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Review

Eliminating vicine and convicine, the main anti-nutritional factors restricting faba bean usage

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ABSTRACT

Background: Faba bean (*Vicia faba* L.) seeds are an excellent source of plant-based protein. In spite of the vast nutritional and environmental benefits provided by faba bean cultivation, its use as a food crop has been restricted, primarily due to the presence of the pyrimidine glycosides vicine and convicine (v-c). Ingestion of v-c can cause favism in individuals with a genetically inherited deficiency in glucose-6-phosphate dehydrogenase (G6PD). In monogastric animals, v-c can cause decreased feeding efficiency. The elimination of these glucosides is a goal of most faba bean breeding programs worldwide.

Scope and approach: Our review focuses on the current genetic, molecular and biochemical knowledge surrounding the accumulation of v-c in faba beans. The gap between the current knowledge and what remains unknown is discussed. This review also explores historical and obscure information on v-c in faba bean.

Key findings and conclusions: A low-v-c faba bean line was identified in the 1980s and this trait has been introduced into several modern cultivars. It has been shown that low-v-c faba beans are safe for G6PD-deficient individuals. A robust molecular marker is now available for marker-assisted breeding to reduce levels of v-c. The biosynthetic pathway of v-c is not yet understood and is currently under investigation. An international coordinated effort, led by the authors of this paper, is making progress towards full elucidation of the pathway. Further efforts in this direction could lead to lower levels of these compounds than the current low v-c genotypes offer, perhaps even complete elimination.

1. Introduction

Cultivation of faba bean (*Vicia faba* L.) as a legume crop delivers generous economic and environmental benefits, stemming from its low reliance on nitrogen inputs and consequent reduced greenhouse gas emissions. Production of plant-based protein has a much lower environmental cost than that of animal-based protein. Faba bean provides symbiotically fixed nitrogen, thus improving soil fertility for itself and successive crops. As a non-host of many cereal pathogens, it can break the cycle of soil-borne diseases in cereal crops (Köpke & Nemecek, 2010). Faba bean is a versatile crop used globally as food, feed, forage, and medicine and as a cover crop. Its seeds help meet the basic dietary needs of millions of people and animals worldwide, as they are a rich source of plant protein, nutrients and dietary fiber (Duc, 1997; Multari, Stewart, & Russell, 2015; Warsame, O'Sullivan, & Tosi, 2018). The immature green pods and seeds of faba bean are marketed as a fresh or frozen vegetable. The dry seeds are widely cooked directly or first canned. Faba bean has a wide adaptability across various global agro-ecological zones including the northern temperate, Mediterranean and sub-tropical savannah. In 2016, faba bean global production was 4.5

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million tons (Tg) from 2.4 million ha (M ha). China was the major producer, with 1.6 M ha (36% of the world production) followed by Ethiopia (0.88 M ha) and Australia (0.42 M ha) (FAOSTAT, 2017). In the European Union, its cultivated area was nearly 0.66 M ha with a production of 2.0 Tg (Eurostat, 2019).

In spite of the many benefits of faba bean, its cultivation and consumption has been historically restricted due to the pyrimidine glycosides vicine and convicine (v-c), which are stored in cotyledons of most faba beans at about 1% of dry matter (Khamassi et al., 2013; Purves, Zhang, Khazaei, & Vandenberg, 2017). The presence of v-c causes favism, an acute haemolytic anaemia, in human individuals who have an X chromosome-inherited glucose-6-phosphate dehydrogenase (G6PD) deficiency (Luzzatto & Arese, 2018). V-c also cause a significant reduction in the efficiency of production systems for broiler chickens, laying hens and pigs (Vilariño, Métayer, Crépon & Duc, 2009; Crépon et al., 2010; Grosjean et al., 2000; Lessire et al., 2017). Though still a matter of speculation, the ability to synthesize v-c and accumulate high concentrations in the seed is thought to have evolved because of a beneficial antibiotic effect during seed germination (Griffiths & Ramsay, 1992; Pavlik, Vanova, Laudova, & Harmatha, 2002; Ramsay & Griffiths, 1996). The level of v-c in faba bean seeds was shown to be the main mortality factor for *Callosobruchus maculatus* (L.) larvae and some other phytophagous pests (Desroches, El Shazly, Mandon, Duc, & Huignard, 1995).

Vicine and convicine are thermostable, but their concentration can be greatly reduced by soaking the seeds in water or in a weak acid solution (Hegazy & Marquardt, 1983; Jamalian & Ghorbani, 2005) prior to cooking. Thermal processing such as boiling, roasting, microwave irradiation, and frying can reduce the v-c content in faba bean seeds (Hussein, Motawei, Nassib, Khalil, & Marquardt, 1986; Ganzler & Salgó, 1987; Muzquiz et al., 2012; Cardador-Martinez et al., 2012). In addition, the combination of enzyme treatment with fermentation (Pulkkinen et al., 2019) or of alkaline extraction with acid precipitation can reduce v-c content by more than 99% (Vioque, Alaiz, & Girón-Calle, 2012). However, removal or destruction of v-c by dry milling for protein concentration on an industrial scale is problematic because air classification of faba bean protein (Tyler, Youngs, & Sosulski, 1981) concentrates the v-c up to nearly four-fold in the protein fraction (Fig. 1). Pitz, Sosulski, and Hogge (1980) reported a similar trend. Wet processing methods for protein purification, e.g., isoelectric precipitation, can remove anti-nutritional factors such as v-c from protein fractions, but these methods are costly and energy-intensive (*reviewed in* Singhal, Karaca, Tyler, & Nickerson, 2016). The best solution for the reduction of v-c is breeding for low v-c faba beans, and the discovery of a low-v-c accession with up to 95% reduction in v-c content compared to wild type has enabled the transfer of the low v-c trait to faba bean cultivars by sexual crosses (Duc, Sixdenier, Lila, & Furstoss, 1989).

Genetic approaches to reducing v-c levels have been challenging because of the mixed breeding system of the crop (Drayner, 1956) and the lack of any knowledge regarding the biosynthetic pathway of v-c. In the last few years, there has been significant investment and progress in molecular breeding and functional genomics of v-c in faba bean (Maalouf et al., 2018). This has been driven mainly by the need to extend crop rotations because of environmental concerns and by the globally increasing demand for plant-based protein. An additional motivation has been the rotational benefits of the crop in legume-supported cropping systems (Watson et al., 2017). In this review, we bring together the current genetic, molecular, and biochemical knowledge surrounding the accumulation of v-c with perspectives on how this knowledge can be used to breed new solutions to a very old problem.

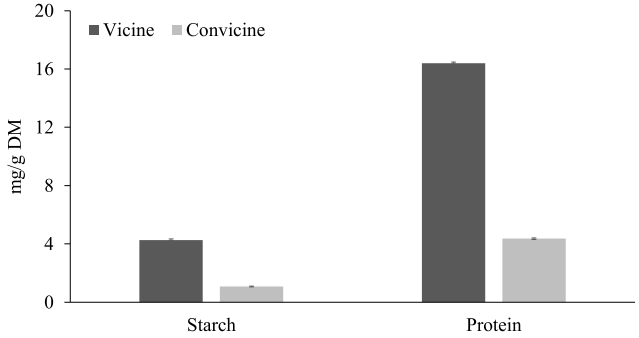

Fig. 1. Vicine and convicine concentrations in the air classification fractions (dry processing), starch and protein of faba bean cv. CDC Snowdrop. The vicine and convicine detection method is described in Purves et al. (2017).

2. The souls of the dead: faba beans

Faba bean has had a controversial history in its centre of diversity, the Mediterranean region. Although it was one of the earliest domesticated crops of Near Eastern agricultural systems (Caracuta et al., 2015), some cultures in the ancient world considered it an undesirable crop. In some regions, in historical periods, its cultivation was banned. Herodotus, the Greek historian of the 5th Century BCE, wrote that Egyptians refused to cultivate faba bean. In Rome, priests of Jupiter were not allowed to touch or even mention them, due to their association with death and decay (Johnson, 1963). The Greek philosopher, Pythagoras, banned his followers from eating faba beans and he was often illustrated avoiding them (Fig. 2). It is claimed that this aversion caused his life to end tragically because he did not want to walk into a faba bean field when he was pursued by the people of Crotonia in

Fig. 2. An illustration showing Pythagoras avoiding faba bean plants. “Do Not Eat Beans” [fol. 25 recto], 1512/1514. Photo © National Gallery of Art, Washington D.C.

Southern Italy and he was killed at the field's edge (Arie, 1959; Meletis; Konstantopoulos, 2004). The faba bean was, however, highly regarded in other historical periods and regions for the benefits it provided. For example, the Fabaria was a feast celebrated in ancient Rome during the month of June in celebration of Carna, the goddess of vital organs and health (Johnson, 1963).

This historical suspicion of faba bean may be rooted in its pathological effects in individuals susceptible to favism, which is induced in humans that carry G6PD deficiency. The deficiency is common in the Mediterranean basin, Middle East, North Africa, and other areas in which malaria is or has been endemic (Luzzatto & Arese, 2018). Surveys of G6PD deficiencies in malaria-endemic countries indicate an average frequency of 8%, reaching over 20% in India, China, Pakistan and Nigeria (Howes et al., 2012). Around the Mediterranean basin, frequencies range from under 1% to over 20% (Al-Musawi et al., 2012). In Rome, a large survey revealed a G6PD deficiency frequency of 1.1%, whereas in Sardinia the frequency averaged 7.5% with a maximum of 33% (Maffi et al., 2014). By comparison, in the Sassari province of Sardinia, favism occurs at the frequency of 1.2 cases per 10,000 individuals (Meloni, Forteleoni, Dore, & Cutillo, 1983) compared to Northern Europe and the United States, with only 1 case per 50,000 (Kalfa, 2016). Awareness of the size of the at-risk population in Italy, coupled with rare reports of individual cases of haemolytic crisis in G6PD-deficient subjects said to have been brought on by exposure to faba bean pollen (e.g. Brodribb, 1966), led to local ordinances still in force in numerous areas stipulating that flowering faba bean crops can be required to be destroyed if grown within 300 m of a residence or public place frequented by a sufferer of favism; furthermore, displays of fresh faba beans must be restricted to specially labelled, closed containers. This type of ordinance appears to treat all living faba bean plant parts as potential contact allergens, despite the well-understood etiology of favism having nothing to do with the immune system. In fact, there is no evidence that v-c are contained in any appreciable level in pollen, which is not wind-borne, nor is there a means of ingestion of pollen such that an interaction with red blood cells could take place. The 'pollen favism' myth has been categorically refuted (Luzzatto & Arese, 2018), so we posit that such restrictive ordinances are untenable and should be repealed.

3. Etiology of favism

G6PD deficiency, at a global average frequency of 4.9%, is widespread in the human population, affecting more than 400 million people worldwide (Nkhoma, Poole, Vannappagari, Hall, & Beutler, 2009). The G6PD gene is on the X chromosome, so the deficiency is more frequently and severely expressed in men. Favism is an acute hemolytic anemia that occurs after the consumption of faba beans containing high levels of v-c. In red blood cells, v-c are metabolized into their aglycone derivatives, divicine and isouramil, which in turn oxidize intracellular glutathione (Winterbourn, 1989). In order to preserve redox homeostasis, oxidized glutathione must be reduced back to glutathione, a process that requires reducing equivalents from the co-factor NADPH. Since the only source of NADPH in red blood cells is the pentose phosphate pathway, of which G6DP is a crucial enzyme, G6PD-deficient individuals cannot regenerate the oxidized glutathione efficiently. The resulting oxidative damage causes the affected red blood cells to aggregate before they can be removed by the immune system. The G6PD enzyme deficiency is caused by various mutant alleles of the G6PD gene. There are various levels of deficiency, from severe (potentially fatal) to an extreme overexpression (Cappellini & Fiorelli, 2008).

It has been widely shown that G6PD deficiency reduces the severity of malaria (Arese, 2006; Louicharoen et al., 2009; Mbanefo et al., 2017; Ruwende & Hill, 1998). The malaria parasite's development in mutant red blood cells causes the oxidation level to increase. Since the deficient individuals cannot rapidly reduce glutathione in the red blood cells, the immune system identifies these cells as damaged and removes them before the parasite can reproduce. Thus, G6PD deficient individuals exhibit reduced severity of malaria due to the parasites being removed by the immune system before it undergoes schizogenesis (Cappellini & Fiorelli, 2008).

Other than avoiding the ingestion of v-c from faba bean or oxidizing drugs such as quinone (Arese, Gallo, Pantaleo, & Turrini, 2012), there is no treatment for favism, and prevention depends on the subjects knowing that they are G6PD-deficient. This is not generally the case unless and until they experience a haemolytic crisis that is correctly diagnosed. An alternative approach to prevention could be the development and use of faba bean cultivars with low levels of the glucosides. Such varieties have been developed and are commercially available in some countries (Khamassi et al., 2013; Purves et al., 2017). Importantly, a recent study confirmed that consumption of large portions of low-v-c faba beans by G6PD-deficient men was safe and favism did not develop in any of the men (Gallo et al., 2018).

4. V-c in other plant species

Vicine was first isolated from seeds of common vetch (*Vicia sativa* L.) in the late 1800s (Ritthausen & Kreusler, 1870), and in 1914 the compounds were discovered in faba bean (Johnson, 1914). V-c have also been found in other *Vicia* species, such as *V. narbonensis* (Pitz et al., 1980; Griffiths & Ramsay, 1996), but their synthesis is not unique to the *Vicieae* tribe. Vicine was also isolated from the seeds of bitter melon or gourd (*Momordica charantia* L.), a member of the Cucurbitaceae family (Dutta, Chakaavarty, Chowdhury & Pakrash, l981; Zhang, Wang, Zhang, Liu, & Hu, 2003; Lucas, Dumancas, Smith, & Arjmandi, 2010). Our data revealed that bitter melon had higher vicine concentration in its seed (1–2% of the seed by dry weight) than faba bean, but that the convicine concentration was very low (Table 1). A draft genome sequence of bitter gourd has been published (Urasaki et al., 2017) and could be helpful in investigating the biosynthetic pathway of v-c. There are contrasting findings on the presence of v-c in beetroot (Chenopodiaceae, Von Lippmann, 1896; Pitz et al., 1980) and in *Lathyrus* species (Fabaceae, Jamalian, Aylward, & Hudson, 1977; Pitz et al., 1980; Griffiths & Ramsay, 1992).

5. Sites of synthesis and accumulation of v-c

V-c are concentrated in the cotyledons of both fresh and dry seeds of faba bean (Crépon et al., 2010). It was suggested that v-c synthesis occurs within the developing pod (Brown & Roberts, 1972). Pitz, Sosulski, and Rowland (1981) and Ramsay and Griffiths (1996) reported that v-c were possibly synthesized during the seed filling stage, most likely in the testa of the seed. The hypothesis was that v-c are synthesized in the testa and then transported to the cotyledons,

Table 1
Vicine and convicine concentration in bitter melon (*Momordica charantia*) and faba bean (*Vicia faba*) seeds at maturity stage.

	Vicine (mg/g dry weight)	Convicine (mg/g dry weight)	Origin
M. charantia			
BT-1	21.35	0.006	USA
BT-3	18.27	0.016	China
BT-4	17.92	0.012	China
BT-5	11.29	0.007	Jade Dragon
V. faba			
ILB 938/2	4.64	3.090	Columbia
Mélodie/2[a]	0.29	0.014	France
CDC Snowdrop	5.64	0.940	Canada

Components were measured following Purves et al. (2017).
[a] Marketed as low v-c.

resulting in fresh faba bean seeds having the highest v-c concentration (Pitz et al., 1981; Ray & Georges, 2010). V-c are also present in roots of faba bean (Ramsay & Griffiths, 1996), while stem, petiole, and leaf tissue accumulate only low amounts (Griffiths & Ramsay, 1996; unpublished data of the authors). The v-c concentration in the seed decreases rapidly with seed development (Burbano, Cuadrado, Mercedes, & Cubero, 1995; Hussein et al., 1986; Jamalian & Bassiri, 1978). Burbano et al. (1995) found slightly more than an 8-fold reduction of v-c concentration during seed development, with the highest concentration in young, fresh seeds at 80% moisture content.

6. Discovery of genetic sources of low v-c

From the 1970s to the mid-1980s, a wide range of faba bean germplasm was screened worldwide with the aim of finding a reliable source of low v-c (Bjerg, Norgaard, Olsen, Poulsen, & Sorensen, 1985; Collier, 1976; Engel, 1970; Gardiner, Marquardt, & Kemp, 1982; Hussein et al., 1986; Jamalian, 1978; Pitz et al., 1981). The efforts revealed little variation for v-c, with a mean concentration of approximately 1% of seed DM. No source lacking v-c was found. Later, Duc et al. (1989) reported a low v-c genotype [line 1268 (4)(1)] after screening a germplasm collection of 919 accessions. Line 1268 (4)(1) was an introduction from a collection of the Department of Plant Genetic Resources, Radzikov, Poland. The low v-c trait was found to be inherited as a single recessive Mendelian character that was associated with a nearly zero v-c content in seeds; it was designated "*vc*$^{-}$". The discovery of this gene opened a realistic possibility for breeding low v-c faba beans.

A second gene for reduced v-c content, *vcr*, was reported after screening 6700 M_2 mutants from the cv. Troy (the first faba bean bred by NPZ and promoted in UK in the 1980s) by Ramsay, Griffiths, and Dow (1991). The *vcr* gene had up to a 20-fold reduction in v-c concentration with respect to wild-type faba bean, similar to *vc*$^{-}$. It was suggested that the *vcr* gene from mutant plant MTG77 was dominant. Another mutation program in Greece, which used gamma-ray irradiation of the Greek cultivar Polycarpe, resulted in a source of low vicine levels (Kara, Lithourgidis, Tsaftaris, Psomas, & Tzavella-Klonari, 1988). The latter two potential sources of low v-c are not available for further investigations from genebanks or host institutes. The genetic interactions among these reported sources remain unresolved.

6.1. Low v-c germplasm catalogue

Low v-c cultivars have been released mainly by INRA (Institut National de la Recherche Agronomique) in France and marketed by Agri-Obtentions (https://www.agriobtentions.com/pulses/field-bean.html). Existing registered low v-c faba cultivars in the French catalogue are Disco, Mélodie, Divine, Dixie, Fabelle, Medina, AO 1155, and Betty. The level of v-c in seeds of most of these cultivars was reconfirmed by Khamassi et al. (2013) and by Purves et al. (2017). The cultivars Lady, Mandoline, Nakka, and Tiffany from NPZ (Norddeutsche Pflanzenzucht, https://www.agriobtentions.com/pulses/field-bean.html) have also been commercially released as low v-c cultivars (Puspitasari, 2017). All the above-mentioned cultivars carry the *vc*$^{-}$ gene. Segregating populations have been developed using this gene for genetic studies (Khazaei, Link, Street, & Stoddard, 2018a; Khazaei, Stoddard, Purves, & Vandenberg, 2018b). Cultivation of the low v-c faba bean varieties remains limited. For example, they represented only 13% of French faba bean production in 2014 (Lessire et al., 2017). Low v-c faba bean cultivars are under development in many other countries, including Finland, Denmark, the UK and Canada.

7. V–c phenotyping

Accurate and rapid phenotyping of v-c concentration is important in faba bean genetic improvement programs. Initially, colorimetric and spectrophotometric methods (Collier, 1976; Kim, Hoehn, Eskin, & Ismail, 1982; Olsen & Anderson, 1978; Sixdenier, Cassecuelle, Guillaumin, & Duc, 1996) were developed, but they are most accurate at high v-c concentrations and lack specificity or sensitivity. More selective liquid chromatographic (LC) methods, typically using UV detection (Pulkkinen et al., 2015; Quemener, 1988), have also been employed for the determination of v-c concentration. Although these methods are more selective and robust, levels of convicine in low v-c genotypes were frequently reported as zero or undetectable. Because small, polar analytes such as v-c are well suited for use with hydrophilic interaction liquid chromatography (HILIC), the use of a HILIC-UV method enabled quantification of convicine in low v-c seeds (Purves, Khazaei, & Vandenberg, 2018a). However, the combination of chromatographic methods with tandem mass spectrometry (more specifically, selective reaction monitoring, or SRM) led to large improvements in sensitivity and selectivity, thereby enabling the routine detection of much lower v-c values that could be used even for complex biological extracts (Purves et al., 2018a).

Unfortunately, for rapid analysis, the relatively long acquisition time required when using chromatographic methods (typical methods require 10–30 min per sample) can be a major limitation. Because tandem mass spectrometry is highly sensitive and selective, the analysis of plant seeds was investigated using flow injection analysis (FIA) instead of chromatographic separation (Purves, Khazaei, & Vandenberg, 2018b). Although a 1 min FIA-SRM method was successful for screening faba bean seeds, its key limitation was that the presence of vicine interfered with the determination of convicine concentration. To improve method selectivity without increasing analysis time, the use of ion mobility was investigated. Ion mobility involves interactions between gas-phase ions (such as v-c ions produced during MS analysis) and neutral gases, such as air, and can be used to separate these ions (Purves, 2018).

One such technique, high-field asymmetric waveform ion mobility spectrometry (FAIMS) acts as an ion filter; ions have specific conditions in which they will be successfully transmitted through the FAIMS device and into the mass spectrometer with the parameter that controls ion selection being called the compensation voltage, CV (Purves, 2018). FAIMS conditions were determined in which v-c and L-DOPA were transmitted at different CV values, thereby enabling gas-phase ion separation and accurate determination of all three compounds (Purves et al., 2017). One of the benefits of FAIMS is that analytes of interest are separated using voltages in the gas-phase on a millisecond time scale. Thus, unlike an LC analysis where the user must wait for the compound to elute (separation based on time), with FAIMS, the CV value(s) is readily cycled to detect the ion(s) of interest. The analysis time for the FIA-FAIMS-SRM method for the rapid high-throughput phenotyping of v-c and L-DOPA was < 1 min (Purves et al., 2017).

Thus, at present, FIA-FAIMS-SRM is undeniably the most efficient and accurate approach for quantifying v-c that is available. The initial set-up cost for such an instrument is substantial, but when used in a high-throughput manner, subsequent cost is on the order of a few USD per sample. Although less expensive LC-UV instrumentation can also be used to distinguish between high and low v-c cultivars, throughput is much lower and v-c values for low v-c cultivars will not be as accurate, especially for convicine.

8. Genotype by environment effects on v-c concentration

There is no clear trend in v-c concentration in faba bean seeds produced in different environments. Previous studies on genotype × environment interactions did not establish the mechanisms that influence environmental effects on v-c concentrations (Gardiner et al., 1982; Pitz et al., 1981; Pulkkinen et al., 2015). Since v-c are measured as a concentration, the value is affected by the concentration of other components of the seed such as starch and protein that are also environmentally influenced. V-c were reported to have fungicidal effects

on pathogens (Pavlik et al., 2002), suggesting that their concentration may increase in wet conditions that promote fungal growth (Pulkkinen et al., 2015).

9. Genetics and molecular marker development

9.1. Maternal effects

Duc et al. (1989) reported no effect of the embryo genotype on the v-c seed phenotype and there is agreement in the literature that the v-c concentration in faba bean cotyledons is maternally determined (Duc et al., 1989; Khamassi et al., 2013; Khazaei et al., 2017; Ramsay et al., 1991; Ramsay & Griffiths, 1996). This consensus fits with the data showing that v-c are synthesized in the maternal plant tissues (particularly seed coats as stated above) and then imported into the cotyledons.

9.2. Morphological markers

Hilum color in faba bean (which can be colourless or black) is determined by a single gene under maternal control, with black hilum being dominant over colourless (Erith, 1930; Khazaei et al., 2015; Sirks, 1931). Due to the fortuitous in-phase linkage of the recessive *vc*$^-$ locus with a recessive pale hilum variant in the original *vc*$^-$ donor material, the use of the visible hilum color marker to track *vc*$^-$ in early generations of crosses between *vc*$^-$ and black hilum genotypes became the established method for breeding low v-c faba beans. However, this linkage is now known to be of 5–10 centimorgans distance (Duc, Marget, Page, & Domoney, 2004; Khazaei et al., 2015); given cv. Betty as an example of *vc*$^-$ with a black hilum, colourless hilum is no longer a reliable marker for low v-c (Khazaei et al., 2017; Purves et al., 2018a). Other sources of colourless hilum are associated with the wild-type *Vc*$^-$ allele.

9.3. Marker-assisted selection

Faba bean is a diploid species with six large chromosome pairs and a huge genome of ~13 Gbp in the haploid complement (Soltis, Soltis, Bennett, & Leitch, 2003). Molecular breeding in faba bean is relatively less advanced than in other legume species, but significant progress has been made to enrich genomic regions linked to the v-c. PCR-based marker systems including RAPD (random amplification of polymorphic DNA), AFLP (amplified fragment length polymorphism) as well as isozymes were used to map v-c in BCF_2 (backcross) and F_6 RIL (recombinant inbred line) populations of faba bean (Ramsay, Griffiths, Waugh, & Powell, 1995). Three genomic regions were found to be associated with low v-c, one with a very large effect as well as two minor QTLs.

Gutiérrez et al. (2006) reported two RAPD markers, converted to CAP (cleavage amplified polymorphism) markers, which were linked to the low *vc*$^-$ gene using an F_2 segregating population (Vf6 × line 1268). Khazaei et al. (2015) employed SNP (single nucleotide polymorphism) markers, developed for use in comparative mapping between the model legume *Medicago truncatula* and faba bean in an F_5 RIL population from Mélodie/2 × ILB 938/2. The distribution of v-c concentrations in the RIL progeny was bimodal, which was consistent with the detection of a single major QTL at the previously reported *vc*$^-$ locus on chromosome 1, explaining 76% of the total phenotypic variation. Later, a robust, breeder-friendly and high-throughput KASP marker (Kompetitive Allele Specific PCR) was developed and validated for this region (Khazaei et al., 2017). Recently, the interval of the *vc*$^-$ region was saturated with additional SNP markers by the development of a 50 K Axiom SNP genotyping array, assisting to narrow this region; it is also being used as a basis for the ongoing positional cloning of the gene (O'Sullivan et al., 2018).

Subsequent studies found that concentrations of v-c were not completely controlled by one locus. A genome-wide association study detected v-c marker (SNP and AFLP) associations in a relatively large set of faba bean accessions with normal levels of v-c (Puspitasari, 2017). Apart from the expected associations of markers near the *vc*$^-$ locus, an AFLP marker (E40M59-387) was found tightly associated with a minor v-c gene. This marker was on the faba bean chromosome 5 consensus genetic map (Webb et al., 2016). Notably, this region was within the flanking region of the second QTL for v-c which has a minor effect that explains 15% of phenotypic variation (Khazaei et al., 2015). This second QTL was located in linkage group 2 (Khazaei, O'Sullivan, Sillanpää, & Stoddard, 2014), which is a region syntenic with *M. truncatula* chromosome 7. This suggests that at least one minor QTL on chromosome 5 modulates v-c content in addition to the *vc*$^-$ locus. This additional minor QTL needs further investigation and validation.

9.4. Candidate gene approach

In the absence of an annotated faba bean reference genome, most attempts at candidate gene discovery have been until now based on comparative genomics approaches. The challenge remains that other model legumes or plants do not synthesize v-c, and only a handful of *Vicia* species make these metabolites. Ray, Bock, and Georges (2015) reported six candidate sequences that may be involved in the v-c biosynthetic pathway, and one (contig 4518) of these was used to develop a robust molecular marker for low v-c (Khazaei et al., 2017). The BLAST search confirmed that the sequence of contig 4518 was matched to the Medtr2g009270 gene on *M. truncatula* chromosome 2. The Medtr2g009270 gene is annotated as *3,4-dihydroxy-2-butanone 4-phosphate synthase*. The sequence of this gene is found within the bifunctional riboflavin biosynthesis protein *RIBA1* in other sequenced grain legume species such as soybean (*Glycine max* [L.] Merr.) and chickpea (*Cicer arietinum* L.) (Khazaei et al., 2017).

Gutiérrez, Fernández-Romero, Atienza, Ávila, and Torres (2017) used comparative genomic approaches, KASPar SNP genotyping assays and high-throughput genome profiling DarTSeq of an F_2 cross (Vf6 × *vc*$^-$) to fine-map the *vc*$^-$ gene. The target region was narrowed to an interval of conserved synteny with *M. truncatula* chromosome 2 between the Medtr2g008210 and Medtr2g010180 gene models. In *M. truncatula*, this region contains 136 genes, but the exact gene content and order of the corresponding *Vicia faba* interval and the identity of the causative gene remains unknown.

10. Elimination of v-c in a food matrix

It is possible to degrade v-c in a food matrix. Treatment of a flour-water mixture (50:50) with a β-glucosidase-carrying strain of *Lactobacillus plantarum* resulted in deglucosylation of the v-c and complete degradation of the divicine and isouramil within 48 h (Rizzello et al., 2016). Treatment of cooked faba beans with ground raw almond (*Prunus dulcis* L.) powder, which is a rich source of β-glucosidase, also resulted in the hydrolysis of the v-c. The degree of hydrolysis ranged from partial to complete and was dependent on appropriate time, temperature, and pH conditions (Arbid & Marquardt, 1985). The utility of the end-product in the food chain has not been tested and some modification of the procedure may be necessary to make it industrially applicable. Because vicine and convicine are relatively small molecules in comparison to proteins, it may be possible to remove them by ultrafiltration, as is done for lactose from milk.

11. Conclusions

An international coordinated effort, led by the authors of this paper, is making progress in identifying the biosynthetic pathway of v-c. Further effort in this direction could lead to lower levels of these compounds than the current *vc*$^-$ genotypes offer, perhaps even complete elimination. At the same time, food scientists are investigating ways of

eliminating v-c and their aglycones from a food matrix, so that protein isolates and products derived from them can be labelled as v-c-free before low-v-c cultivars are available outside of their current region of adaptation in France and Germany. The combination of these efforts will enable the widespread use of faba bean as a valuable source of high-quality protein that can be produced in a sustainable manner for the expanding plant-based food chain. It will provide plant protein products suitable for consumption both by those with soybean allergy or intolerance and by those with a desire to consume local or regional plant products. Increased usage of faba bean in the food and feed industries can contribute to reduced demand for imported soybean, thereby lessening the pressure for land-use change in South America and other warm-climate regions from which plant proteins are exported to cool-climate regions.

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