IMPROVING SUGARBEET RESISTANCE TO CERCOSPORA LEAF SPOT THROUGH GWAS AND DOUBLED HAPLOID

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Introduction

Cercospora leaf spot (CLS), caused by the fungus *Cercospora beticola* Sacc., is the most widespread foliar disease in sugar beet (*Beta vulgaris* L.). Significant losses can occur under warm and humid environments with yield losses as high as 42 - 50% (Verreet et al., 1996). Application of fungicide and growing resistant cultivars are two main measures for controlling the disease but using host resistance would be more effective with a lower cost. Vogel et al. (2018) found that recent breeding efforts have made CLS resistant cultivars comparable to susceptible ones in terms of yield performance, consequently, the resistant cultivars thus have a relatively better economic performance since no fungicide needs to be applied.

Many studies were conducted to identify germplasms resistant to CLS and some accessions of *Beta. vulgaris* spp. *maritima*, the wild ancestor of sugar beet, were found to have a high level of resistance and were used as a source of CLS resistance (Leuterbach et al., 2004). Meanwhile, genetic studies suggested that CLS resistance was complicatedly inherited. However, major genes conferring CLS resistance were also reported. Smith and Gaskill (1970) have assumed that CLS resistance is controlled by at least four or five genes with effects varied depending on the severity of infection. Taguchi et al. (2011) reported four QTLs conferring the resistance carried by the line 'NK-310mm-O'. Abd El-Fatah et al. (2020) reported some molecular DNA and isozyme markers showed obvious association with sugarbeet resistance to CLS. Thus, evaluation of CLS resistance in sugarbeet genetic resources followed by genome-wide association studies (GWAS) will be an efficient way of identifying resistance genes and developing markers to assist selection by pyramiding resistance genes from different sources to achieve long-lasting resistance.

However, genetic heterozygosity and heterogeneity are common attributes of sugarbeet germplasms due to the self-incompatibility nature of the species, which greatly increase difficulties in genetic and breeding research such as gene identification, marker development and estimation of allele effects. Haploids and doubled haploids (DHs) only carry one set of chromosomes from their diploid parents. This eliminates the interactions between different homologous alleles in haploid/DH plants since either only one copy of homologous alleles exists in haploid or two identical copies of alleles present in DH for each locus. Also, the DH method only takes one year to develop completely homozygous and genetically stable genotypes, which greatly accelerates germplasm development in sugarbeet.

Sugarbeet haploid plants can be induced through gynogenesis by culturing unfertilized female gametophytes (ovules) (Hosemans and Bossoutrot, 1983) with the haploid induction efficiency varying from 1 to 15% (Pazuki et al., 2018). Therefore, the ovule culture is the most promising technique for DH development in different genetic resources.

The objectives of this research will include: 1) using SNP (single nucleotide polymorphisms) markers from GBS (genotype-by-sequencing) to genotype all available genetic resources of sugarbeet in the US, and then conducting genetic diversity analysis to reveal the potential of those resources for broadening the genetic base of sugarbeet; 2) conducting GWAS to identify genomic regions associated with CLS resistance; and 3) using different resistant resources to develop new DH breeding lines with stable resistance.

Materials and methods

A total of 1,935 *Beta vulgaris* germplasm lines were collected for this research, which included 1,080 accessions of sugarbeet, 86 accessions of fodder beet, 67 accessions of leaf veg, 82 accessions of root veg, and 595 accessions of wild relatives but were mainly from *B. maritima* (Table 1).

Table 1. List of accessions in *Beta vulgaris* collection used in this research.

Species		Number of accessions
Beta atriplicifolia		6
Beta macrocarpa		11
Beta macrorhiza		2
Beta palonga		1
Beta patula		3
Beta procumbens		1
Beta webbiana		1
Beta maritima L.		595
Beta vulgaris L.		1315
Fodder beet	86	
Leaf beet	67	
Root veg	82	
Sugarbeet	1,080	
Total		1,935

Whole genomic DNA was extracted from leaf samples using a DNA purification system from King Fisher, Inc., and DNA samples were co-digested with two restriction enzymes NsiI (recognizes ATGCA^T sites) and BfaI (cuts C^TAG sites) to develop sequencing libraries. An Illumina HiSeq 2000 was used to sequence about 100-bp from both directions of enzyme cutting sites. SNP calls were made using the reference-based TASSEL pipeline with EL10.2 assemblies from the sugarbeet line EL10 as the reference genome, to obtain SNPs covering the whole genome. Genetic diversity analysis was conducted using computer program TASSEL v5.0 (https://tassel.bitbucket.io/) and a phylogenetic tree was drawn by the online tree drawing tool iTOL v6 (https://itol.embl.de/). The Population structure of the collection was analyzed using the computer program STRUCTURE v2.3.4 (https://web.stanford.edu/group/pritchardlab/structure.html)

Preliminary GWAS analysis was conducted through computer program TASSEL v5.0 using the SNP data from this research and the existing historic CLS disease severity data collected from 797 *B. vulgaris* germplasms and stored in the U.S. NPGS (National Plant Germplasm System, https://www.ars-grin.gov/npgs/).

For haploid/DH development, sugarbeet lines F1024 and F1042 were used for setting up the DH procedures. Briefly, the unfertilized flowers were stored in a fridge at 4 ˚C for a week and ovules were dissected after flowers were sterilized in 20% bleach solution for 25 minutes. Ovules were then cultured on the MS growth media containing sucrose (60 g/L), 6-BAP (1 mg/L) and kinetin (1 mg/L) for over 4 weeks under light at 27 ˚C. The enlarged and germinated ovules were transferred to new growth media to promote callus growth and seedling regeneration. The induced seedling was then moved to rooting media that contained sucrose (30 g/L) and NAA (5 mg/L). Once the root was developed, the seedling was transplanted into the soil with 16 hr of day length under 25 ˚C. Root tips were collected for chromosome counting after being stained by Feulgen staining. Colchicine treatment for chromosome doubling was conducted either using callus tissue or the seedlings that have been treated at 4 ˚C for over three months for inducing reproduction. If callus tissue was used for colchicine treatment, 40 µl colchicine solution (33 mg/ml) was added to a mini cup containing ten callus pieces in 2 ml liquid media (growth media with no agar added). The mini cup was kept at 18 ˚C overnight and callus tissue pieces were then transferred to rooting media on the next day. Once planets with roots were induced from the rooting media, they were transplanted into the soil in a growth chamber, followed by cold treatment for three months and then moved to a greenhouse till matured seeds were obtained.

If seedlings were used for colchicine treatment, the cold treated seedlings were pulled out from soil and the root was cleaned in water. The seedlings were then transferred into 50-ml centrifuge tubes with each containing about 40 ml

1.5 g/L colchicine solution. The tubes along with seedlings were put into a centrifuge and spun at 50 G for 3 minutes. After dumping the colchicine solution, the tubes and seedlings were spun again at the same speed for 3 minutes to remove the extra colchicine solution from seedling leaves. The seedlings were then transplanted back to soil in a growth chamber for two weeks and the recovered seedlings were moved to a greenhouse till matured seeds were obtained. All plants from colchicine treatment were individually bagged and all seeds harvested from each plant were DHs.

Results & discussion

A total of 148,137 SNPs were obtained in the germplasm collection and covered the whole sequenced genome according to EL10.2 assemblies (Table 2). SNP coverage was uniform on each chromosome, which indicated that SNP markers in the collection were suitable for genetic diversity analysis and GWAS.

Table 2. SNPs from GBS in the collection of 1,935 *B. vulgaris* germplasms.

Chromosome /scaffold	Number of SNPs	Covering region (Mb)*
Chr. 1	15,746	64.1
Chr. 2	14,674	56.8
Chr. 3	15,466	57.1
Chr. 4	16,987	66.1
Chr. 5	19,115	67.7
Chr. 6	19,140	72.2
Chr. 7	15,693	60.9
Chr. 8	16,666	61.8
Chr. 9	14,277	55.6
Scaffold_10	73	0.3
Scaffold_11	124	0.8
Scaffold_12	16	0.6
Scaffold_13	105	0.6
Scaffold_14	74	0.5
Scaffold_16	111	0.2
Scaffold_17	34	0.1
Scaffold_18	16	0.06
Total	**148,137**	**565.46**

* According to McGrath et al. (2020), the whole sequenced genome of EL10.2 assemblies have a total of 580 Mb. The nine scaffolds were not anchored to any chromosome yet with each covered the genomic region ranging from 0.1 to 1.0 Mb.

Structure analysis based on SNPs indicated that five sub-populations were included in the germplasm collection with accessions tended to be clustered according to their usage (Fig. 1a). However, the genetic background in the four clusters had a high level of admixture, which agreed with the expected low genetic diversity among sugarbeet germplasms in those clusters (Fig. 1b).

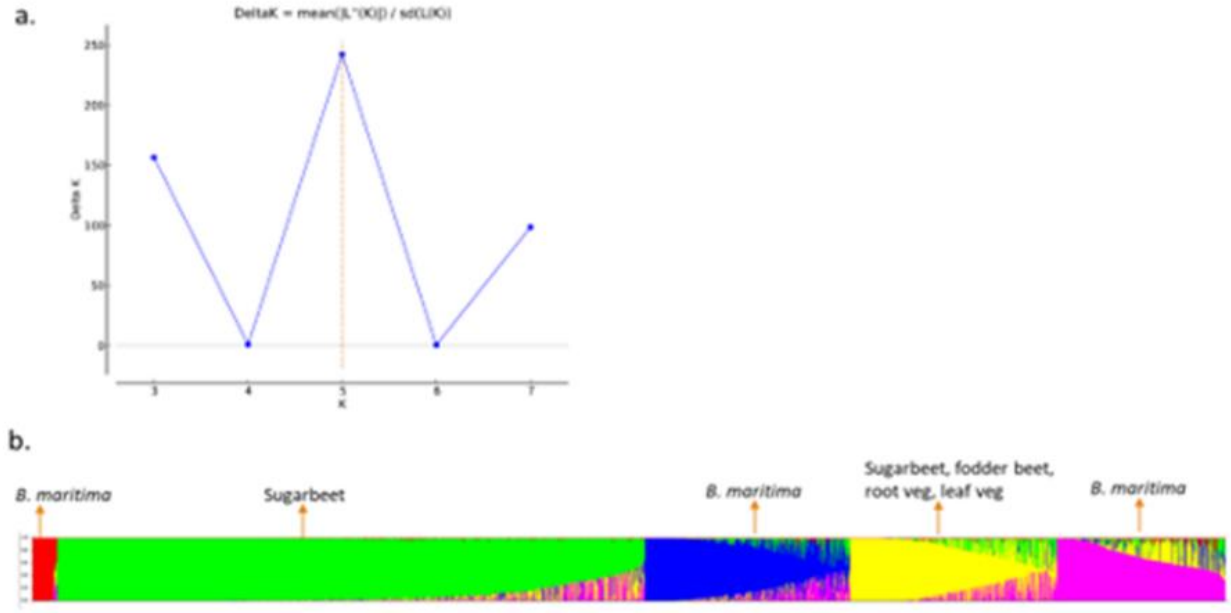

Fig. 1. Structure analysis in the *B. vulgaris* germplasms using the computer program STRUCTURE v2.3.4. (a) Analysis from STRUCTURE indicated five sub-populations in the germplasm collection. (b) Genetic admixture among sub-populations.

Phylogenetic tree analysis using the tools TASSEL and iTOL further supported the results from population structure analysis (Fig. 2). Except for a cluster of 355 accessions mainly from *B. maritima* that showed a more distinct genetic distance from the others, the rest of the germplasms were closely related and confirmed the narrow genetic diversity in sugarbeet germplasms.

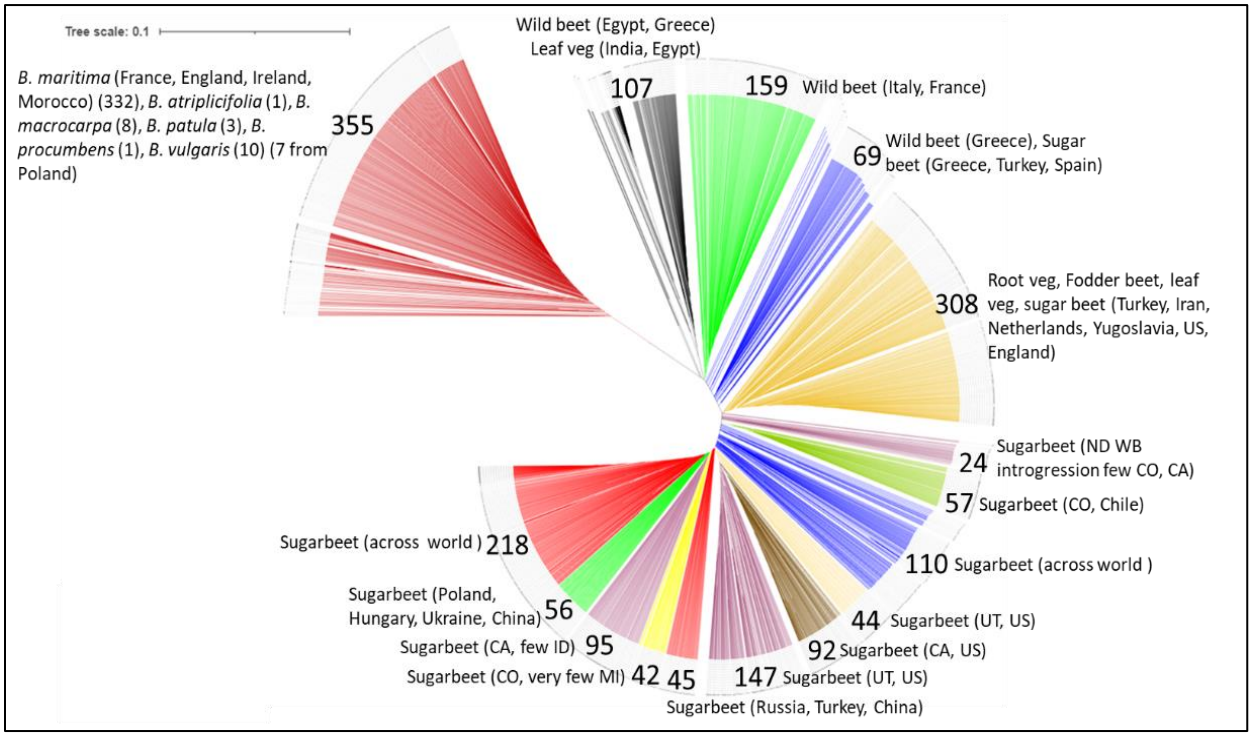

Fig. 2. Phylogenetic tree of the *B. vulgaris* germplasms obtained using computer program TASSEL v5 and iTOl v.6. The number in each cluster indicates the number of accessions in the cluster.

From the database of NPGS (https://npgsweb.ars-grin.gov/), the historical CLS data have been collected from 797 accessions of *B. vulgaris* (Table. 3). The CLS ratings were recorded in a 0 – 9 system where 0 is immune to CLS, 1 – 3 as resistant, 4 - 6 as moderately susceptible, and 7 - 9 as susceptible to CLS.

Table 3. The historical CLS data that have been collected from 797 accessions of *B. vulgaris.* *

Species	Number of accessions	Average CLS ratings	Range of CLS ratings
Beta atriplicifolia	4	4.5	3 - 8
Beta macrocarpa	1	9.0	9.0
Beta maritima L.	390	4.7	0 - 9
Beta vulgaris L.	402	6.4	1 - 9
Fodder beet	75	7.3	1 - 9
Leaf veg	37	6.5	3 - 9
Root veg	56	6.5	3 - 9
Sugarbeet	234	6.0	3 - 9

* Data was downloaded from database of NPGS (https://npgsweb.ars-grin.gov/)

Among germplasms been rated for reaction to CLS, *B. maritima* accessions showed the better CLS resistance with 131 out of 390 accessions were CLS resistant, whereas only 12 out of 402 cultivated beets lines were rated as “3” or below (Fig. 3), indicated that some *B. maritima* accessions will be more promising for using as the CLS resistance sources.

Fig. 3. CLS rating distribution in accessions of *B. maritima* (left) and cultivated *B. vulgaris* (right).

GWAS in the 797 accessions found genomic regions on chromosomes 1, 4, 5, 6, 7, and 8 were significantly associated with the resistance (Fig. 4 and Table. 4) and each region explained 4 - 5% of trait variations. However, since those CLS reaction data were collected at different times under different environments, the GWAS results presented here mostly indicated the ability of GWAS for identifying the CLS resistance genes, therefore, the resistance associated genomic regions identified from the historic data needs to be validated using new disease data from well-designed experiments to be conducted during the 2022 crop season.

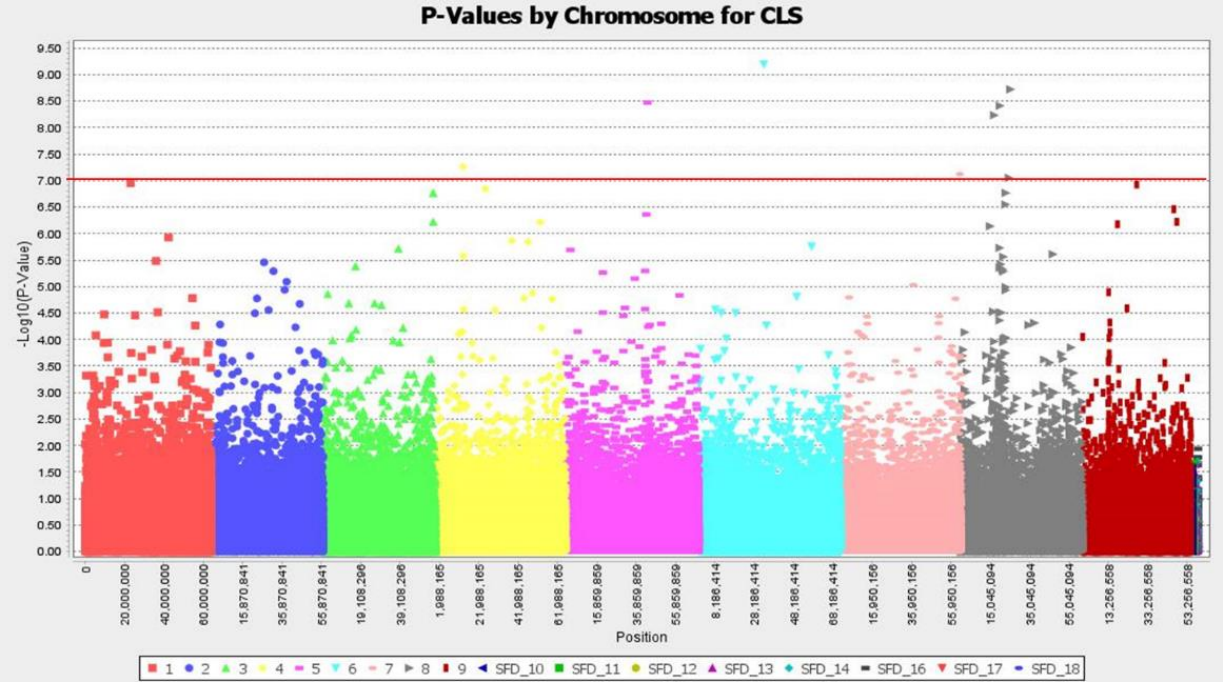

Fig. 4. Manhattan plot of association mapping of CLS resistance in 797 accessions of *B. vulgaris* using the historic disease data. The threshold was set as LOD = 7 that is indicated using a red horizontal line.

Table 4. List of SNP markers significantly associated with CLS resistance in the historic data

SNP Marker	LOD	Additive effect	Dominant effect	Marker R^2	Allele effect			
S1_23048025	7	-2.8	3.13	0.04	A	-5.9	G	0.3
S4_13904356	7.3	-1.5	0.82	0.04	A	-2.3	G	0.7
S5_41539664	8.5	-2.1	2.07	0.05	A	-4.1	G	0
S6_32978686	9.2	-2.9	3.01	0.05	C	-5.9	G	0.1
S7_60416461	7.1	0.8	1.28	0.04	A	0.4	G	-2.1
S8_16880753	8.2	-1.6	1.34	0.05	A	-2.9	G	0.2
S8_19832249	8.4	-1.8	1.6	0.05	A	-3.43	G	0.2
S8_24350838	7.1	-1.2	-0.98	0.04	A	-0.2	G	2.1
S8_25199724	8.7	-2	2.09	0.05	C	-4.1	G	0

For DH production, over 5,000 unfertilized ovules from sugarbeet lines F1024 and F1042 were cultured, and callus tissue was successfully induced from 27 individual ovules with an induction rate of 0.5%. Seedlings were regenerated from all callus tissues and chromosome counting using root tip cells confirmed they are haploids. Colchicine treatment using callus tissue was conducted and seedling regeneration from the treated callus is ongoing. The colchicine treatment on haploid seedlings will be conducted once the seedlings finished the vernalization processes. Fig. 5 shows the procedures of haploid induction through ovule culture and chromosome counting confirmed nine chromosomes carried in each observed cell.

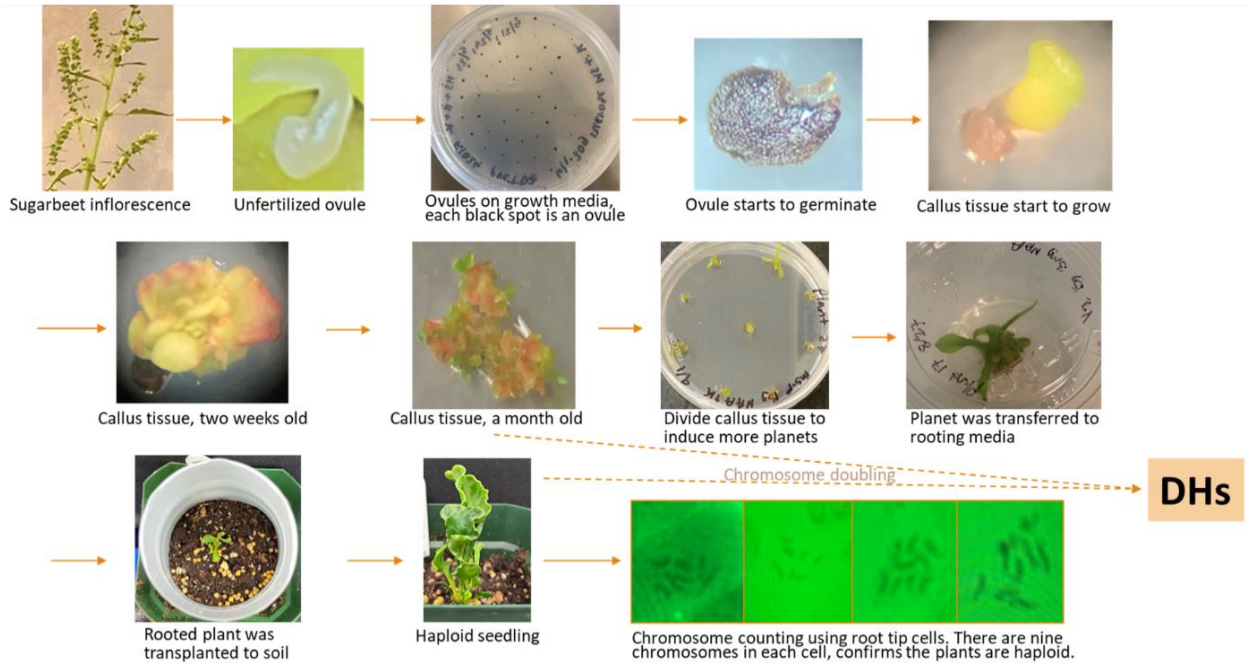

Fig. 5. The procedure of doubled haploid production in sugarbeet by ovule culture. Chromosome counting using root tip cells confirmed the plants regenerated from ovule callus are haploids.

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