Genetic study of the resistance of faba bean (Vicia faba) against the fungus Ascochyta fabae through a genome-wide association analysis

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Abstract
Ascochyta fabae is a fungal pathogen responsible for marked yield losses in spring and winter faba beans worldwide. The aim of this genome-wide association study (GWAS) using 188 diverse winter faba bean inbred lines was to exploit earlier Ascochyta blight resistance studies and to identify new resistance loci. Phenotyping after artificial inoculation under controlled conditions revealed significant variation for all eight scored disease traits. This GWAS was based on 1829 AFLP marker and 229 SNP marker, including 17 so-called ‘guide’ SNP markers. The latter were identified by map fragment alignments between the consensus smap of Webb et al., (2016, Plant Biotechnology Journal, 14, 177–185) and three earlier published Ascochyta blight resistance studies. A total of 12 markers were found significantly associated with six traits, explaining 5.6% to 21.7% of the phenotypic variance. One ‘guide’ SNP on chromosome III co-localizes with the known resistance QTL Af1 on chromosome III. Probably nine new resistance trait-associated marker loci were identified which will improve resistance breeding on winter faba beans and support a broader inclusion of the crop into rotations.

KEYWORDS
ascochyta blight, Ascochyta fabae resistance, GWAS, Vicia faba

1 | INTRODUCTION

Faba bean is a diploid legume crop adapted to cool-season regions. It offers nutritious seed (starch 45%, protein 30%) for humans and animals (e.g. Duc et al., 2015). Its agronomic benefits are supportive of a sustainable agriculture system as faba beans are capable of symbiotic fixation of atmospheric nitrogen and as being a useful break crop in cereal-dominated rotations (Angus et al., 2015). Major growing countries are China with about 933,000 ha, Ethiopia (519,000 ha), Australia (138,000 ha) and France (68,000 ha) (Rawal & Navarro, 2019). In Germany, cultivation area steadily increased during the last decade, from 16,300 hectares in 2010 up to 59,500 hectares in 2020 (Destatis, 2020; Zerhusen-Blecher et al., 2018). Yet faba bean production is affected by biotic stress as: Ascochyta blight (Ascochyta fabae), chocolate spot (Botrytis fabae), downy mildew (Peronospora viciae), rust (Uromyces viciae-fabae), foot rots (Fusarium spp.), and, in Mediterranean region, also by broomrape (Orobanche crenata) (Torres et al., 2006). Ascochyta blight is a serious disease, at times even a devastating threat to faba bean, the fungus Ascochyta fabae (its teleomorph is named Didymella fabae; Jellis &
Punithalingam, 1991) attacks both spring beans and winter beans. Ascochyta blight is found globally (Bond & Pope, 1980; Geard, 1961; Hawtin & Stewart, 1979). The disease symptoms are observed on leaves, stems, pods and seeds. Faba bean yield losses may rise to 90% for susceptible cultivars, aggravated by wet weather conditions (Ahmed et al., 2016; Davidson & Kimber, 2007; Hanounik & Robertson, 1989; Omeri et al., 2012). Ascochyta spores are carried and distributed by infected faba bean seeds and crop debris; rain and wind disperse the spores (Hanounik & Robertson, 1989; Rashid et al., 1991). Fungicides, appropriate crop rotation and the use of clean seed help in reducing Ascochyta incidence (Ahmed et al., 2016; Davidson & Kimber, 2007; Hanounik & Robertson, 1989; Omeri et al., 2012). The development of resistant cultivars is the most effective control option. Reduced lesion numbers, lesion size, lesion area and absence of pycnidia in lesions are indicative of Ascochyta resistance mechanisms (Maurin & Tivoli, 1992; Ondrej, 1993; Rashid et al., 1991). Several sources of partial resistance of faba bean against Ascochyta blight have been reported (Bond & Pope, 1980; Hanounik & Robertson, 1989; Rubiales & Fondevilla, 2012; Siddique et al., 2013; Sillero et al., 2001). There is limited, even conflicting information on the genetic basis of resistance against Ascochyta blight. Both polygenic (Roman et al., 2003) and major gene inheritance (Rashid et al., 1991) have been reported (cf. Avila et al., 2004; Hanounik & Robertson, 1989; Kohpina et al., 2000; Roman et al., 2003; Sillero et al., 2010; Stoddard et al., 1999). Moreover, the genetics of resistance in the stems and leaves of faba beans differ. Avila et al., (2004) described QTL specific to Ascochyta blight isolates and plant organs; and Kaur et al., (2014) found, in an artificial inoculation trial, four QTL for either leaf or stem necrosis. The small-seeded winter-type, inbred line 29H is highly resistant; A. fabae hardly penetrates its tissues (Bond et al., 1994; Maurin & Tivoli, 1992; Sillero et al., 2001, 2010; Tivoli et al., 1987, 2006).

The big genome size of faba bean (13,000 Mb) is a hurdle for genomic studies (Satovic et al., 2013). Actually, still no faba bean genome sequence is publicly available; but for the model legume Medicago truncatula, with its small genome of 500 Mb, sequence data are available (Burstin et al., 2007; Djemel et al., 2005; Duc, 2004; Gnanasambandam et al., 2012; Rispail et al., 2010). M. truncatula shows high synteny with faba bean; high synteny has also been reported from other legumes such as Cicer arietinum and Lupinus albus (Phan et al., 2007). Meanwhile, a large number of SNP and SSRs have been developed for Cicer arietinum and Lupinus albus (Phan et al., 2007). Meanwhile, a large number of SNP and SSRs have been developed for Lens culinaris, Cicer arietinum and Lupinus albus (Phan et al., 2007). Meanwhile, a large number of SNP and SSRs have been developed for Lens culinaris (Kaur et al., 2011; Sharpe et al., 2013), Pisum sativum (Kaur et al., 2012) and Cicer arietinum (Gujaria et al., 2011; Hiremath et al., 2011; Jhanwar et al., 2012; Stephens et al., 2014).

Various genetic faba bean maps have been published based on pertinent bi-parental mapping populations (Vf6 × VF136, 29H × VF136; Ascot × Icarus) (e.g. Gutierrez et al., 2013; Satovic et al., 2013; Webb et al., 2016). Most SNP used in Webb et al., (2016) can be assigned to a gene of M. truncatula. Hence, this data allow synteny-based transfer of genetic information from the genomic sequence of M. truncatula to the consensus faba bean linkage map (Sullivan & Angra, 2016; Webb et al., 2016). At the start of this study, the densest map available for us was Webb et al., (2016), containing 687 SNP markers. Meanwhile further such consensus maps have become known (e.g. Carrillo-Perdomo et al., 2020; Ocaña-Moral et al., 2017; Sudheesh et al., 2019). Although previously various types of markers were employed to detect and map QTL for Ascochyta resistance in faba beans (Avila et al., 2004; Cruz-Izquierdo et al., 2012; Díaz-Ruiz et al., 2009; Ellwood et al., 2008; El-Rodeny et al., 2014; Kaur et al., 2014; Roman et al., 2003), today, mainly SNPs are used for such analyses.

Already in 2003, Roman et al. detected two resistance QTL (Af1, Af2) in a faba bean study with artificial Ascochyta fabae inoculation in an F2 population derived from resistant line Vf6 and susceptible line VF136. QTL Af1 showed additive gene action and was located on chromosome III. QTL Af2, located on chromosome II, displayed dominant gene action for resistance. QTL Af1 explained 25.2% and QTL Af2 as 21% of the phenotypic variation in that study. Avila et al., (2004) continued the work of Roman et al., (2003). They suggested the presence of six QTL (Af3-Af8) based on off-spring from the cross-resistant line 29H × susceptible lineVF1136. Their QTL Af3 was located on chromosome III, just as QTL (Af1) mentioned above. Later, Díaz-Ruiz et al., (2009) confirmed, based on informative, common markers that QTL Af1 is located in the same chromosomal region as Af3. Furthermore, Díaz-Ruiz et al., (2009) confirmed the two QTL Af1 and Af2 in a recombinant inbred line (RIL) population derived from the material previously used by Roman et al., (2003). Both QTL together explained 24% of the phenotypic variation for disease severity on leaves and 16% of the phenotypic variation for disease severity on stem. Atienza et al., (2016) recently corroborated the QTL Af1 in a RIL population developed from the Avila et al., (2004) cross and judged it as identical to Af3. While Avila et al., (2004) assessed resistances after artificial inoculation in growth chamber, Atienza et al., (2016) tested in greenhouse and field. They re-identified Af2 on chromosome II and considered it the same as reported previously (Díaz-Ruiz et al., 2009; Kaur et al., 2014; Roman et al., 2003).

Kaur et al., (2014) employed SNP markers and reported four Ascochyta QTL in a RIL population derived from crossing Icarus (susceptible) × Ascot (resistant) and tested under controlled conditions. Both belong to Australian-bred germplasm, seemingly different from the genotypes used in previous, Spanish studies. Their QTL-3 and the prior reported QTL Af2 (Díaz-Ruiz et al., 2009; Roman et al., 2003) were both located on chromosome II; however, lack of common markers prevents more definite conclusions (Avila et al., 2004; Díaz-Ruiz et al., 2009; Roman et al., 2003). Ascochyta blight resistance QTL-2 and QTL-4, detected first by Kaur et al., (2014), were confirmed in a biparental (Nurah × Farah) RIL population by Sudheesh et al., (2019), on chromosome I and VI, respectively. Since both parents of that population, Nurah and Farah, were resistant to strain1, that RIL population specifically segregated for resistance to Ascochyta blight strain 2.

Breeding for improved resistance to Ascochyta blight is thought to significantly support winter faba beans in Central Europe, in Germany and neighbouring countries. In times of global warming,
winter beans may offer advantages over spring beans and increase the diversity of crop types to choose from. The Göttingen Winter Bean Population is a promising and highly relevant, diverse germplasm pool for this objective; it is used for breeding and research (Ali et al., 2016). In the present study, a panel of 188 winter faba bean lines, derived from that germplasm pool, is tested for Ascochyta blight resistance under controlled conditions and artificial inoculation. Phenotypic data on symptom expression are used for genome-wide association study to identify QTL for Ascochyta blight resistance in faba beans. Literature is thoroughly studied to exploit existing QTL data.

The study aimed to:

1. exploit existing data on Ascochyta blight resistance QTL from earlier studies in Mediterranean types and spring types of faba bean and
2. identify new markers associated with Ascochyta resistance in the Göttingen Winter Bean Population.

2 | MATERIAL AND METHODS

2.1 | Genetic material

The plant material consisted of \( N = 188 \) homozygous lines (association set; A-set) of faba bean (\( Vicia faba \) L.). These lines were bred via single-seed descent (SSD) without selection from the Göttingen Winter Bean Population (Link & Araboui, 2006) to generation \( F > 9 \). The GWBP was created in 1989 by combining 11 founder lines: Webo/1, Wibo/1, Hirvena/1, L79/79, L977/88/S1wn and L979/S1/1/sn (German lines), Côte d’Or/1, Arrisot (French lines), Banner/1, Bourdon/1 and Bulldog/1 (UK lines), through eight generations of open pollination (Gasim, 2003). A total of 400 SSD lines were developed of which the A-set was randomly taken and used for earlier analyses (Ali et al., 2016) and for the current association study.

2.2 | DNA markers and GWAS

Genotyping of the A-set lines as well as the 11 founder lines yielded a total of 2058 polymorphic markers, including 229 SNPs. Judged from the SNPs, the average degree of homozygosity was 98.8%, very high as expected. A number of 1,451 of these markers were mapped to 1,159 loci by Welna (2014). The 12 linkage groups of this author could be unambiguously assigned (Welna, 2014; pages XXVII–XXXV; cf. Ali, 2015) to the six \( Vicia faba \) chromosomes following the notation of Webb et al., (2016). After deleting markers with allele frequencies (MAF) ≤5%, a total of 1,355 markers were available for GWAS (1,147 AFLPs, 208 SNPs). The AFLP bands were scored as present/absent; the very few expected cases of wrongly scoring a heterozygous ALFP locus as homozygous dominant were tolerated. All AFLP markers and the majority of SNPs had been employed by Ali et al., (2016) for the same set of lines. All SNPs were chosen from the SNPs used by Webb et al., (2016). The SNPs contained 19 random SNPs and the so-called guide SNP marker set; these were not included in Ali et al., (2016). The 1,355 markers thus dissociate into two sets: R-set (randomly chosen markers set) and G-set (guide markers set). The R-set enclosed all AFLPs and the SNPs as taken at the onset of this research from Ali et al., (2016), plus the 19 additional, random SNPs. The G-set contained, after deleting three markers for their MAF ≤5%, 14 of initially 17 so-called ‘guide’ SNP markers. The new SNPs (19 plus 17) were analysed as reported by Ali et al., (2016).

2.3 | Development of the guide marker set (G-Set)

As all SNPs used here, the G-set markers can be found in Webb et al., (2016). The G-set markers were chosen during this study by exploiting prior literature about Ascochyta QTL (Table 1). DNA markers linked to Ascochyta resistance QTL published in linkage maps (Kaur et al., 2014; Atienza et al., 2016; Satovic et al., 2013; more over Roman et al., 2003; Díaz-Ruiz et al., 2009, and Avila et al., 2004) were noted. The guide SNP markers (Table 1) were defined by alignments of locus positions of the referenced maps with the map of Webb et al., (2016).

Five SNP markers were clearly identified in the map of Webb et al., (2016) as potentially being linked to two Ascochyta QTL in the map by Kaur et al., (2014) based on their sequence data and their BLAST-based physical position in the Medicago truncatula genome (Table 1). Four guide markers were defined from cross-inspection of QTL markers in the map of Atienza et al., (2016) and Webb et al., (2016) (Table 1), with 0.06 cm to 1.07 cm distances in Webb et al., (2016) between two initial markers (as reported by Atienza et al., 2016) and these four here-defined guide markers. Further guide markers were defined from alignment with the linkage map of Satovic et al., (2013). However, the distance between QTL and the common markers in the map of Satovic et al., (2013) was high and no direct picking was possible. Therefore, linear regression of cM data was applied with the positions of sets of common markers, to roughly predict the positions of two of the QTL markers of Satovic et al., (2013) in the map of Webb et al., (2016). A total of eight further guide SNP markers (four predicted to be near to Af2 and the other four predicted to be near to Af1) were thus defined from the mapped SNPs at Webb et al., (2016). All were predicted to be between 0.2 and 4.2 cm from Af1 or Af2, respectively.

The investigations of the maps of Roman et al., (2003), Díaz-Ruiz et al., (2009) and of Avila et al., (2004) did not reveal common markers, thus no additional guide SNP. Our association study is thus a study in two layers: a genome-wide association study with all 1,355 markers, and, included, a guided approach based on the 14 G-set markers.

Genome-wide association analyses were carried out using TASSER version 3.0 (Bradbury et al., 2007). The mixed linear model (MLM) procedure of TASSER was used with an optimum level of compression and re-estimation of the variance component estimates of
A kinship matrix was employed, which was developed by using the average genetic similarity among the 11 founder lines as a threshold to define unrelatedness (Ali et al., 2016). A false discovery rate of 20% (FDR = 0.20) was used to test the statistical significance of marker–trait associations (Benjamini & Hochberg, 1995; Benjamini & Yekutieli, 2005).

Based on GWAS results and marker genotype, a marker score was calculated for the trait 'number of lesions per leaflet'. For this, for each inbred line, the sum of the effects of its markers with favourable allele present was calculated.

### 2.4 | Phenotyping

The experiments were conducted under semi-controlled conditions in greenhouse in 2017 and 2018. The line 29H was included as phenotypic check. Plants were grown in pots of 13 × 13 cm² size filled with 1.6 kg sand–soil mixture. One pot with one plant was the experimental unit. The 188 faba lines were grown in six experiments with two replications each. Plants were inoculated at four expanded leaf stage.

Fungal material had been collected and purified from leaf lesions of A-set lines grown in season 2015 at the local experimental faba bean nursery (Göttingen, Germany) and from a faba bean nursery of the breeding company NPZ Lembke near Eckernförde, Germany, yielding a total of 56 Ascochyta isolates. Leaf material showing typical lesions interspersed with pycnidia was air dried after sampling and subsequently incubated in humidity chambers for sporulation. Spores were picked from the ostiolum of a single pycnidium with the help of a sterile needle and transferred to V8-agar plates amended with 100 ppm streptomycin. In order to ensure to work with defined fungal genotypes, this procedure was once again repeated after one cycle of subcultivation. Two isolates named as number 50 and number 51 were used in the current study for being both, highly virulent yet differently responding to two rather susceptible and two rather resistant genotypes (Remer et al., 2016). The conidiospores were grown on V8 Agar media for the current analyses. Spore suspension was prepared with autoclaved tap water. The concentration of spore suspension was measured using a Fuchs Rosenthal hemocytometer under a microscope and further diluted to create the intended spore concentration (1 × 10⁶ conidia spores per ml). Spore suspension of isolates 50 and 51 was mixed as 1:1 ratio for inoculation. A fresh spore suspension was prepared for each inoculation event.

### Table 1 - G-set of SNP (guide) markers as picked from the map by Webb et al., (2016)

| No | Marker name in Webb et al., (2016) | Chromosome No. | Position (cM) | Qualification of marker |
|----|-----------------------------------|----------------|-------------|------------------------|
| QTL assignment from Kaur et al., (2014) | | | | |
| 1 | Vf_Mt5g098420_001 | I | 170.86 | SNP_50000451 | QTL−2 |
| 2 | Vf_Mt5g098060_001 | I | 171.49 | SNP_50000451 | QTL−2 |
| 3 | Vf_Mt4g091530_001 | VI | 90.81 | SNP_50002192 | QTL−4 |
| 4 | Vf_Mt4g092850_001 | VI | 93.44 | SNP_50002192 | QTL−4 |
| 5 | Vf_Mt4g092750_001 | VI | 93.44 | SNP_50001976 | QTL−4 |
| QTL assignment from Atienza et al., (2016), yet distances (cM) from Webb et al., (2016) | | | | |
| 6 | Vf_Mt1g086810_001 | III | 95.81 | 0.65 cm from ♦ LG31 | A/3 |
| 7 | Vf_Mt1g088190_001 | III | 97.53 | 1.07 cm from ♦ LG31 | A/3 |
| 8 | Vf_Mt8g091280_001 | VI | 49.99 | 0.06 cm from *RNAR | Field-DSP1 |
| 9 | Vf_Mt8g093440_001 | VI | 50.55 | 0.50 cm from *RNAR | Field-DSP1 |
| QTL assignment from Satovic et al., (2013), yet distances (cM) from Webb et al., (2016) | | | | |
| 10 | Vf_Mt3g096560_001 | II | 107.21 | 0.33 cm | A/2 |
| 11 | Vf_Mt3g095660_001 | II | 106.66 | 0.86 cm | A/2 |
| 12 | Vf_Mt3g094760_001 | II | 110.86 | 1.92 cm | A/2 |
| 13 | Vf_Mt3g098530_001 | II | 101.59 | 2.13 cm | A/2 |
| 14 | Vf_Mt1g012610_001 | III | 186.87 | 0.31 cm | A/1 |
| 15 | Vf_Mt1g013400_001 | III | 189.72 | 2.54 cm | A/1 |
| 16 | Vf_Mt1g016390_001 | III | 195.46 | 2.99 cm | A/1 |
| 17 | Vf_Mt1g014230_001 | III | 188.26 | 4.21 cm | A/1 |

*RNAR; ♦ LG31: common markers between map by Webb et al., (2016) and map by Atienza et al., (2016); I 14 SNP markers available after applying limit of MAF(5%).
level of run-off. Inoculated plants were enclosed in a plastic foliar tunnel for 72 hr to maintain high humidity and favourable conditions for fungal growth. Afterwards, plants were visually scored eight times for each replication. The first scoring was performed 7 days after inoculation. Further assessment followed alternately after 3 and 4 days, making a total scoring period of 30 days. A total of eight resistance-related traits were assessed at each scoring: 'number of lesions per leaflet', 'length of biggest lesion per leaflet' (mm; lesions are typically circular to oval), 'area covered by lesions per leaflet' (%), 'presence or absence of pycnidia per leaflet' (1 for yes, 0 for no), 'number of lesions at stem', 'length of biggest lesion at stem' (mm), 'area covered by lesions at stem' (%) and 'presence or absence of pycnidia at stem'. All leaf observations were recorded from the single, most infected leaflet per plant found at each visit for scoring, to efficiently differentiate between degrees of susceptibility. Only the main stem of plants was monitored while any tillers were removed. This pragmatic and efficient approach allowed conducting the experiments with its high number of genotypes and replicates. The eight consecutive data points per trait were summed up into one aggregate value, basically following the concept of ‘Area under Disease Progress Curve’ (Campbell & Madden, 1990). Thus, for instance, a value of 2.0 for ‘presence of pycnidia per leaflet’ accrued from finding pycnidia only at two of eight scoring dates (in such case at the ultimate and penultimate date).

The statistical analyses were performed with PLABSTAT (version Dez2012; Utz, 1991). The six experiments, each with its two replications, were randomized as an alpha lattice design and their results, as first step, analysed accordingly, to acquire lattice-adjusted values for the 12 replicates. The lattice-adjusted figures were then used for analysis of variance with the 12 replicates based on this linear model:

\[ Y_{ij} = \mu + g_i + r_j + gr_{ij} \]

where \( Y_{ij} \) is the phenotypic value of a trait for inbred line \( i \) in replicate \( j \), \( \mu \) is the general mean, \( g_i \) and \( r_j \) are the main effects of genotypes and replications, respectively; and \( gr_{ij} \) is genotype × replication interaction of genotype \( i \) with replication \( j \).

This procedure allowed the software to estimate substitutes for the 0.92% of missing data points based on all 11 remaining replicates rather than based only on the one remaining of the two replicates per experiment. Variance components for genotypes for genotypes were estimated from means squares of the analyses of variance as:

Variance component for genotypes = \((MS_g - MS_{gr})/12\);

Repeatability \( h^2 \) was estimated from mean squares (MS) and expressed in per cent:

\[ h^2 = (MS_g - MS_{gr})/MS_g \]

Spearman’s rank correlation coefficients were used to examine correlations between traits.

## RESULTS

Data adjustments due to the randomization as alpha lattice were small, with lattice efficiencies between 100% (i.e. no adjustment) and 113% (across the eight traits and six experiments); the mean efficiency was 104%. Analyses of variance revealed highly significant variation (\( p < .01 \)) for all eight traits due to replicate, genotype and replicate × genotype effects. The genotype effect had F-values between 1.95 and 7.50, depending on trait (data not shown). Genetic variation within this panel of 188 inbred lines was high, as indicated by wide ranges of results (Table 2). Repeatability of the variation among the 188 faba bean inbred lines was high for their leaf symptoms (84.15% < \( h^2 < 86.66\% \)) except for ‘presence of pycnidia per leaflet’ (\( h^2 = 48.70\% \)). Repeatability of stem traits was lower than that of leaf traits (Table 2).

Highly significant correlations (\( p < .01 \)) were observed among all traits (Table 3). Correlations within stem traits (0.72** \( r < 0.91**\)) and within leaf traits (0.52** \( r < 0.92**\)) were higher than between leaf and stem traits (0.44** \( r < 0.61**\)) (Table 3). The highest correlation was found for ‘number of lesions per leaflet’ with ‘area covered by the lesions per leaflet’ (\( r = 0.92**\)). However, moderate correlations were observed for the presence of pycnidia and other traits on leaflets (0.52** \( r < 0.66**\)), whereas ‘presence of pycnidia on stem’ was highly correlated with the other stem traits (0.72** \( r < 0.84**\)).

Association analysis was performed for the 188 A-set lines using their means across lattice-adjusted data from the 12 replications. The average LD among the 1,355 markers employed for GWAS was \( r^2 = 0.0075 \) (cf Ali et al., 2016). Among the 12 markers that were significantly associated with traits, the averaged LD value was \( r^2 = 0.0067 \), ranging from 0.0000 < \( r^2 < 0.108 \). A total of 12 markers, including nine AFLP and three SNP markers, displayed a statistically significant association with six of the eight traits; four of these 12 markers were associated with two or more traits (Table 4). One significantly associated SNP marker (marker 5; Vf-Mt1g014230-001) belongs to the G-set of markers. According to Satovic et al., (2013; Table 1), marker Mt1g014230-001 is located in the genomic vicinity of Ascochyta blight resistance A1; here, it was significantly associated with two leaflet traits: ‘number of lesions’ and ‘area covered by lesions’, explaining 7.56% and 8.23% of the phenotypic variance, respectively. Four of the 11 further markers could not be mapped. Of the other mapped markers, AFLP marker E36M56-356 (marker 1; Table 4) was significantly associated with ‘number of lesions per leaflet’ and explained the highest percentage (21.71%) of the phenotypic variance of all associated markers. Most marker–trait associations were detected for leaf traits. For stem traits, only AFLP marker E40M59-281 (marker 8) appeared, being associated with ‘length of the biggest lesion’ and ‘presence of pycnidia’, two traits which were highly correlated.
This one, marker 8 (E40M59-281), and the SNP Mt1g014230-001 (marker 5, associated with Af1) were in much higher LD than any other pair of markers ($r^2 = 0.108$). The AFLP marker E40M59-281 (marker 8) was furthermore associated with two leaf traits ('number of lesions per leaflet' and 'area covered by lesions per leaflet'; Table 4), which were as well highly correlated ($r = 0.92**$, Table 3). The explained phenotypic variance of marker 8 was, however, low; between 6.2% and 8.9% for its four associated traits. With the exception of marker 1 (E36M56-356), this range of explained variance is similar to that of other associated markers in the current study. For two stem-related traits (number of lesions and area covered by lesion), no significant marker--phenotype associations were detected.

The marker score for the trait ‘number of lesions per leaflet’ was correlated with the phenotypic result of this trait by $r = 0.295**$.

### 4 | DISCUSSION

Ongoing climate warming, although a huge problem, may offer opportunities for new crop types. In Germany, where nearly exclusively spring faba beans are grown, winter faba bean is a novelty, a striking innovation for breeders and policymakers. The improvement of winter faba beans’ genetic defence against biotic stresses such as Ascochyta blight will allow their inclusion into German crop rotations. This GWAS study is the first with a focus on Ascochyta blight...
in *Vicia faba*. It revealed new putative resistance loci in the Göttingen Winter Bean Population, which recommends it as a possible germplasm source for resistance breeding.

The analyses were based on 188 faba bean inbred lines, two strains of *Ascochyta fabae* and a total of 1,355 DNA markers. Ali et al., (2016) certified the A-set of lines as showing no marked deviations, and 10 of them were significant in the GWAS analyses. The Q-Q plot supports the notion that the database can be used for the applied analysis.

The average LD among all markers was very low \(r^2 = 0.0075\). Given this very small LD, the available number of markers is likely a limiting factor. Indeed, two of eight traits were not associated with any of the marker, even though literature had been exploited for so-called ‘guide’ SNP markers.

This GWAS could be carried out with phenotype data of, mostly, high repeatability. The highest values \(h^2\) were found for ‘area covered by lesions per leaflet’ \((h^2 = 86.66\%)\) and ‘number of lesions per leaflet’ \((h^2 = 86.57\%)\). Accordingly, the highest number of marker associations was detected for these two traits.
significant markers were found for 'area covered by lesion per leaflet' and 10 markers for 'number of lesions per leaflet' (Table 4). The AFLP locus E40MS59-281 with its allele '0' (band absent) was associated with a decrease in 'number of lesions per leaflet', in 'area covered by lesion per leaflet', in 'length of the biggest lesion per leaflet', in 'length of biggest lesion at stem' and in 'presence of pycnidia at stem'. Another AFLP locus, E44MS58-177, was, with its allele '1', associated with a decrease in 'number of lesions per leaflet' and 'length of the biggest lesion per leaflet'. This is in accordance with the strong correlation between these two traits. A statistically significant SNP (Vf-Mt1g014230-001) was shared by 'number of lesions per leaflet' and 'area covered by the lesion per leaflet', and explained 7 to 8% of phenotypic variance. The exploitation of literature was successful insofar as this 'guide' SNP marker was picked for being listed in Webb et al., (2016) as near to the inferred position of a QTL marker of Satovic et al., (2013), when projecting that marker's position from the latter to the former map; even though the SNP was more than 4cM distant from that projected position. With one in 14 guide markers being significantly associated with resistance, this proportion was higher than for the genome-wide markers employed here.

Previously, QTL for Ascochyta blight have been reported on chromosomes II, III and VI. The QTL on chromosome III (Af1) has been reported and validated several times (Atienza et al., 2016; Avila et al., 2004; Díaz-Ruiz et al., 2009; Roman et al., 2003; Satovic et al., 2013). Kaur et al., (2014) assumed that their QTL3 in cross (Icarus ×Ascot) was identical with QTAL2 on chromosome II; Af2 was reported by Díaz-Ruiz et al., (2009) and by Roman et al., (2003). Despite a lacking physical Vicia faba map, Atienza et al., (2016) confirmed congruence of QTAL3 and Af2 on chromosome II based on synteny to Medicago truncatula. Further QTL were reported on chromosome VI by Atienza et al., (2016) and by Avila et al., (2004). However, these QTL are not yet independently validated. Interestingly, the RNAR-marked QTL reported by Atienza et al., (2016) on chromosome VI shares this chromosome with our two significant SNP markers Vf_Mt8g106690_001 and Vf_Mt8g086470_001, which were located on that chromosome at 6.0 and 65.4 cM, at distances of 44.5 and 15.3 cM from the RNAR marker (Webb et al., 2016) albeit with nearly zero LD to each other in the A-set of lines. Probably, these two SNPs do not mark the same QTL, yet Vf_Mt8g086470_001 (marker 11; Table 4) could still be associated with the RNAR-marked QTL (although the two employed G-set markers, at 0.06 and 0.50cM distance from RNAR, were not significant). Altogether, for nine of the 12 markers, excluding markers 5, 8 and 11, there is currently no evidence of redundancy among them or of identity with known, published QTL for Ascochyta resistance.

For 'number of lesions per leaflet', the naïve sum of the 10 $R^2$ values (Table 4) is 85.6%. This is probably overly high, although not higher than the repeatability of that trait ($h^2 = 86.6\%$). Yet, one cannot expect that the current analyses detected all QTL for Ascochyta blight. A multiple regression of the phenotypic values of the lines on the 10 significant markers resulted in a multiple value of $R^2 = 29.7\%$; markedly lower than 85.6%. The sum of the effects of these 10 significant markers was 111.3 lesions per leaflet, which is higher than the maximum numbers (72.6 to 91.5) found in the most susceptible lines S_232, S_060, S_168; these are indications of overestimation in the data. Furthermore, the correlation between the marker score for 'number of lesions per leaflet' and the phenotype itself was small, $r = 0.295^{**}$, indicating that the marker score contains less information than what naïve interpretation suggests. The allele phases of two of the ten markers, marker 5 (presumably Af1) and marker 8, were associated (LD value of $r^2 = 0.108$), redundancy cannot fully be ruled out. With a false discovery rate of 20%, about two in 10 markers are not expected to be sustained as positives, and with the limited number of 188 lines, overestimation of effect sizes has to be anticipated (Josephs et al., 2017; Vales et al., 2005). Epistasis, as it is statistically presented as interaction, might be a further explanation for shrinking effects when joining markers (unless epistasis is specifically implemented in the statistical model; Göring et al., 2001).

Although the findings here are bound to the greenhouse conditions and the two fungal strains used, the currently most promising parents among the A-set lines to combine in a cross for breeding seem to be line S_150 and line S_162. This is because S_150 is, except for marker 4 (E44MS58-177), homozygous for the resistance-associated allele ('number of lesions per leaflet') at the other nine markers loci (Table 4); it is ranked as 26th best for the phenotypic trait value and on position 1 for marker score. The 25 lines that ranked phenotypically better than S_150 carried, at only three to eight marker loci, the resistance-associated allele; line S_150 was the highest-ranked line with nine. Line S_162 is, except for marker 5 (Vf-Mt1g014230-001) and marker 10 (E41M55-177), homozygous for the resistance-associated allele at the other eight loci. Line S_162 ranks third best for its marker score and first for its phenotypic value. Markers 4 and 5, although mapped on the same chromosome (Table 4), are barely linked. Marker 10 is unmapped, yet with its LD values of $r^2 = 0.005$ and $r^2 = 0.013$ to markers 4 and 5, it is probably not strongly linked to them. The cross of lines S_150 with S_162 would allow a complementation of the three genetic gaps (markers 4, 5, 10), thus roughly (1/2) of the RIL lines from this cross should have all 10 markers as desired.

Based on 'number of lesions per leaflet', three A-set lines (S_162, S_009; S_123) were more resistant than the highly resistant check line S_29H showing 2.07 'lesions per leaflet'. Hence, novel promising donors of high resistance levels are available for validation and use. Marker-assisted introgression of resistance into elite genetic material is supported by conversion of AFLP-derived results into SNP-supported data. Currently, an Affymetrix 50K chip is under development and will be publicly available on short notice (O’Sullivan, 2020; personal communication). Genotyping the A-set lines with that tool promises marked advance for applied breeding and for genetic analyses.

5 CONCLUSIONS

Substantial and significant genetic variation for Ascochyta-resistance traits was detected, and all eight assessed traits were
seemingly genetically related. LD in this set of Göttingen Winter Bean lines was very low; the number of markers probably did not match such high genetic resolution. To reduce these limitations and to steer the focus towards Ascochyta blight resistance genes, a so-called guided marker approach was conducted in addition to the default genome-wide analyses. A total of 12 markers, including nine AFLP and three SNP markers displayed significant associations with six traits; nine of these markers probably stand for new resistance genes. Significant SNP markers were found at chromosomes III and VI in the descendants of the Göttingen Winter Bean Population. The guided approach was successful: one of 14 guide marker (VF-Mt1g014230-001) was found significant and it is hypothesized that this SNP at chromosome III validates the previously reported QTL (Af1; chromosome III). The significant SNP found at chromosome VI should be validated in future studies. Applied marker-assisted selection for Ascochyta-resistance relies strongly on the transfer of genetic results among different faba bean populations, depending on further saturation of QTL bearing chromosomal regions.

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CONFLICT OF INTEREST
Authors declare: no conflict of interest.

AUTHORS CONTRIBUTIONS
Rabia Faradi conducted the experiments, gathered the data, conducted the statistical analyses, contributed to the exploitation of literature, wrote the initial version of the manuscript and contributed to the finalization of the manuscript. Birger Koopmann supervised the experiments, contributed the phytopathology expertise and contributed to later versions of the manuscript. Antje Schierholt contributed to the exploitation of literature, to the interpretation of data and results and to later versions of the manuscript. Mohamed B. Ali conducted the definition of G-set markers, including the alignments of linkage map fractions, contributed to the exploitation of literature and to the interpretation of results. Stefanie Apel gathered, purified and described the fungal strains and contributed to the manuscript. Wolfgang Link devised the project, designed the experiments, supervised the analyses of data, contributed to the interpretation of results and supervised the latest versions of the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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