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GENETIC STOCK

Registration of Sugarbeet Doubled Haploid Line KDH13 with Resistance to Beet Curly Top

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Abstract

KDH13 (Reg. No. GS-10, PI 663862) is a sugarbeet (*Beta vulgaris* L. ssp. *vulgaris*) doubled haploid line released as a genetic stock by USDA-ARS in cooperation with the Beet Sugar Development Foundation, Denver, CO. KDH13 is resistant to beet curly top (BCT) caused by *Beet curly top virus*, which is transmitted by the beet leafhopper (*Circulifer tenellus* Baker). This line was extracted from the BCT-resistant parental line C762-17 (PI 560130) that segregates for BCT resistance and genetic male sterility. Intensive phenotypic screening was used to select plants from C762-17 that showed no BCT symptoms under controlled infection conditions in the greenhouse. Seed from a single plant was used as a donor of floral buds for isolation of unfertilized ovaries to regenerate KDH13 via gynogenesis. It was confirmed a diploid by flow cytometry. This line's genome was sequenced via next-generation sequencing, and its assembly was designated *BvvSeq-1*. Homozygosity of KHD13 was confirmed using single nucleotide polymorphisms. It is monogerm and self-fertile, and it requires at least 90 d of vernalization to induce bolting. KDH13 showed no BCT symptoms in the greenhouse and showed better resistance than commercial checks field screening experiments. In crosses with a BCT-susceptible line, KDH13 demonstrated its suitability as a donor parent for introduction of curly top resistance genes.

Doubled haploid lines facilitate fundamental genetic studies, breeding approaches, and molecular genetics research in sugarbeet (*Beta vulgaris* L. ssp. *vulgaris*). Hammond (1966) reported production of the first homozygous diploid sugarbeet line, designated C600. This line was produced by treating vegetative flower stalks with colchicine. C600 was further characterized, field tested, and released as PI 590806 in 1988 by R. T. Lewellen (USDA-ARS, Salinas, CA) with its cytoplasm male sterile (CMS) equivalent as PI 520748. Forster et al. (2007) reported that sugarbeet doubled haploids parents have been used in breeding, including the production of F_1 hybrids. Sugarbeet is recalcitrant in response to androgenic or gynogenic in vitro culture (Van Geyt et al., 1987; Zakhariev and Kikindonov, 1997). In general, gynogenesis proved to be the most successful method to produce haploids in sugarbeet (Lux et al., 1990). Despite these constraints, some commercial sugarbeet seed companies (e.g., SES VanderHave N.V. Tienin, Belgium) have developed proprietary protocols to produce doubled haploid lines and populations. There have been limited public attempts to produce sugarbeet doubled haploid lines via in vitro culture of unfertilized ovaries, anther, and microspore-culture (Bossoutrot and Hosemans, 1985). Lux et al. (1990) reported that genotypic effect is the major factor that determines the rate of success in generating doubled haploid lines. Successful attempts to produce doubled haploids can be as low as 2.2% as reported by Gürel & Gürel (1998). However, the rate of success can be increased to as high as 13% from some genotypes with improved protocols (Gürel et al., 2000). Recombinant inbred lines could facilitate genetic research similarly to doubled haploid lines, but the process in sugarbeet is prolonged by the biennial nature of the plant. Currently, the publically available sugarbeet gene pool is deficient of inbred lines, mapping populations, genetic stocks, near isogenic lines, and doubled haploid lines.

Beet curly top (BCT) is a major disease in the semiarid and arid sugarbeet production areas worldwide. Panella (2005) reported that the disease dates back to the start of the industry in the United States in the 1920s and significantly reduced yield to the

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Abbreviations: BCT, beet curly top; BCTV, *Beet curly top virus*; ELISA, enzyme-linked immunosorbent assay; SNP, single nucleotide polymorphism.

level of threatening the industry survival. The first cultivar resistant to BCT, designated US1, was released in 1933. This disease is caused by *Beet curly top virus* (BCTV) and is known to possess a number of strains: Cal/Logan (CA/Logan), CO (CO), Mild (Mld), Severe (Svr), Severe pepper (SvPep), Spinach (SpCT), and Worland (Wor), as recently reclassified molecularly by Varsani et al. (2014). Infected sugarbeet plants commonly carry more than one strain of BCTV (Strausbaugh et al., 2008). The virus strains are vectored by beet leafhopper (*Circulifer tenellus* Baker) populations that carry the strains in unknown proportions and random distribution. Breeding efforts, specifically mass selection, successfully provided resistance to BCT in commercial varieties in the western United States. In general, there is sparse knowledge available on the mode of inheritance of resistance to BCT in sugarbeet and no established consensus inheritance model (Bennett, 1979). The earlier findings (1950s) on linkage of BCT resistance to the R (red hypocotyl) gene are not obvious in the current modern hybrids as well as public germplasm. However, most current resistance to BCT that has been utilized apparently is quantitative and controlled by a few minor and major genes (R.T. Lewellen, personal communication, 2011). As BCTV infected other crops, including common bean (*Phaseolus vulgaris* L.), researchers studied the inheritance of resistance in common bean; Larsen and Miklas (2004) reported complete dominance of resistance as shown in symptomless F_1s. In the same study, they were unable to confirm a segregation pattern in recombinant inbred lines because of possible skewed results due to death of infected plants. However, they identified a single DNA marker (SCAR) that can be used in marker-assisted selection. For effective control, commercial cultivars (hybrids) that provide moderate to low levels of BCT resistance would require insecticide application (Strausbaugh et al., 2012, 2014). Host resistance is crucial to supplement the chemical control and reduce economic damage at any stage of the plant growth. Homozygous parental lines with resistance to BCT could facilitate inheritance studies as well as molecular dissection of resistance to this disease. Additionally, such lines could be used to transfer the resistance genes to elite parental lines. To this end, KDH13 (Reg. No. GS-10, PI 663862), a doubled haploid line with exceptional resistance to BCT, is being registered. To facilitate molecular research on BCT resistance genes, KDH13 genome has been sequenced and assembled (http://www.ncbi.nlm.nih.gov/assembly/GCA_000397105.1#/st) and genotyped with single nucleotide polymorphism (SNP) markers.

Methods

Generation of Doubled Haploids

Parental line C762-17 (PI 560130) released in 1989 by R.T. Lewellen (1994), with high BCT resistance, was selected as donor plant for production of the doubled haploids. After several cycles of phenotyping of PI 560130 for BCT, a single plant was selected for doubled haploid production. Unopened floral buds were collected from four selected, healthy donor plants (C762-17-S1) growing in the greenhouse. Floral buds (approximately 1000 from each plant) were surface disinfected by immersion in 70% ethanol for 30 s, followed by 15 min in 15% Clorox bleach (8.25% NaOCl), and finally rinsed three times with sterile-distilled water (5 min each time). After sterilization, the unpollinated ovaries were dissected from the floral buds under a stereo microscope, transferred onto Petri dishes containing freshly prepared semisolid embryo initiation medium, and subsequently placed in a growth room with a photoperiod of 16 h/8 h (day/night) at temperatures of 24°C/18°C (day/night). Induced embryos were transferred to new growth MS medium (Murashige and Skoog, 1962) for embryo germination and further shoot growth and development. Only 20 plantlets were viable and with the potential to grow normally. These plantlets were transplanted into a mixture of 1:1 of potting mix: SunShine Professional Growing Mix 1 (supplied by Sun Gro Horticulture) and commercial play sand. The plantlets were grown in a growth chamber with high humidity and optimum growth conditions.

Determination of Ploidy Level

The ploidy level of the 20 plants was determined using flow cytometry (Costich et al., 1993). Leaf samples from the 20 plants were sent to the Flow Cytometry Facility at Iowa State University (Ames, IA). A suspension of leaf nuclei was prepared by placing approximately 0.5 g of freshly chopped leaf tissue in a 60- by 15-mm polystyrene dish containing 1 mL of nuclei-stabilizing buffer (15 mM HEPES [Sigma-Aldrich], 1 mM ethylenediaminetetraacetic acid [EDTA], 80 mM KCl, 20 mM NaCl, 300 mM sucrose, 0.2% Triton X-100 [Sigma-Aldrich], 0.5 mM Spermine tetrahydrochloride, and 0.25 mM PVP [Sigma-Aldrich]). The homogenized suspension was supplemented with an additional 2.5 mL of nuclei-stabilizing buffer, passed through a 20-μm nylon mesh filter (Small Parts Inc.) into a 12- by 75-mm polystyrene tube. The suspension was then centrifuged for 6 min at 100 × *g*. The nuclei pellet was resuspended in 250 μL of staining buffer (10 mM $MgSO_4$, 50 mM KCl, 5 mM HEPES, 0.1% dl-dithiothreitol, 2.5% Triton X-100, and 100 μg/mL propidium iodide). At least 5000 gated nuclei events were collected and analyzed for each sample. A 10-μL aliquot of the cell suspension from each sample was analyzed by flow cytometry (BD Biosciences) for relative nuclei DNA content.

Beet Curly Top Disease Resistance Evaluation

KDH13 was included in 2 yr (2013 and 2015) of testing in the BCT nursery at the USDA-ARS Northwest Irrigation and Soils Research Laboratory, Kimberly, ID. The BCT nursery protocol and experimental design was described by Eujayl and Strausbaugh (2010). The BCTV infection was facilitated by the release of viruliferous leafhoppers in the nursery when the plants were at four- to six-leaf growth stage. The field symptoms of BCT were rated using the 0-to-9 rating scale developed by Mumford (1974), with 0 = no visible symptoms and 9 = completely dead. Leaf samples were collected from the BCT nursery for enzyme-linked immunosorbent assay (ELISA) analysis. The samples, including KDH13, K19-19, and the BCT nursery resistant and susceptible checks, were analyzed by the ELISA protocol reported by Durrin et al. (2010). KDH13 also was screened for BCT in the greenhouse, using a complete randomized design experiment. The plants were infected at the four-leaf growth stage with six leafhoppers that were allowed to feed for 5 d in

leaf clip-cages to transmit the virus by means of a modified method as described by Wintermantel and Kaffka (2006) and were rated using the same 0-to-9 scale.

Characteristics

Three of the 20 plantlets were diploid. Diplodization was due to spontaneous chromosomal doubling, an established phenomenon in tissue culture (Svirshchevskaya and Dolezel, 2000). The remaining 17 haploid plants were treated with colchicine (Hansen et al., 1995) for diploidization but without success. The three doubled haploid plants grew normally in the greenhouse. They were designated as KDH04, KDH09, and KDH13. These plants were vernalized (at 4°C with 24 h light) for 120 d and subsequently flowered and produced seeds. KDH04 and KDH09 were found to be identical (using SNP markers) and showed a lower level of resistance to BCT. KDH13 was highly resistant to BCT in the greenhouse and field trials. Its reaction to BCT is shown in Table 1 as a result of analysis of 2 yr data of screening at the BCT nursery, USDA-ARS, Kimberly, ID. Means of KDH13 were lower than HPM90, the resistant check, based on ANOVA analysis, but the differences between lines were not significantly different at $P = 0.05$. The inner whorl of the young leaves of KDH13 shows natural curling that could be misphenotyped as mild curly top symptoms and led to scores of up to 3.5 in the field compared with the greenhouse, where we did not observe symptoms (data not shown). In 2015, the ELISA results (Table 1) showed that the BCT virus titer in KDH13 (0.27) was significantly lower than HPM90 (0.56).

KDH13 has a compact canopy and narrow upright small leaves, similar to its donor plant C762-17-S1, but with light green leaves. It is monogerm, with green hypocotyl, is self-fertile, and requires at least 90 d of vernalization to induce bolting. In field performance trials (2010–2013, data not shown), it did not show economical yield potential. KDH13 is susceptible to rhizomania (*Beet necrotic yellow vein virus*), Rhizoctonia root rot caused by *Rhizoctonia solani* Kühn, and powdery mildew caused by *Erysiphe polygoni* DC.

Table 1. Reaction of germplasm lines in the beet curly top (BCT) field nursery in Kimberly, ID. The BCT rating (scale 0 to 9) and enzyme-linked immunosorbent assay (ELISA) titer means were extracted from a dataset that contained 16 experimental entries in both years.

Entry	BCT rating	ELISA titer		
	2013			
KDH13	3.2	1.39		
K19-19	8.7	2.02		
HPM90 (resistant check)	4.1	1.36		
Monohikari (susceptible check)	7.7	2.49		
Trial mean	6.8	1.85		
LSD (0.05)	1.1	0.46		
Field ANOVA source	**df**	**MS†**	**F ratio**	**Prob > F**
Block	5	1.3	1.5	0.192
Entry	15	26.8	31.5	<0.0001
Error	75	0.9		
Corrected total	95			
ELISA ANOVA source				
Block	5	0.9	5.8	0.0001
Entry	15	0.7	4.5	<0.0001
Error	75	0.2		
Corrected total	85			
	2015			
KDH13	3.4	0.27		
K19-19	6.8	0.83		
HPM90 (resistant check)	3.7	0.56		
SV2012RR (susceptible check)	7.2	0.95		
Trial mean	5.6	0.66		
LSD (0.05)	1.0	0.21		
Field ANOVA source	**df**	**MS†**	**F ratio**	**Prob > F**
Block	5	3.6	4.9	0.0006
Entry	15	9.7	13.1	<0.0001
Error	75	0.7		
Corrected total	95			
ELISA ANOVA source				
Block	5	0.4	12.0	<0.0001
Entry	15	0.3	7.5	<0.0001
Error	75	0.0		
Corrected total	95			

† MS, mean squares.

Transfer of Beet Curly Top Resistance in Experimental Hybrids

KDH13 was used as a female parent (green hypocotyl) in a cross with K19-19, a BCT susceptible pollen donor (red hypocotyl) from the sugarbeet breeding program at USDA-ARS Northwest Irrigation and Soils Research Laboratory, Kimberly, ID. K19-19 was extracted from C5944 (PI 663873), a composite population released by R.T. Lewellen in 2011. K19-19 was self-pollinated for four generations and consistently rated between 7 and 9 in the greenhouse or field screening trials indicating susceptibility. F_1 seed from the cross KDH13/K19-19 was harvested from KDH13 (mother parent). The F_1 seedling was confirmed to be a true hybrid if it expressed the red hypocotyl dominant gene from the pollen donor (Panella et al., 2008). Ninety-three F_1 plants were infected with BCT using six infected leafhoppers in a clip-cage, clipped to plants at the four-leaf growth stage in the greenhouse. The mean value for the F_1 population was 3.4, which was lower than midparent value of 4.5 (Fig. 1). There were 56 F_1s rated below the midparent value and 37 rated above the midparent value. The majority of the F_1 plants from this cross showed a resistant phenotype rated below 4.0. That 40% of F_1s had susceptible rating suggests that K19-19 may still be segregating for genes impacting BCT despite four generations of selfing. Apparently, the inheritance of BCT resistance in sugarbeet may fit into a codominant additive mode of inheritance. However, there is a logical need to explore the BCT inheritance mode using more crosses, further segregating populations, and progeny tests.

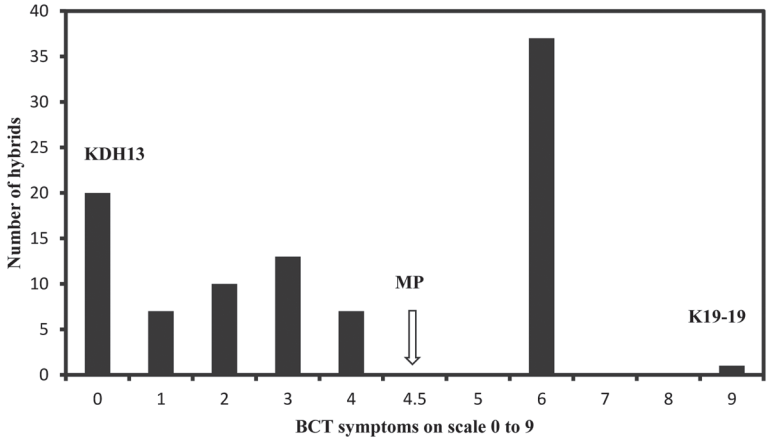

Fig. 1. Reaction of hybrids of KDH13/K19-19 to beet curly top (BCT) controlled infection (clip-caged six leafhoppers) in the greenhouse. Parental lines KDH13 and K19-19 were rated 0 (no leaf curl symptoms) and 9 (dead plant), respectively. MP, midparent.

Availability

Seed of KDH13 was deposited in the National Center for Genetic Resources Preservation, where it is available for research purposes and development of commercial sugarbeet cultivars. Small quantities of seed are also available for distribution by both the corresponding author and the USDA-ARS, Western Regional Plant Introduction Station, Pullman, WA. All seed will be distributed without cost to requesters. It is requested that appropriate recognition be made if KDH13 contributes to the development of new breeding materials.

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