010; Kulak et al. 2013). However, its usability as food and feed is hampered by the antinutritive seed compounds vicine and convicine (VC), which can have negative impacts on health and performance of livestock such as laying hens and broilers (Guillaume and Bellec 1977; Halle 2006; Larbier and Leclercq 1994). Moreover, VC can cause hemolytic anemia (favism) in genetically predisposed humans after consumption of VC-containing faba beans, caused by the metabolic products divicine and isouramil (Luzzatto and Arese 2018), a condition which is potentially fatal.

Even though a few spring bean varieties with low VC (LVC) amounts exist (among others Tiffany, Allison, and Bianca, see Bundessortenamt 2021), more high-yielding faba beans displaying this characteristic are needed. Winter beans especially are known to be high yielding and are therefore a good germplasm for breeding LVC cultivars.

To facilitate the breeding progress and to better describe this sought-after phenotype, more intimate knowledge of the LVC feature is necessary. Closely linked genetic markers are important to speed up and simplify breeders' selection. Additionally, it is important to understand potential indirect effects of the LVC feature: Which traits differ between faba beans with high VC (HVC) amount vs. LVC faba beans? Might such concomitant differences cause disadvantages to a LVC type? This is an important question because literature suggests that VC might have fungistatic effects (see Chapter 3.1.; Bjerg et al. 1984; Pavlik et al. 2002).

Therefore, our main objectives in this work were:

1 "To develop SNP markers with close linkage to the VC locus via fine-mapping and to locate and preferably to identify the VC gene in our genetic material" (Chapter 2.2),

2 "Search for differences between LVC faba beans and HVC faba beans in agronomic traits other than seed quality" (Chapter 3.1).

Chapter 2 of this thesis was concerned with development of new SNP markers as well as the search for the gene that should be mainly responsible for the seed LVC phenotype. Also, we directly started breeding a LVC winter faba bean variety.

The group of Khazaei et al. (2015) was able to present a region on chromosome 1 of the faba bean genome which most likely comprises the major VC locus and they narrowed the interval in which this

locus was probably located down to about 4 cM, while the group of Gutiérrez et al. (2016) reported similar findings (see also Chapter 2.2). Our analysis reported in Chapter 2 of this thesis placed the putative locus of the VC gene in the same chromosomal region and narrowed the size of that interval down to only 0.13cM. This then relatively small interval yielded a set of DNA-markers very closely linked to the VC locus (see Tables 5A, 5b, and 7 in Chapter 2). These new markers were subsequently tested for usage in actual breeding, started with a cross between a winter faba bean line (Hiverna/2) and a LVC spring bean line (Mélodie/2); the markers worked fine and yielded good results (see Table 6 in Chapter 2). Therefore, these markers were further used in our winter faba bean breeding ventures for selection of LVC genotypes.

In 2021, Björnsdotter et al. published a paper in which they identified the first enzyme associated with vicine and convicine biosynthesis and named it VC1 (Björnsdotter et al. 2021). They identified the gene encoding this enzyme by combined gene expression analysis and metabolite profiling of eight different tissues of the inbred line Hedin/2 and subsequently analyzing gene-to-metabolite correlations. With this approach, they identified the bifunctional gene RIBA1, encoding (in the N-terminal section) an isoform of 3,4-dihydroxy-2-butanone-4-phosphate synthase, and (in the C-terminal section) a GTP cyclohydrolase II, as the most likely candidate for the gene responsible for VC. RIBA1is a bifunctional enzyme generally known to be involved in riboflavin biosynthesis (Björnsdotter et al. 2021). Additionally, they performed a fine-mapping analysis with a population of 1157 F_2 individuals from a cross between a HVC (Hedin/2) and a LVC (Disco/1) inbred line. With this, they reduced the size of the interval already defined by Khazaei et al. (2017) and Gutiérrez et al. (2016) from app. 4 cM down to 0.21 cM. In this interval, they were able to identify eight genes in the syntenic and sequenced genome of *Medicago truncatula*, one of which encodes the same isoform of bifunctional 3,4-dihydroxy-2-butanone-4-phosphate synthase/GTP cyclohydrolase II. This gene was consequently identified as the sought-for VC1 (Björnsdotter et al. 2021).

The methodology utilized by us to search for the gene responsible for VC content lacks the gene-to-metabolite analysis. Our fine-mapping analysis, though, resembles the fine-mapping analysis performed by Björnsdotter et al. (2021). We used not one but two mapping populations of 751 and 899 F_2 individuals, respectively. The two mapping populations demonstrate different genetic backgrounds on the HVC-side. Importantly, both mapping populations are not directly obtained from a cross between a LVC and a HVC inbred line. To get as little as possible disturbance from segregating background, we used inbred near-isogenic lines originating from an F_5 individual which still segregated for the seed VC content feature. This F_5 individual itself originated from a cross between an inbred LVC and an inbred HVC line.

With this material, i.e. with the near-isogenic lines, mRNA expression experiments were conducted. To this purpose, immature cotyledon tissue in developmental stages four, five and six was collected and used for the analyses. Two different transcriptome sequencing techniques were employed (Tacke et al. 2022): RNASeq, addressing the entire mRNA sequence and MACE, focusing on the 3´-region of the mRNA templates. Within the above-described small interval of 0.13 cM identified by us to contain the VC gene, we identified a set of putative candidate genes which were located in the chromosomal region of *Medicago truncatula (M.t.)* and *Cicer arietinum (C.a.)* syntenic to the interval identified in *Vicia faba* (see Table 8 in Chapter 2). Of the contigs derived from our transcriptomics analysis (AboVici 2022), only two could be found to simultaneously map into this narrow region and be differentially expressed. A SNP used in our study was even found to locate directly in one of these contigs. Both contigs matched the gene "3,4-dihydroxy-2-butanone 4-phosphate synthase" in the *M.t.* genome (or in *C.a.* "bifunctional riboflavin biosynthesis protein RIBA 1") – the same gene identified by the group of Björnsdotter et al. (2021) in their analyses. As we arrived at the same conclusion as Björnsdotter et al. (2021) regarding the putative gene for VC content, this result is hence very strong.

Furthermore, Björnsdotter et al. (2021) confirmed the identity of VC1 as the causative gene for VC content. They transformed hairy roots of LVC inbred line Mélodie/2 with the VC1 coding sequence, which led to a significant increase in vicine levels of roots. Since LVC varieties express a non-functional version of VC1 with a frame shift insertion, it was expected – and confirmed – that overexpression of the *Agrobacterium*-transferred wild-type VC1 in the LVC genotype Mélodie/2 would lead to an increase in its vicine levels if the gene was indeed causative for the wild-type VC phenotype.

Previously, it had been hypothesized that VC might be formed as part of the orotic pathway, since vicine and convicine are pyrimidin derivates (Brown and Roberts 1972)). The identified gene VC1, however, encodes the enzyme GTP cyclohydrolase II, which is involved in the riboflavin biosynthesis pathway starting with the purine GTP (Björnsdotter et al. 2021). In feeding studies with C- and N-labelled GTP, Björnsdotter et al. (2021) could show that GTP is indeed a precursor of vicine in faba bean. They additionally proved that GTP can also be converted into vicine in the distantly related species of bitter melon (*Momordica charantia*), one of the few species which also produces vicine (Gauttam and Kalia 2013; Khazaei et al. 2019).

Interestingly, Björnsdotter et al. (2021) state that they noticed a similarity between unstable intermediates of the riboflavin pathway, built following the catalytic activity of GTP cyclohydrolase II (VC1) and VC. They hypothesize that VC might be built from these unstable compounds (2,5-diamino-6-143 ribosylamino-4(3*H*)-pyrimidinone 5'-phosphate, i.e. DARPP; and 5-amino-6-ribosylamino-2,3(1H,3H)-pyrimidinedione 5'-phosphate, i.e. ARPDP; Björnsdotter et al. 2021) by three steps. The first step

should be a hydrolysis which has recently been shown to be facilitated by a N-glycosidase in bacteria and plants (Björnsdotter et al. 2021, Frelin, O. *et al.* 2015). This N-glycosidase is described by Frelin et al. (2015) to be important in directed overflow metabolism and the pre-emption of damage from reactive metabolites. Vicine and convicine, hence, may be formed in situations where an overflow of the reactive, unstable intermediate compounds within the riboflavin pathway (DARPP, ARPDP) are formed and the action of GTP cyclohydrolase II cannot be stopped.

Vicine and convicine might not have genuine, critical roles for plant vitality, since tentatively they are understood as the outcome of directed overflow metabolism. This assumption is supported by another finding of this thesis, namely by results reported in Chapter 3: The field tests Legume fatigue trial 1 and 2 were performed to determine whether a very low level of VC (not only in seed but in other organs including roots) might have negative consequences for faba bean plants, since VC had been described in literature to possibly have fungistatic effects (Pavlik et al. 2002). However, none of the field trials showed any significant disadvantages of LVC faba bean cultivars and lines as compared
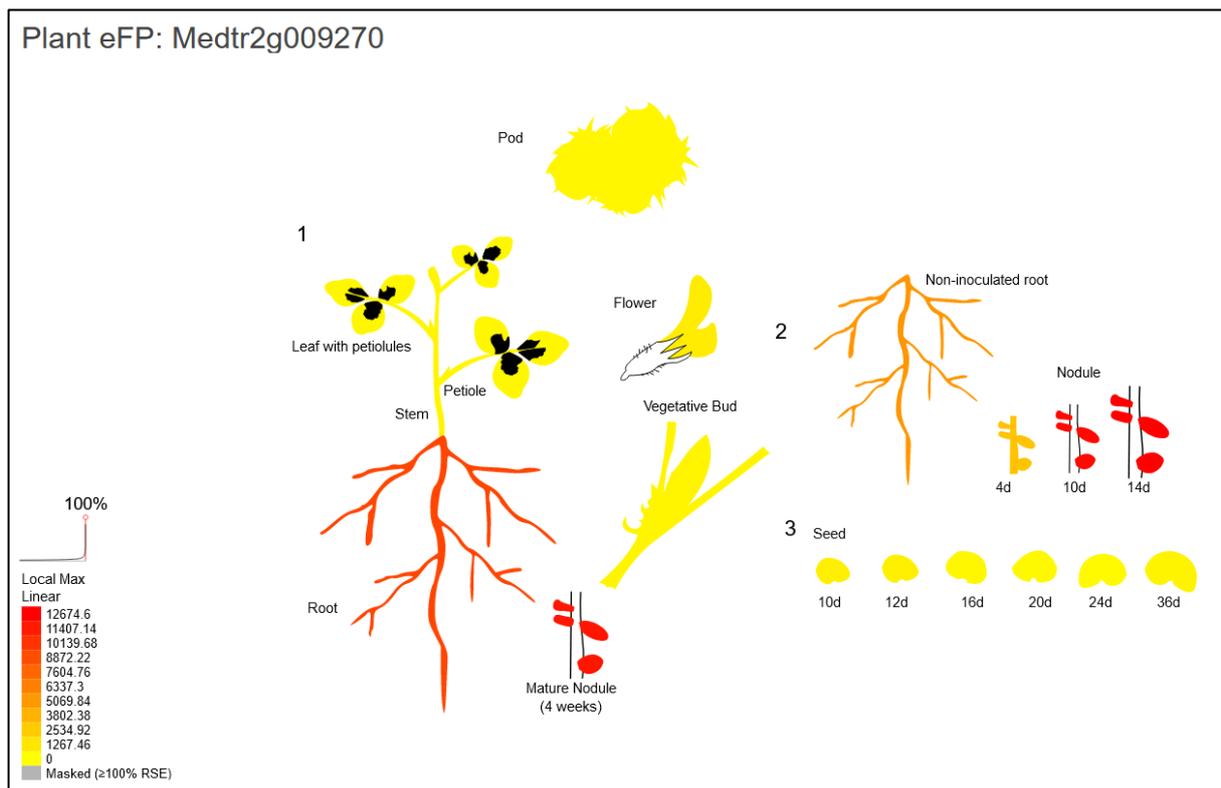

Figure 1. Expression profile of GTP cyclohydrolase II in *Medicago truncatula* as obtained from Plant eFP. Information on website: "Data a gene expression atlas of the model legume *Medicago truncatula*: Udvardi et al. 2008, The Plant Journal 55:504-513. Data normalized by MAS 5.0 and RMA methods. TGT value of 100. All tissues sampled in triplicate. This image was generated with the Plant eFP at bar.utoronto.ca/eplant by Waese et al. 2017." Visible are the different plant organs of *Medicago truncatula* and their respective expression profiles of the GTP cyclohydrolase II, partly also to different timepoints during development.

to their HVC counterparts (see Figures 5 and 6 and Tables 8 and 9 of Chapter 3). These findings suggest that VC might not play a general, vital role for faba bean fitness, vigor and agronomic performance.

Another intriguing question is, where the very low, non-zero amount of VC stems from in LVC genotypes. These types carry a frameshift insertion in the VC1 gene and should, theoretically, not be able to produce any VC at all. In our data, two contigs could be found with the same annotation as VC1, namely "RibA" (pers. communication with Michael Höfer from AlPlanta, see also AboVici 2022). They show a slight deviation from the sequence of VC1 and map to the same region of *M.t.* on Chromosome 4 (regions 37271855 – 37270593, with a length of 1262 bp; and 37267131 – 37271512, with a length of 4381 bp; pers. comm. with Michael Höfer, 2022). These contigs have been found to be non-differentially expressed between HVC and LVC samples. This might point to the existence of a second GTP cyclohydrolase II (or RibA) gene. This might explain the residual (yet very low) amounts of VC found in LVC faba bean genotypes. It might also solve another riddle: Since riboflavin or vitamin B_2 is an essential micronutrient, a total lack of this compound would most probably be lethal for the plant (see also AboVici 2022; pers. comm. with Michael Höfer 2022), so maybe a non-zero activity of a RibA gene is vital, and is accompanied by a non-zero amount of VC.

We observed in our inheritance studies of VC in Chapter 3 that seeds of plants carry the same VC phenotype as the plants they emerge from (Table 7) – i.e. the VC amount is maternally inherited, as is reported in literature (Griffiths and Ramsay 1992; Duc et al. 1989; Brown and Roberts 1972).

From our distribution studies on VC in the plant (Chapter 3.3.1), it is clearly visible that VC amount is highest in the roots of faba bean plants as compared to shoot and leaves plus flowers (Table 6). Also, the amount of VC seems to decrease over time, since VC values are lower in plants after flowering than before flowering over all three tested plant organs. A survey of GTP cyclohydrolase II expression in *M.t.* on the Plant eFP platform (see Figure 1) reveals that this enzyme is most abundantly expressed in the roots. Interestingly, the platform also shows that GTP cyclohydrolase II is very highly expressed in nodules (Figure 1). Correspondingly, it is described in literature that GTP cyclohydrolase II might indeed play an important part in symbiotic interactions between rhizobia and their host plants (García Angulo et al. 2013; Pankhurst 1974; Streit et al. 1996). It is stated that riboflavin availability might influence the capacity of rhizobia to colonize roots and that it can influence the onset of rhizobial interactions (García Angulo et al. 2013). Examples are reported by Pankhurst (1974), who reported that a *Rhizobium trifolii* strain which is auxotrophic for riboflavin, requires the addition of riboflavin to be able to facilitate an effective symbiosis with red clover plants (Pankhurst 1974, García Angulo VA et al. 2013). Also, Streit et al. (1996) report that the addition of riboflavin to the rhizosphere can increase colonization of roots with rhizobia.

This indicates that the increased presence of VC in the roots as compared to the other organs of the plant as observed in our analysis of faba bean might merely be a by-product of increased GTP cyclo-hydrolase II activity to facilitate the symbiosis with rhizobia. It has been reported that VC in faba bean is also present in the embryo (see Chapter 1.5.2 and 2.2). It might be possible that the immediate presence of riboflavin in the embryo of faba bean gives the plant an edge in fast establishing a symbiosis with rhizobia directly after germination.

In conclusion, VC are probably formed as compounds in a directed overflow metabolism. Also, the agronomic performance of LVC plants will most probably not suffer from their much-reduced amount of VC as confirmed in our Legume fatigue trials 1 and 2 (Chapter 3.3.3 and 3.3.4). These experiments did not show decreased yield for LVC phenotypes. This makes the breeding of LVC cultivars and especially LVC winter faba bean cultivars, a good venture.

Consequently, we can confirm with that our breeding venture to create LVC winter faba bean lines to be able to later establish a LVC winter faba bean cultivar is a promising way to go. Utilizing the eight SNP markers which are suitable for LVC marker-assisted selection (MAS) in a winter faba bean background (Table 6 of Chapter 2 and Chapter 2.4.4) and which we established during our fine-mapping analyses, we developed LVC winter faba lines with a backcrossing-program. First, this program consisted of lines with a Hiverna/2 (HVC) * Mélodie/2 (LVC) background which were backcrossed to Hiverna/2 to increase the winter faba bean genome dose. After arriving at backcrossing generation BC_3F_2, the genetic diversity of the program was widened. We replaced Hiverna as backcross-parent and instead backcrossed with other winter faba bean lines (S_062, S_300, S_306 and S_340; Gasim and Link, 2007), which originated from the Göttinger Winter Faba Bean Population and exhibit superior winter hardiness and agronomic performance in Göttingen (see Chapter 2.4.6). Finally, we were able so sow the first generation of an experimental synthetic variety (Syn-0) in October 2019; it meanwhile serves as research and breeding germplasm pool and thus LVC feature is successfully established in winter faba beans.

The new breeding material, the realization that LVC phenotypes do not show lower field performance and lower fungal resistance and the very closely linked markers for the LVC trait newly developed, by us as well as by Björnsdotter et al. (2021), facilitating MAS, all these achievements will for sure advance faba bean breeding for the LVC trait in general and, specifically, will advance winter faba bean breeding.

This question needs to be answered: What is the economic significance of faba beans with LVC content. As part of the project Abo-Vici, feeding experiments with laying hens were performed at Friedrich Löffler Institute Braunschweig (Halle, 2020). During these experiments, Halle substituted 15-

30% of soybean coarse meal in the feed ratio by HVC faba beans. She could show that this led to reduced daily feed intake by the hens (www.proteinmarkt.de 2022). This, in turn, reduced the number of laid eggs. It also led to reduced egg weight and egg mass production (Halle 2020). Substituting the same amounts of soybean coarse meal with LVC faba beans, however, had no measurable negative consequences (Halle 2020). The PorReE-project (Porree 2022: https://www.youtube.com/watch?v=4j2j-md-dmA) was, among others, also asking how a faba bean diet in German local chicken races might influence their performance; and whether LVC faba bean cultivars made superior diets compared to HVC cultivars. The group of Nolte et al. (2020a; 2020b) found enlightening results: In terms of egg production and chicken bone stability, limited dietary effects could be observed in local chicken breeds fed with 20% HVC faba beans. These dietary effects constituted a significant reduction of egg weights as well as a significant reduction of eggshell stability (Nolte et al. 2020a). However, for the parameters chicken body weight development, chicken carcass qualtity and fattening parameters as well as general health of the chickens, no significant differences could be detected for animals fed with HVC and LVC faba bean proportion in the feed (Nolte et al. 2020b).

The findings show a mixed picture of the importance of LVC faba bean genotypes. In conclusion, though, feeding chicken with the risk of undesirable outcome due to the utilized feedstuff seems undesirable and usually leads to a lower acceptance of faba bean as a replacement for soybean. Therefore, LVC faba bean varieties are a promising option to introduce more faba beans into chicken feed.

The LVC faba beans discussed here are not "zero VC" phenotypes but rather phenotypes with a very low amount of VC (below 0.05%, a 20-fold reduction of their natural amount; Duc et al. 1989). It will be difficult to advance faba bean breeding in a "zero VC" phenotype direction, since such a mutation might possibly prove lethal (see above). Yet, with respect to the discussed findings of the impact of LVC faba beans on chickens, this does not seem to be a top priority, at least for the use of faba beans in chicken feed. Additionally, it has been shown that such LVC varieties are safe for consumption by humans suffering from genetic deficiency of G6PD as described in Chapter 1.5.2 (Khazaei et al. 2019). Concerning the topic of human consumption of faba beans, an interesting idea might be to introduce a label for LVC faba beans, calling attention to the specific nutritional benefits of the LVC feature.

Another promising approach into the direction of "zero VC" usage of faba beans unrelated to breeding might be a chemical and/or bioprocessing approach and has been introduced by the group of Rizzello et al. (2016). They showed that cost-effective bioprocessing techniques can be applied to detoxify faba bean for industrial applications. Additionally, the group of Pulkkinen et al. (2019) shows the possibilities to reduce VC in suspensions and sourdoughs and finds that is it possible to greatly

reduce the two compounds as well as the presence of their resulting aglycones, which cause the oxidative stress leading to favism.

In the future, hopefully the breeding of high performing LVC faba beans – and especially high yielding LVC winter faba beans – will lead to increased faba bean cultivation and faba bean utilization for chicken feed instead of mostly imported soybean. Moreover, the usage of faba bean for human consumption might be increased if the values of the LVC feature and the beneficial properties of faba bean such as high-quality protein content are more widely introduced to the public. Promising steps to this account have already been taking in ventures such as the "Demonstrationsnetzwerk Erbse / Bohne" (demoneterbo.agrarpraxisforschung.de).

Finally, it can be stated that our research about the VC gene was greatly facilitated by our ability to use the 50k SNP chip (Angra and O´Sullivan, 2021; personal communication). This technique enabled us to perform our isogeneity analysis of Chapter 4 of this thesis. In the future, the 50k SNP Chip and its follow-up tools will lead to important new fine-mapping and MAS possibilities and might therefore greatly advance research for unsolved problems in faba beans. One such unsolved problem is resistance to diseases. A few DNA markers have been developed for resistance to rust, ascochyta blight (Faridi et al. 2021), and broomrape, but resistances against other diseases or pests as well as suitable markers for these resistances are still lacking. A few examples are pests and diseases such as *Aphis fabae, Bruchus rufimanius* and *Sitona lineatus* (Rubiales and Khazaei 2022). Another great remaining problem are lacking resistances and employable markers to abiotic stresses such as heat, drought, frost and water logging (Adhikari et al. 2021).

A nutritional problem of faba bean that is solved, from a breeding perspective, is the presence or absence of the antinutritive compound tannin in the seed coat of faba bean. Recent studies concerning the complementary genes z-1 and zt-2, which control the absence of tannin in faba bean, made significant advances. Hou et al. (2018) were able to create a linkage map for zt-1 and identified an SSR marker that was able to predict the zt-1 genotypes with absolute accuracy while the group of Gutierrez et al. identified the bHLH transcription factor VfTT8 as the gene underlying the locus for zt-2. Corresponding tannin-free spring and winter bean cultivars exist and compete with wild-type cultivars on the market (i.e. Bianca in Germany, Bundessortenamt 2022).

Ultimately, what is needed to greatly advance faba bean breeding are more genetic tools such as the already mentioned 50k SNP Chip. Adhikari et al. (2021) discuss in their systematic review article about conventional and molecular breeding tools, among others, the great advancement that the physical DNA-sequence of the 13 Gb diploid genome of faba bean will bring to breeding (Adhikari et al. 2021; Faba Bean Genome Project: fabagenome.dk). Even though successful gene discoveries have

been made in the absence of such a faba bean reference genome, tools such as this facilitate genotyping approaches greatly and make methods such as genotyping-by-sequencing much more cost-effective (Adhikari et al. 2021; Khazaei et al. 2021). These resources, among many others, pave the way to advanced breeding techniques such as genomic selection and site-specific gene editing (Adhikari et al. 2021; pflanzenforschung.de). Combined, the newly available genomic resources, markers, and breeding techniques make it highly possible to greatly advance faba bean breeding and increase the still small area of this highly advantageous crop.

5.1 Literature

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Summary

Presented here is a thesis on a genetic and breeding project addressing the anti-nutritional seed constituents vicin and convicin in faba bean. Faba bean (*Vicia faba* L.) is a traditional grain legume of the Old World; vicin and convicin (VC) hinder the usefulness of this crop because they are antinutritional for non-ruminant animals such as poultry, and can be dangerous to humans with a genetic deficiency of the enzyme glucose-6-phosphate dehydrogenase. One goal of this work was to identify new DNA markers that are tightly linked to the VC locus and therefore useful for breeding. Additionally, it was aimed to find the major gene for the genetic difference between normal (HVC) and genetically very low (LVC) VC levels. A further objective was to realize for the first time the genetically very low VC content of the LVC type in winter field beans. In addition, field trials were conducted over several years to test whether LVC field beans might have agronomically detectable disadvantages compared to HVC types. Fungistatic effects of vicin and convicin, which suggest a higher susceptibility of LVC types to soil-borne fungi, have been reported in the past. Since winter faba beans have a higher yield than spring faba beans, breeding of a first winter faba bean variety with low VC content was aimed at here. Since major parts of the here-conducted research depended on the employment of near-isogenic lines, such pairs of lines were produced and their degree of isogeneity was analyzed. This was important to indicate the relevance of the obtained results.

The central work package was the fine mapping of the major locus for vicin and convicin. For this aim, two biparental F2 families were utilized, both resulting from crosses of two near-isogenic lines, one was a HVC type, the other was a LVC type. The respective parents of these two crosses and other closely related pairs of close-isogenic lines were used to perform two transcriptome sequencing experiments (employing RNAseq and MACE), the results of which were used to develop 58 novel VC-associated SNP markers. The two biparental F2 families mentioned above were then genotyped with these 58 new markers and with further markers which had previously been developed by other groups. Genetic maps were generated accordingly. Phenotypic data was used for the subsequent fine mapping; the data on VC seed contents of the genotyped F2 individuals were derived from HPLC analyses and employed for this fine mapping. A comparably very small region for the possible location of the VC gene (the main VC locus mentioned above) on chromosome 1 of *Vicia faba* of about 0.13 cM was finally identified.

In addition, based on these fine mapping results, the synteny to *Medicago truncatula* and *Cicer arietinum* and supported by the previous results of the Björnsdotter et al. (2021) group, the candidate gene RIBA 1 was identified as the causal gene of LVC vs. HVC. The markers most closely linked to this gene were tested on yet another F2 family, which had not yet been present in the analyses (this F2 family resulted from a cross of an HVC winter faba bean line with an LVC spring faba bean line). Eight

SNP markers suitable for breeding winter faba beans for low VC content were hence identified. Using these eight markers, novel LVC winter faba bean lines were developed and grown as Syn-0 in 2020 and Syn-1 in 2021 to establish them as a LVC synthetic breeding population or cultivar candidate.

LVC spring faba bean varieties have already been bred. Seeing the higher yield potential of winter beans, it seems advantageous to realize the LVC trait in winter faba beans, too. However, it is important to investigate whether a LVC content could have disadvantages under field conditions, especially for winter beans (which have longer growing seasons), for example higher susceptibility to soil-borne fungi. This must also lead to the consideration of VC content in roots, i.e. plant organs other than seeds. Therefore, specific studies were conducted to investigate the agronomic performance of faba beans (spring and winter) on legume-fatigued soils, to look at VC content in different plant organs, and to analyze the relationship between VC type in sown seed and VC type in harvested seed.

The field trials, which were performed to study the agronomic differences between LVC and HVC faba beans, were named legume fatigue trials 1 and 2. These trials were conducted in a field plot where a faba bean monoculture had been grown prior to these trials for at least three years. This monoculture induced the accumulation of faba bean-specific soil-borne pathogens. Legume fatigue trial 1 was performed with diverse faba bean lines and cultivars; specifically, HVC and LVC types, as well as winter and spring faba bean cultivars. These faba bean lines and cultivars were evaluated for their agronomic performance, specifically for their yield and yield components. The results did not indicate any disadvantages of the LVC lines and cultivars compared to the HVC lines and cultivars. Since the issue of agronomical performance of LVC faba beans compared to HVC faba beans is very important for breeding and recommending LVC winter faba bean cultivars, legume fatigue trial 2 was conducted in addition. For this, very near-isogenic line pairs, differing within one pair mainly in their VC content, were grown in the same field plot as legume fatigue trial 1. These near-isogenic line pairs were hence examined for agronomic differences in the same way as lines and cultivars in legume fatigue trial 1. Resulting from the very near-isogenic status of these pairs, detected differences in performance, if present, should now only be due to the different VC genetics and the resulting different VC status of the plants. However, the results of the legume fatigue trial 2 also showed no significant differences in yield between faba bean types with HVC and LVC. Conclusively, LVC plants seem to have no disadvantages compared to HVC plants in a field situation with high pathogen pressure from soil-borne pathogens. Therefore, breeding of LVC winter bean lines and varieties should not lead to agronomic problems caused by the LVC status of the plants.

Additionally, isogenic lines were used for distribution and inheritance studies on VC, owing to the importance of obtaining more insights into the trait VC content. These studies revealed the highest VC content to be found in the roots of faba beans, as compared to shoots and leaves and flowers. Seeds

were shown to have VC types similar to the genotype of their respective maternal plants (i.e., LVC, HVC, or intermediate), confirming seed VC content to be maternally determined.

Since knowing the actual degree of isogeneity was important in assessing the validity of the results described above, one work package concentrated on this. Therefore, all near-isogenic lines utilized in the project were genotyped using the 50k Affymetrix chip for faba bean in collaboration with O'Sullivan in Reading (UK). Within the isogenic pairs, 34320 SNP markers were analyzed and used for isogeneity level analysis. The results of these calculations showed a very high level of isogeneity for all tested near-isogenic pairs (in absolute terms, and compared to the expectation of isogeneity based on the pedigree of the tested lines). Therefore, it can be deduced that the agronomic inference from of our experiments regarding the differences between HVC and LVC lines are solid.

In conclusion, our research on the agronomic performance of LVC faba beans, the development of new markers closely linked to and the identification of the gene responsible for VC, and our initial approaches to breeding LVC winter type lines paved the way for breeding LVC winter field bean varieties.

Zusammenfassung

Vorgelegt wird eine genetisch-züchterische Arbeit zu den antinutritiven Sameninhaltsstoffen Vicin und Convicin bei der Ackerbohne. Die Ackerbohne (*Vicia faba* L.) ist eine traditionelle Körnerleguminose der Alten Welt; Vicin und Convicin (VC) behindern die Verwendbarkeit dieser Kulturpflanze, da sie für Nichtwiederkäuer wie z.B. Geflügel antinutritive Faktoren sind und zudem für Menschen mit einem genetischen Mangel des Enzyms Glucose-6-Phosphat-Dehydrogenase gefährlich sein können. Ein Ziel dieser Arbeit war es, neue DNA-Marker zu identifizieren, die mit dem genetischen VC-Status eng gekoppelt und daher züchterisch gut nutzbar sind. Außerdem sollte das Hauptgen für den genetischen Unterschied zwischen dem normalen (HVC) und dem genetisch sehr niedrigen (LVC) Gehalt an VC gefunden werden. Darüber hinaus war ein weiteres Ziel, erstmalig die genetisch sehr niedrigen VC-Gehalte des LVC-Typs in Winterackerbohnen zu realisieren. Es wurden zusätzlich mehrjährige Feldversuche durchgeführt, um zu prüfen, ob LVC-Ackerbohnen im Vergleich mit HVC-Ackerbohnen agronomisch feststellbare Nachteile aufweisen. In der Vergangenheit wurde über die fungistatische Wirkung von Vicin und Convicin berichtet, die auf eine höhere Anfälligkeit der LVC-Typen für bodenbürtige Pilze schließen lässt. Da Winterackerbohnen einen höheren Ertrag als Sommerackerbohnen haben, wurde hier die Züchtung einer ersten Winterackerbohnensorte mit niedrigem VC-Gehalt vorangetrieben. Da ein großer Teil der hier durchgeführten Forschung von der Verwendung nah-isogener Linien abhing, wurden solche Linienpaare hergestellt und ihr Isogenitätsgrad analysiert. Dies war wichtig, um die Relevanz der erzielten Ergebnisse aufzuzeigen.

Das zentrale Arbeitspaket war die Feinkartierung des Hauptlokus für Vicin und Convicin. Zu diesem Zweck wurden zwei biparentale F2-Familien verwendet, die beide aus je einer Kreuzung von zwei nah-isogenen Linien hervorgingen, je ein Kreuzungspartner war dabei ein HVC-Typ, der andere ein LVC-Typ. Die jeweiligen Eltern dieser beiden Kreuzungen und andere eng verwandte Paare nah-isogener Linien wurden zur Durchführung von zwei verschiedenen Transkriptom-Sequenzierungstechniken (RNAseq und MACE) verwendet, deren Ergebnisse zur Entwicklung von 58 neuen, VC-assoziierten SNP-Markern genutzt wurden. Die beiden oben erwähnten biparentalen F2-Familien wurden dann mit diesen 58 neuen und anderen Markern, die zuvor von anderen Arbeitsgruppen entwickelt worden waren, genotypisiert. Es wurden entsprechend genetische Karten erstellt. Phänotypischen Daten wurden für die anschließende Feinkartierung verwendet; diese Daten zum VC-Samengehalt der genotypisierten F2-Individuen wurden aus HPLC-Analysen abgeleitet und für diese Feinkartierung verwendet. Es wurde schließlich eine vergleichsweise sehr kleine Kernregion für das VC-Gen (für den o.g. Hauptlokus) auf Chromosom 1 von *Vicia faba* von etwa 0.13 cM identifiziert.

Darüber hinaus wurde auf Basis der Feinkartierung, mittels Syntenie zu *Medicago truncatula* und *Cicer arietinum* und unterstützt durch die früheren Ergebnisse der Gruppe Björnsdotter et al. (2021)

das Kandidatengen RIBA 1 als das ursächliche Gen für LVC vs. HVC identifiziert. Die am engsten mit diesem Gen gekoppelten Marker wurden an einer weiteren F2-Familie getestet, die noch nicht in den Analysen enthalten war (diese F2-Familie entstand aus einer Kreuzung einer HVC-Winterbohnenlinie mit einer LVC-Sommerbohnenlinie). So wurden acht SNP-Marker identifiziert, die sich für die Züchtung von Winterackerbohnen auf einen niedrigen VC-Gehalt eignen. Mit Hilfe dieser acht Marker wurden neue LVC-Winterbohnenlinien entwickelt und 2020 als Syn-0 und 2021 als Syn-1 angebaut, um sie als synthetische LVC-Zuchtpopulation oder als Sortenkandidaten zu etablieren.

LVC- Sommerackerbohnen sind bereits gezüchtet worden. Angesichts des höheren Ertragspotenzials von Winterackerbohnen erscheint es vorteilhaft, einen LVC-Gehalt auch bei Winterackerbohnen zu etablieren. Es ist jedoch wichtig zu untersuchen, ob ein LVC-Gehalt unter Feldbedingungen Nachteile insbesondere für Winterackerbohnen haben könnte (die eine längere Vegetationsperiode haben), z. B. eine höhere Anfälligkeit für bodenbürtige Pilze. Dies führt unter anderem auch zur Betrachtung des VC-Gehalts in Wurzeln, d. h. in anderen Pflanzenorganen als Samen. Daher wurden spezielle Studien durchgeführt, die die agronomische Leistung von Ackerbohnen (sowohl Sommer-als auch Winterackerbohnen) auf Leguminosen-müden Böden untersuchten, den VC-Gehalt in verschiedenen Pflanzenorganen betrachteten und die Beziehung zwischen dem VC-Typ im Saat-Samen und dem VC-Typ im geernteten Saatgut analysierten.

Die Feldversuche, die zur Untersuchung der agronomischen Unterschiede zwischen LVC- und HVC-Ackerbohnen durchgeführt wurden, werden als Leguminosen-Müdigkeitsversuch 1 und 2 bezeichnet. Diese Versuche wurden auf einem Feldstück durchgeführt, auf dem zuvor mehrere Jahre lang eine Ackerbohnen-Monokultur angebaut worden war. Diese Monokultur führte zu einer Anreicherung von ackerbohnenspezifischen Bodenpathogenen. Der Leguminosen-Müdigkeitsversuch 1 wurde mit verschiedenen Ackerbohnenlinien und -sorten durchgeführt; insbesondere mit HVC und LVC-Typen sowie mit Linien, die einen normalen Tanningehalt in den Samenschalen aufwiesen oder genetisch Tannin-frei waren, sowie mit Winter- und Sommerackerbohnensorten. Die Ackerbohnenlinien und -sorten wurden hier hinsichtlich ihrer agronomischen Leistungen, insbesondere auf ihren Ertrag und ihre Ertragskomponenten, untersucht. Die Ergebnisse ließen keine Nachteile der LVC-Linien und -Sorten im Vergleich zu den HVC-Linien und -Sorten erkennen. Da die Frage der agronomischen Leistung von LVC-Ackerbohnen im Vergleich zu HVC-Ackerbohnen für die Züchtung und Empfehlung von LVC-Winter-Ackerbohnensorten sehr wichtig ist, wurde zusätzlich der Leguminosen-Müdigkeitsversuch 2 durchgeführt. Zu diesem Zweck wurden sehr nah-isogene Linienpaare, die sich innerhalb einer Paares hauptsächlich in ihrem VC-Gehalt unterschieden, in derselben Parzelle wie der Leguminosen-Müdigkeitsversuch 1 angebaut. Diese nah-isogenen Linienpaare wurden in gleicher Weise auf agronomi-

sche Unterschiede untersucht wie die Linien und Sorten im Leguminosen-Müdigkeitsversuch 1. Aufgrund des sehr nah-isogenen Status dieser Paare sollten die festgestellten Leistungsunterschiede, sofern vorhanden, nur auf die unterschiedliche VC-Genetik und den daraus resultierenden unterschiedlichen VC-Status der Pflanzen zurückzuführen sein. Die Ergebnisse des Leguminosen-Müdigkeitsversuchs 2 zeigten jedoch ebenfalls keine signifikanten Ertragsunterschiede zwischen Ackerbohnenlinien mit HVC und LVC. Zusammenfassend lässt sich sagen, dass LVC-Pflanzen im Vergleich zu HVC-Pflanzen in einer Feldsituation mit hohem Erregerdruck durch bodenbürtige Krankheitserreger offenbar keine Nachteile haben. Daher sollte die Züchtung von LVC-Winterackerbohnenlinien und -sorten nicht zu agronomischen Problemen führen, die durch den LVC-Status der Pflanzen verursacht werden.

Zusätzlich wurden isogene Linien für Verteilungs- und Vererbungsstudien über VC verwendet, da es wichtig ist, mehr Erkenntnisse über das Merkmal VC-Gehalt zu gewinnen. Diese Untersuchungen ergaben, dass der höchste VC-Gehalt in den Wurzeln der Ackerbohnen zu finden ist, verglichen mit Stängeln und Blättern und Blüten. Es zeigte sich außerdem, dass die Samen den gleichen VC Typ aufwiesen wie ihre jeweiligen Mutterpflanzen (d.h. LVC, HVC oder intermediär), was bestätigt, dass der VC-Gehalt der Samen mütterlicherseits bestimmt ist.

Da die Kenntnis des tatsächlichen Isogenitätsgrades für die Bewertung der Qualität der oben beschriebenen Ergebnisse wichtig war, konzentrierte sich ein Arbeitspaket darauf. Daher wurden alle im Projekt verwendeten nah-isogenen Linien in Zusammenarbeit mit O'Sullivan in Reading (UK) mit dem 50k Affymetrix-Chip für Ackerbohnen genotypisiert. Innerhalb der nah-isogenen Paare wurden 34320 SNP-Marker analysiert und für die Analyse des Isogenitätsgrades verwendet. Die Ergebnisse dieser Berechnungen zeigten ein sehr hohes Maß an Isogenität für alle getesteten nah-isogenen Paare (in absoluten Zahlen und im Vergleich zur erwarteten Isogenität auf der Grundlage des Pedigrees der getesteten Linien). Daraus lässt sich ableiten, dass die Schlussfolgerungen aus unseren Experimenten hinsichtlich der Unterschiede zwischen HVC- und LVC-Linien solide sind.

Zusammenfassend lässt sich sagen, dass unsere Forschung zur agronomischen Leistung von LVC Ackerbohnen, die Entwicklung neuer Marker, die eng mit dem VC-Gen verbunden sind, und die Identifizierung des dafür verantwortlichen Gens sowie unsere ersten Ansätze zur Züchtung von LVC-Linien den Weg für die Züchtung von LVC-Winterackerbohnensorten geebnet haben.

Acknowledgements

Curriculum vitae

Name:	Rebecca Tacke
Date of birth:	28.07.1990
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	29574 Ebstorf
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Education

2017-2023

Promotion

Georg-August-Universität Göttingen

Graduiertenschule
Forst- und Agrarwissenschaften (GFA)

Division of plant breeding methodology

Topic of dissertation: "Breeding and agronomy of novel, low-vicine faba beans and adaption as domestic protein feed"

Vicin and convicin are antinutritional compounds of faba bean (*Vicia faba* (L.)) with partly negative consequences when used as feed e.g. for laying hens. The aim of this work was to precisely localize the main gene for these compounds by fine mapping and, with the help of the genetic markers thus generated, to breed the first winter-hardy faba bean variety low in vicin and convicin. (see also project "Abo-Vici", https://www.uni-goettingen.de/de/abo-vici-projekt/559637.html)

Tasks: Supervision and execution of field experiments, crossing work in greenhouse and isolation houses, statistical evaluation of experiments, sampling and evaluation of genetic markers, execution of fine genetic mapping, supervision of 5 bachelor students as well as one master student, participation in various conferences including poster presentations (u.a. GPBC 2018, ICLGG 2019, CiBreed 2019, GPZ 2020), co-organization of the CiBreed Conference 2019 in Göttingen

2014 – 2017

Master of Science

Georg-August-Universität Göttingen

Master's Degree Program: Agricultural Sciences

Focus on: Crop Sciences

Topic of Master thesis: „Cross-fertilization studies of faba bean"

Vicia faba (L.) is a partial cross-fertilizer. Its cross-fertilization and self-fertilization levels vary between 40 - 60%. The work carried out here investigated the extent to which the cross-fertilization levels of eight genotypes differ from each other in a polycross in the field.

2010 – 2014	**Bachelor of Science**

Georg-August-Universität Göttingen

Bachelor's Degree Program: Biology

Focus on: Molecular Biosciences

Topic of Bachelor's thesis: "Analysis of Putative Members of the Arabidopsis Hop/Sti1-Hsp90 Complex"

2003 – 2010	**Abitur**

Lessing Gymnasium, 29575 Uelzen

Advanced courses: Biology, Mathematics, English

Practical Experiences

2017 – 2020	**Employee of the Division „Plant breeding methodology"**

In working group „Züchtungsforschung Ackerbohne"; employed for project „Abo-Vici"

2017 – 2018	**Tutor and guest lecturer in the module „Genetische Grundlagen der Pflanzenzüchtung"**

Georg-August-Universität Göttingen

Master's Degree Program: Agricultural Sciences

Focus on: Crop Sciences

Tasks: Supervision of students in tutorials, joint solving of tasks set by lecturer; teaching of certain lecture topics, e.g. genetic drift and genomic selection.

2015 – 2016	**Assistant Scientist at the Institute for Bioinformatics**

Georg-August-Universität Göttingen

Tasks: Tutor of module "Applied Bioinformatics I" (topics among others: Creating and extensive use of databases, information theory), tutor of module "Basics of Biostatistics with R" (topics among others: Processing of large data files, descriptive statistics, statistical methods from 2-sample tests to ANOVA and post-hoc tests), participation in projects and in two publications (see below).

2013	**Participation in „Summer School of Green Genetics"**
	University of Wageningen, Netherlands
2010	**2-week internship at Bioplant Biotechnologisches Forschungslabor GmbH**
	Brüggefeld 44
	29574 Ebstorf
2009	**4- week internship at Mingan Island Cetacean Studies (MICS)**
	Longue-Pointe de Mingan, Kanada

Scientific Publications

2022 Tacke, R.; Ecke, W.; Höfer, M.; Sass, O.; Link, W. (2022): Fine-mapping of the major locus for vicine and convicine in faba bean (*Vicia faba*) and marker-assisted breeding of a novel, low vicine and convicine winter faba bean population. Plant Breeding 141: 644-657. DOI: 10.1111/pbr.13039.

2016 Zeidler, S, Meckbach C, Tacke R, Raad FS, Roa A, Uchida S, Zimmermann WH, Wingender E, Gültas M. (2016): Computational Detection of Stage-Specific Transcription Factor Clusters during Heart Development. Front Genet. 23 (7):33. DOI: 10.3389/fgene.2016.00033.

2015 Meckbach, C.; Tacke, R.; Hua, X.; Waack, S.; Wingender, E.; Gültas, M. (2015): PC-TraFF: identification of potentially collaborating transcription factors using pointwise mutual information. BMC Bioinformatics 16:400. DOI: 10.1186/s12859-015-0827-2.

Further Knowledge

Languages:
German – Native language
English – fluent to business fluent in written and spoken language

French – basic skills

Software:
MS Powerpoint, MS Word, MS Excel – adept
R – well-founded
Python – basic skills
SQL – basic skills

Others: Driving license class B

Ebstorf, 18.07.2023