LR-5041
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REVIEW ARTICLE **Legume Research- An International Journal**

Advancement in Genomics and Molecular Marker Technologies for Breeding of Faba Bean with Low Vicine-convicine Content: A Review

Sadhan Debnath, Ng. Tombisana Meetei, Mayank Rai

10.18805/LR-5041

ABSTRACT

Faba bean (*Vicia faba* L.), is a popular legume crop due to its high protein content (22-38.2%), dietary fibre (12%), medicinal and nutritional values and environmental benefits. It is considered as an excellent source of protein, particularly in developing countries where people cannot afford to buy meat. It also has antioxidant, anti-inflammatory, antiviral, anticancer, anti-diabetic and anti-atherosclerotic effects. But the presence of some key anti-nutritional factors, such as vicine and convicine, tannins, phytic acid *etc.* restricts the potential utilization of this crop as food and feed. Vicine and convicine are the thermostable, glucosidic aminopyrimidine derivatives that accumulate in the cotyledons of faba bean during seed development and maturity and cause “Favism” or haemolytic anaemia upon consumption by individuals having deficiency of glucose 6-phosphate dehydrogenase enzyme. As globally millions of people are genetically predisposed to such condition, it is highly desirable to eliminate these compounds from faba bean without compromising yield and other quality characteristics. In this review, we have highlighted the recent advancement in the field of genomics and molecular marker technologies for an easy and efficient selection of faba bean with low vicine and convicine content and the strategies to deploy these efficient tools in the future molecular breeding programs in faba bean.

Key words: Anti-nutritional factors, Faba bean, Favism, VC locus, Vicine-convicine.

Faba bean (also broad bean, horse bean or field bean) is an annual herbaceous species of Fabaceae family and is believed to have originated in the Near East (Cubero, 1974). It is considered as a partially crosspollinated species and the rate of outcrossing ranges from 4 to 84% (Bond and Poulsen, 1983). It grows well at temperature about 22°C and tolerates chilling between 0 to 10°C. It is generally cultivated in warm temperate and subtropical countries in the winter and in northern latitudes in the spring (Duc, 1997). Faba bean is widely grown for food and feed as a generous source of high-quality protein, dietary fibre and other valuable nutrients (Duc, 1997). According to the Food and Agriculture Organization Corporate Statistical Database (2019), faba bean is the fourth most widely grown cool season grain legume globally after pea, chickpea and lentil.

Despite of having high yield potential and several nutritional, medicinal and environmental benefits, faba bean cultivation is mostly sporadic and mainly limited to some of the Middle-Eastern and European countries, which is chiefly because of the presence of some anti-nutritional factors (ANFs) and their effects on human and animals’ health. The most potent ANFs limiting its widespread consumption are vicine-convicine (VC) (Duc *et al*., 1999; Khamassi *et al*., 2013). Several genomics and molecular studies have been conducted to understand the genetics of the locus responsible for VC and elucidate biosynthetic pathways of these compounds. Further, high-throughput molecular markers flanking the allele for VC have been identified (Tacke *et al*., 2021; Björnsdotter *et al*., 2021; Khazaei *et al*., 2015). This review is focused on understanding the origin and domestication, major challenges in improving production and productivity, nutritional and medicinal values and key ANFs in faba bean. Moreover, it also highlights the achievements in genomics and molecular marker technology for improving production of desired quality faba bean, genetics of VC locus and genes for biosynthesis of VC and recent advances in molecular breeding of faba bean for low vicine-convicine (LVC) content.

School of Crop Improvement, College of Post-Graduate Studies in Agricultural Sciences, Central Agricultural University, Umiam-793 103, Meghalaya, India.

Corresponding Author: Sadhan Debnath, School of Crop Improvement, College of Post-Graduate Studies in Agricultural Sciences, Central Agricultural University, Umiam-793 103, Meghalaya, India. Email: debnathsadhan1989@yahoo.com

How to cite this article: Debnath, S., Tombisana Meetei, Ng. and Rai, M. (2022). Advancement in Genomics and Molecular Marker Technologies for Breeding of Faba Bean with Low Vicine-convicine Content: A Review. Legume Research. DOI: 10.18805/LR-5041.

Submitted: 13-09-2022 **Accepted:** 13-12-2022 **Online:** 27-12-2022

Origin and domestication of faba bean

Faba bean is one of the first domesticated food legumes and has a long history of cultivation, started in the early Neolithic times, nearly 8.000 B.C. (Karkanis *et al*., 2018; Torres *et al*., 2006; Cubero, 1974). However, the origin and domestication of faba bean is still debated as no wild progenitor of the crop has been discovered yet, or the progenitor may have been extinct (Cubero, 1974; Maxted, 1993). Based on the seed size, faba bean has four subspecies: *V. faba major* (having large seeds and mainly grown in China and South Mediterranean countries), *V. faba equina* (having medium seeds and mainly grown in North Africa and Middle Eastern countries), *V. faba minor* (having small seeds and generally grown in Ethiopia and North Europe) and *V. faba paucijuga* (which is the primitive form and mainly found from Afghanistan to India) (Cubero, 1974). No successful inter-specific cross between faba bean and other *Vicia* species has been reported (Duc, 1997). Production of faba bean is mainly concentrated in nine major agro-ecological regions: The Nile valley, Mediterranean basin, Central Asia, East Asia, Ethiopia, Oceania, Latin America, North America and Northern Europe (Torres *et al*., 2006). It was introduced into India nearly 3000 B.C. through the Mesopotamia probably after the advent of the Arabian spice trade route (Gol, 2015). Since then, faba bean has become a traditional legume crop in the state of Bihar, India. Moreover, this crop is also grown in small scale in Jharkhand, Eastern Uttar Pradesh, Chhatisgarh, Odisha, Madhya Pradesh and some of the North-Eastern states of India (Gol, 2015).

Major challenges in improving production and productivity of faba bean

Global production of faba bean is estimated to be 4.84 million tonnes from an area of 2.5 million ha (FAO Stat, 2019). China is the largest producer of faba bean (37.3%) followed by Ethiopia, Australia, United Kingdom, Germany, France and Egypt. Productivity is the highest in Europe (3.0 tonnes/ha), followed by Asia, Africa, Australia and the Americas (FAO Stat, 2019). Though production of faba bean has increased since last two decades, which may be due to selection and development of high yielding cultivars through various breeding programs, still there is immense need to increase the production and productivity of good quality faba bean varieties to sustain the global food security and nutrition requirement for an ever-increasing population, which is estimated to reach 9.6 billion by 2050 (FAO, 2020). Major faba bean breeding challenges are mainly due to its mixed breeding system, unknown wild progenitor and large genome size of nearly 13 Gb, which is the largest among diploid field crops (Khazaei *et al*., 2021). Like many other major legume, faba bean yield remains unstable due to biotic and abiotic stresses (Cernay *et al*., 2015). Moreover, studies reported that, the total grain yield of faba bean is positively correlated with seed protein content, which in turn is determined by genotypes (El- Sherbeeny and Robertson, 1992). However, recent studies have reported that several promising genotypes were identified for seed yield and its component quantitative traits that could be used in different faba bean hybridization programs for yield improvement (Dewangan *et al*., 2022; Kubure, 2016).

Commercial importance of faba bean

Faba bean could be eaten in several forms such as vegetable fresh, dry seeds, frozen or canned, snacks, stewed broad bean (Medamis), broad bean cakes (Taamia), stewed broad bean paste (Bissara), germinated broad bean soap, thick gruels, purees *etc.* (Dhull *et al*., 2021, Pasqualone *et al*., 2020). In China, Ethiopia, the Middle-East and the Mediterranean, faba bean is used as a breakfast food as soup, stews and paste, whereas, in India, fresh green podsare mainly cooked and consumed as vegetable. Apart from these, faba bean is also widely used as livestock feed for poultry, pigs and horses in many industrialized countries (Crépon *et al*., 2010, Guillaume and Bellec, 1977).

Nutritional property

Being an excellent source of protein (22-38.2%), faba bean is mainly used as a cheap source of protein in many developing countries where people find difficult to buy meat (Alghamdi *et al*., 2012). Its protein content is higher than other common food legumes (Burstin *et al*., 2011; Griffiths and Lawes, 1978). It is also a good source of carbohydrate (57.3%) with an average starch content of 47%, fibre (12%), lipids (1.2-4.0%), important vitamins (B complex vitamins), bioactive compounds and energy (320 kcal/100 g) (Karaköy *et al*., 2018; Baginsky *et al*., 2013; Ofuya and Akhidue, 2005). Faba bean contains high amount of folic acid (Vitamin B9, 148 mg/100 g), which plays a critical role in synthesis and repair of nucleic acids, amino acid metabolism and prevention of anaemia by helping in production of Red Blood Corpuscles (RBCs) (Singh, 2018). It is also a rich source of mineral elements as it contains good amount of macronutrients like nitrogen (6.40%), phosphorous (0.56%), potassium (1.51%), calcium (0.62%) and magnesium (0.35%) and micronutrients like copper (17.6 mg), zinc (42.7 mg), iron (83.8 mg) and manganese (24.0 mg) per kg (Karaköy *et al*., 2018).

Medicinal significance

Flavonoids, tannins, lignins, gallic acid, stillbenes etc. are some of the phenolic compounds present in faba bean. Among these, flavonoids are the most important compounds as they have antioxidant, antiviral, anticancer, anti-inflammatory and anti-atherosclerotic effects (Nijveldt *et al*., 2001). Faba bean contains L-DOPA (L- 3,4-dihydroxy phenylalanine), which is the precursor of Dopamine (Happiness Hormone) that has the ability to cross blood brain barrier and hence used for treatment of Parkinson’s disease (PD), the second most common neurodegenerative disease in elderly people, leading to disability due to an imbalance between dopamine and acetylcholine in the brain (Topal and Bozoğlu, 2016; Oviedo-Silva *et al*., 2018). The concentration of L-DOPA in dry seeds is nearly 0.07% (Ramya and Thaakur, 2007). It is also a suitable food for diabetic patients, heart and cardiovascular diseases because of its chemical composition (Baginsky *et al*., 2013).

Genetic constitution and genomic resources

Faba bean is a partially allogamous diploid species with six pairs of remarkably large chromosomes (2 n = 12) and has largest known genome (13 Gbp) among legumes (Sato *et al*., 2010; Ellwood *et al*., 2008) and any diploid field crops (Soltis *et al*., 2003) with more than 85-95 % repetitive DNA (Novák *et al*., 2020). The genome of faba bean is about 26, 15.9, 4.0, 3.0, 2.9 times larger than the model legume *M. truncatula*, Chickpea, Human, Lentil, Pea genomes respectively (Khazaei *et al*., 2021). Hence, the large genome size highly complicates the identification and location of important agronomic genes as well as the development of saturated linkage maps to be used as tools for Marker Assisted Selection (MAS). Therefore, genomic resources are relatively less advanced in faba bean compared with other grain legume species (Khazaei *et al*., 2021).

Though initially several genetic maps were developed with the help of morphological characteristics, isozymes, seed protein genes and random amplified polymorphic DNA (RAPD) markers, later faba bean genetic studies and breeding have been enriched due to development of expressed sequence tags (ESTs), microsatellites or simple sequence repeats (SSRs), EST-SSRs, single nucleotide polymorphisms (SNPs) and Kompetitive Allele Specific PCR (KASP) markers (Maalouf *et al*., 2022; Khazaei *et al*., 2021; Zanotto *et al*., 2020; Abuzayed, 2019; Khazaei *et al*., 2017; Kaur *et al*., 2014a;). Several mapping populations were developed for flowering, yield-related traits and plant architecture (Avila *et al*., 2017; Cruz-Izquierdo *et al*., 2012), biochemical and morphological traits (Ramsay *et al*., 1995), seed weight (Vaz Patto *et al*., 1999), drought adaptation-related, morphological traits and vicine-convicine (Khazaei *et al*., 2015, 2014a, 2014b,), rust resistance (Ijaz, 2018), rust, broomrape and ascochyta blight resistance (Román *et al*., 2004). In the absence of a reference genome assembly for this species, high-throughput approaches such as transcriptome analysis are considered as efficient tools for enrichment of genomic resources.

Antinutritional factors restricting faba bean usage

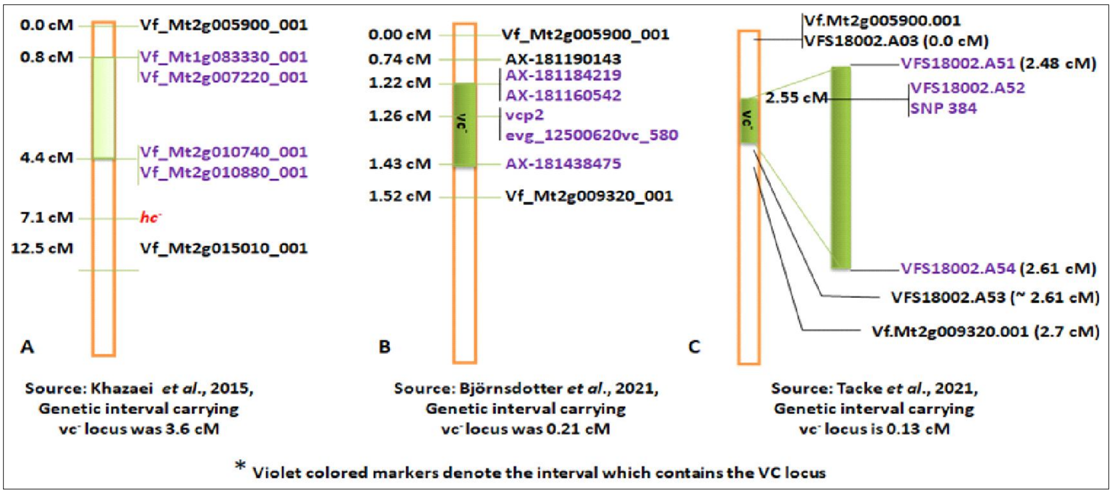
Key anti-nutritional compounds *viz*., vicine and convicine, tannin, phytic acid, saponins, lectins (favin) etc. in faba bean seeds can prevent its potential use as a protein source. VC are the thermostable, glucosidic aminopyrimidine derivatives that accumulate in the cotyledons of faba bean during seed development and maturity (Khamassi *et al*., 2013). The amount of VC in seeds ranges from 3 to 14 g/kg or approx. 0.3 to 1.5% in wild type, however, VC-free genotypes contain only 5-10% (0.3 to 1.4 g/kg) of this amount (Duc *et al*., 1999). Though VC are present in all parts of faba bean plant, seeds contain these compounds in an approximate 2:1 ratio (Goyoaga *et al*., 2008).VC are very much unique to the genus *Vicia*, whereas, *Momordica charantia* (bitter gourd or bitter melon or bitter apple) is the only species outside this genus containing vicine (Khazaei *et al*., 2019; Gauttam and Kalia, 2013).

Faba beans with high levels of VC when consumed by human being, may cause a condition called Favism in individuals having deficiency in G6PDactivity. When these compounds are hydrolyzed by β-glucosidase enzyme, they produce aglycones divicine and isouramil, which cause oxidation of glutathione in RBC, resulting in haemolysis of RBC (Björnsdotter *et al*., 2021). More than 400 million people (~4% of the world population), upon ingestion of faba bean containing high level of VC, will suffer from favism, which is caused by the human X-chromosomal inherited genetic deficiency of G6PD. Tannins interfere with digestive enzymes by forming complexes with nutrient molecules which results in reduced digestibility (Gutierrez *et al*., 2007), whereas, phytic acid reduces the bioavailability of minerals (Deshpande and Cheryan, 1984). For improving quality traits in faba bean, major progress on reduction of VC and seed coat tannins, the main ANFs limiting faba bean seed usage, have been recently achieved through gene discovery (Björnsdotter *et al*., 2021; Zanotto *et al*., 2020; Gutiérrez *et al*., 2020; Gutiérrez and Torres, 2019).

Genetics of vc locus and genes for biosynthesis of VC

Consumption of faba bean is limited by the presence of potent ANF, VC in both fresh and dry seeds. This trait is governed by a locus, designated as VC locus, which has two alleles *viz*., VC$^+$ (wild type) and vc$^-$ (mutant allele). Duc *et al.,* (1989) reported a natural mutant genotype (genebank accession 1268(4) (1), which is a Greek landrace with LVC, after an extensive phenotyping of more than 900 faba bean genotypes. A single recessive allele, vc$^-$ confers the LVC phenotype in faba bean. The allele reduces the concentration of VC by more than 95% (10-20-fold reduction) in seeds of faba bean. Homozygosity at VC locus with mutant allele (vc$^-$/vc$^-$) has the potential to alleviate the genetic disorder of favism in G6PD-deficient individuals and prevent dietary disadvantages when such faba beans are used as feed for animals, whereas, heterozygosity leads to intermediate concentration of VC (Tacke *et al*., 2021; Gallo *et al*., 2018; Crépon *et al*., 2010). Initially, the vc$^-$ locus was mapped on chromosome 1 within an interval of 3.6 cM and ~5-10 cM away from the gene for colorless hilum (*hc*) (Fig 1A), though this marker doesn't give much guarantee of LVC without adequate phenotyping (Khazaei *et al*., 2015; Gutierrez *et al*., 2006). Björnsdotter *et al*., (2021) greatly refined the genetic interval carrying vc$^-$ locus to 0.21 cM by employing a population of 1,157 pseudo-F$_2$ individuals from the cross between Hedin/2 (normal phenotype) and Disco/1 (mutant phenotype with low vicine and convicine) (Fig 1B). Very recently, the same locus was fine mapped and the genetic interval was placed to 0.13 cM by employing another set of 58 newly developed SNPs (Tacke *et al*., 2021) (Fig 1C).

Earlier Duc *et al*., (1989) emphasized that formation of VC occurs in the seed coat of faba bean and thereafter, these compounds are transported to embryo. Several studies reported that the VC content of faba bean seeds is maternally determined (Khazaei *et al*., 2017; Khamassi *et al*., 2013; Ramsay and Griffiths, 1996). Based on a recent gene-to-metabolite correlation study by Björnsdotter *et al*., (2021), it was demonstrated that the contents of these compounds in seed are maternally determined and they are synthesized in maternal tissues and finally transported to embryo. The biosynthetic pathway of these compounds was recently elucidated by Björnsdotter *et al*., (2021), who provided the experimental evidence that, a bifunctional riboflavin biosynthetic protein, RIBA1 plays the principal role in the biosynthesis of VC (Fig 2). The GTP cyclohydrolase II domain of RIBA1, which catalyzes the first step of the riboflavin biosynthetic pathway, also catalyzes the key step in the biosynthesis of VC.



Source: Khazaei *et al*., 2015, Genetic interval carrying vc- locus was 3.6 cM

Source: Björnsdotter *et al*., 2021, Genetic interval carrying vc- locus was 0.21 cM

Source: Tacke *et al*., 2021, Genetic interval carrying vc- locus is 0.13 cM

* Violet colored markers denote the interval which contains the VC locus

Fig 1: Fragments of chr. 1 of faba bean showing the putative regions of VC locus.

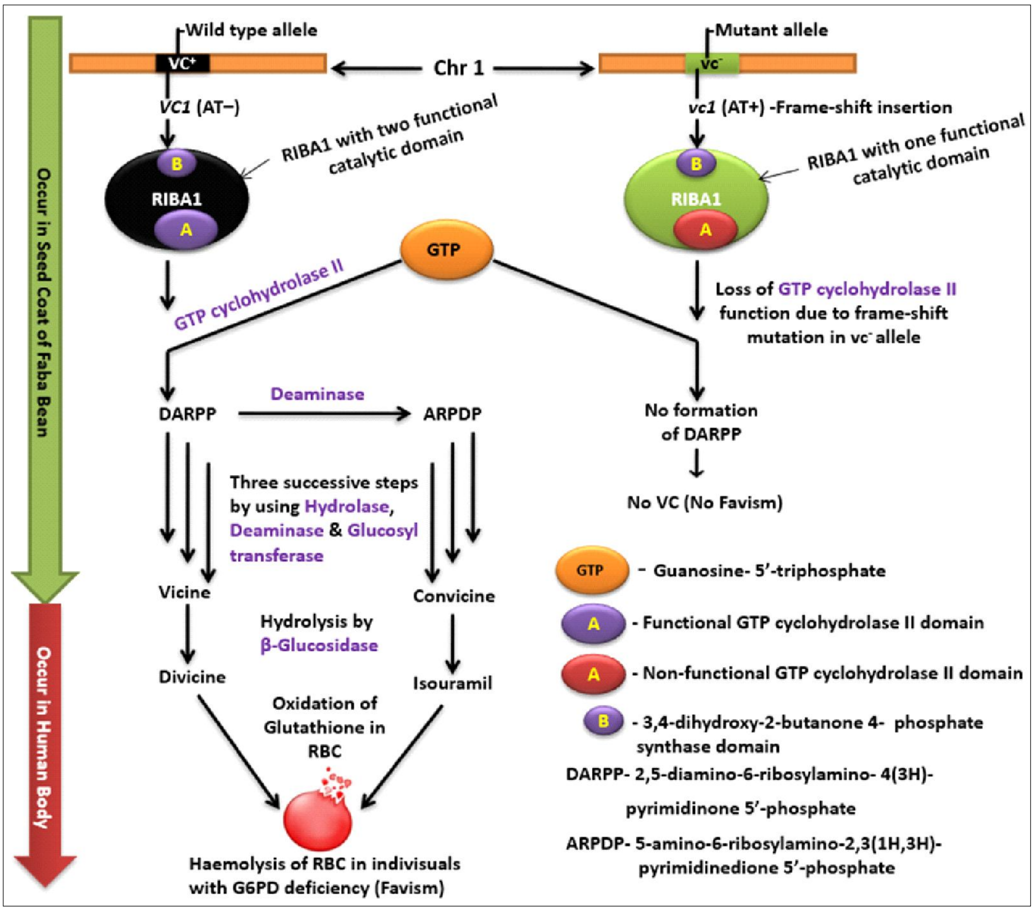


Fig 2: Schematic diagram of biosynthesis of VC in faba bean.

Expression profiling of 20 genes, which were very tightly correlated with vicine revealed that the gene *evg_*1250620 has the highest expression level in whole seeds during the early seed-filling stage (Björnsdotter *et al*., 2021). This gene encodes an isoform of 3,4-dihydroxy-2-butanone-4-phosphate synthase/GTP cyclohydrolase II, which is a bifunctional riboflavin biosynthesis enzyme (designated as VC1). According to Björnsdotter *et al*., (2021), Gene expression profiling revealed that the expression of VC1 was 7.4 times higher in seed coat than embryo. This enzyme activity is very low in young seeds, but at its maximum in matured seeds and again comes down in older seeds (Björnsdotter *et al*., 2021). The enzyme gets inactivated during seed drying process, cooking or by acids treatment similar to adult gastric juice. Their findings clearly suggest that VC are the side products of riboflavin biosynthesis from the purine GTP. They have hypothesized that the cause of the LVC phenotype is a frame shift insertion in the *vc1* gene located at vc allele and simultaneous changes in the amino acid sequence of RIBA1 protein, whose GTP cyclohydrolase II function is destroyed due to the two base pairs (AT) insertional mutation.

Molecular breeding strategy for faba bean with LVC

Faba bean exhibits huge genetic variability for nutritional contents as well as ANFs (Karaköy *et al*., 2018; Baginsky *et al*., 2013; Burstin *et al*., 2011; Duc *et al*., 1999). Faba bean genotypes which have relatively low levels of VC, tannins and phytic acids and high phytase activity are beneficial and highly desirable for their potential use in human and animal nutrition. The discovery of the natural mutant with vc locus had led to an increasing interest among plant scientist in breeding and development of LVC faba bean varieties (Duc *et al.,*1989). But despite of having monogenic inheritance nature of vc$^-$ locus, the initial attempts to develop marker assisted selection strategies had been unsuccessful towards the identification of suitable and reliable molecular markers for the vc$^-$ allele, which is chiefly because of lack of sequence information and limited genomic data on such a huge and complex genome of this crop (~ 13 Gb; Cooper *et al*., 2017, Soltis *et al*., 2003). Consequently, the genomic tools in faba bean are still underdeveloped (Annicchiarico *et al*., 2017) and therefore, the high syntenic correspondence of this crop to the already sequenced genomes of *Medicago truncatula*, *Cicer arietinum* and other legumes of subfamily Faboideae have great importance for genomic analyses (Kaur *et al*., 2014b, 2012; Cruz-Izquierdo *et al*., 2012; Ellwood *et al*., 2008).

Ellwood *et al*., (2008) for the first time provided a sequence-based genetic map of *Vicia faba,* which allowed the global pattern of *Vf -Mt* synteny to be observed. But the most clear and accurate picture of macrosynteny between *Vf* and *Mt* comes from the study of Webb *et al*., (2016). A single consensus map containing 687 SNPs and six linkage groups was constructed by merging six linkage maps. This sequence-based consensus map has been utilized for exploring synteny with the most closely related legume species such as *Medicago truncatula*, *Cicer arietinum, Lens culinaris etc*. (O'Sullivan and Angra, 2016). These SNPs are valuable tools for genotyping unexplored faba bean genotypes and synteny based trait targeting.

As the genetic interval carrying vc$^-$ allele is present at the distal end of the short arm of chromosome 1 of faba bean, the tightly linked high-throughput markers flanking the allelic position are of utmost importance for an efficient marker assisted selection (Webb *et al*., 2016). Although, initially Khazaei *et al*., (2015) genotyped a segregating population of 210 F_5 recombinant inbred lines with a set of 188 SNPs and identified a strong single QTL determining VC concentration on chromosome 1, flanked by markers 1.0 cM upstream and2.6 cM downstream of the QTL (Fig 1A), but unfortunately these markers turned out to be non-diagnostic for LVC in a large set of germplasm. For the first time, Khazaei *et al*., (2017) developed and validated a reliable, low-cost, breeder-friendly, robust and high-throughput KASP marker system (KASP_vcp2) for large scale screening of LVC faba bean. This marker system is a highly efficient tool, because it can detect individual homozygous or heterozygous plants very easily without any phenotyping for VC. Hence, this marker has the potential to achieve rapid homozygosity at VC locus for LVC (vc-/vc-) (Khazaei *et al*., 2017) and facilitate any faba bean breeding program through rapid acceleration in the process of genetic improvement in faba bean.

In another attempt, Björnsdotter *et al*., (2021) narrowed down the interval for vc$^-$ allele by employing SNPs from a previous map based on 3.6 cM interval reported by Khazaei *et al*., (2015) and individual SNPs designed based on markers mined from RNA-seq data. The SNPs (AX-181184219/AX-181160542 and AX-181438475), are flanking the genetic interval carrying VC locus, spanning a genetic distance of 0.21 cM (Fig 1B). The interval also contains the KASP marker (vcp2) previously found to be tightly linked with VC gene as reported by Khazaei *et al*., (2017). Recently, the same locus was fine mapped to a region of 0.13 cM by using 58 newly developed SNPs (Tacke *et al*., 2021). Markers VFS18002.A51 and VFS18002.A54, which span an interval of 0.13 cM are reported to be the final boundary markers which define the core region where the VC locus must be located (Fig 1C). They also reported that, based on the data generated by Song, (2017) and Khazaei *et al*., (2017), markers SNP384, Vf_Mt2g009320_001 and Vf_Mt2g010740_001 and their chromosomal vicinity deemed to be the most likely region which contains the VC gene. These markers could be used as potential diagnostic marker for VC content in faba bean.

CONCLUSION

Faba bean, a highly proteinaceous underutilized legume, has immense potential to fulfil the nutritional requirement and maintain general wellbeing and health of human beings including large number of low-income families of several states of India. The global average yield of faba bean has been increased significantly in the last four decades. Faba bean varieties with improved tolerance to several biotic and abiotic stresses were released in different parts of the world. However, food safety concerns remain as the potential impediment, which is threatening human and animal health and delaying large scale cultivation and commercialization of this crop as food and feed for nutrition. Large genome size and unavailability of a suitable reference genome assembly also complicate the molecular breeding strategy for improving quality characteristics in faba bean.

Recent discovery of the *VC1* gene and fine mapping of VC locus using tightly linked SNPs will greatly facilitate the selection of faba bean for LVC. Tightly linked SNP markers mentioned here could serve as highly useful tools to explore LVC content in large number of local and unadopted germplasms of major faba bean growing regions including North-East India. The significant SNPs near close vicinity to the VC locus or residing at the VC locus could be useful for high throughput SNP genotyping for low content of VC using KASP-assay. Reliable SNPs, which show adequate association with VC phenotyping can also be characterized by using ARMS markers for tetra-primer Amplification Refractory Mutation System PCR (ARMS-PCR) assay. One-step ARMS-PCR is highly efficient and cost effective as this assay could easily identify the genotype of a plant based on homozygous or heterozygous status of an SNP allele. Moreover, a PCR reaction volume of 10 µl is generally sufficient and alleles are easily separated on simple agarose gel. SSRs or microsatellites markers, which were found to be linked with VC content in faba bean, could serve as valuable tools for screening faba bean genotypes for low and high VC content in a limited resourced facility. However, further study needs to be done with these microsatellites markers for generating conclusive data in faba bean.

As the sequence of the VC locus is publicly available now, breeders can easily design primers specific to the two bp insertional mutation, which has led to a substantial reduction in VC content in faba bean. Presently, several collaborative reference genome assembly and pan-genome initiatives are underway and these are expected to provide annotated reference transcriptomes for faba bean and hence, accelerate high-throughput genotyping in this crop. Advanced faba bean genotypes with low vicine and convicine could efficiently be used in future breeding programmes in faba bean worldwide to prevent the metabolic food disorder of Favism in susceptible individuals.

Authors' contribution

All authors contributed equally in literature search, compiling writing and editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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