

Registration of the Oilseed Sunflower Genetic Stocks HA 458, HA 459, and HA 460 Possessing Genes for Resistance to Downy Mildew

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ABSTRACT

Within the sunflower (*Helianthus annuus* L.) breeding community, there is a need for additional sources of downy mildew [caused by *Plasmopara halstedii* (Farl.) Berl. & De Toni] resistance to combat economically damaging races in North America. Our objective was to produce three genetic stocks, each with a different source of downy mildew resistance, in the genetic background of HA 434, a recently released sunflower maintainer germplasm with high oleic fatty acid content in the seeds. We used the backcross and pedigree breeding methods, with HA 434 as a recurrent parent. Gas chromatography analysis of seed oil composition and greenhouse testing of downy mildew resistance were used during the development process and for progeny testing of the finished genetic stocks. As a result, three oilseed sunflower genetic stocks, HA 458 (Reg. No. GS-51; PI 655009), HA 459 (Reg. No. GS-52; PI 655010), and HA 460 (Reg. No. GS-53; PI 655011), have been released which are resistant to downy mildew and possess a high-oleic fatty acid profile in the seed oil. The experimental designations for HA 458, HA 459, and HA 460 were 06GH 116-9, 06GH 113-4, and 05GH 138-4, respectively. These genetic stocks were released by the USDA-ARS and the North Dakota Agricultural Experiment Station, Fargo, ND, to fill the urgent need in the sunflower industry for donor lines with elite genetic background and resistance to common races of downy mildew.

The USDA-ARS and the North Dakota Agricultural Experiment Station, Fargo, ND, have developed and released three oilseed sunflower (*Helianthus annuus* L.) genetic stocks, HA 458 (Reg. No. GS-51; PI 655009), HA 459 (Reg. No. GS-52; PI 655010), and HA 460 (Reg. No. GS-53; PI 655011), resistant to downy mildew [caused by *Plasmopara halstedii* (Farl.) Berl. & De Toni]. These genetic stocks were developed to provide diversity for resistance against downy mildew, a major sunflower disease under moist, cool environmental conditions. The sources of resistance for HA 458 and

HA 459, which are PI 468435 and PI 435434, respectively, had not been used in any public breeding program before this release. The two plant introductions are wild collections from Idaho and Texas, respectively, and while it is unknown whether the two resistances are allelic, they have different resistance profiles, indicating that they are not identical. The resistance present in HA 460 is due to the Pl_8 gene, derived from RHA 340. This registration documents the first time that Pl_8 will be available in a high-oleic, public sunflower maintainer line. All of these genetic stocks are available for use by industry and public researchers to create parental lines or germplasm with a mid-oleic (NuSun) (>550 and <700 g kg⁻¹ oleic acid) or high-oleic (>750 g kg⁻¹) fatty acid concentration in the oil.

Most downy mildew resistance genes in sunflower are the result of genes found in wild *H. annuus*, *H. praecox*, *H. tuberosus*, or *H. argophyllus*, although resistance has also been found in other species (Vear et al., 2008). Most of these are of a vertical nature with complete genetic dominance, although non-race-specific resistance has also been found (Vear et al., 2008). Since *H. annuus* is the domesticated sunflower, genes from other species are introduced by interspecific gene transfer using traditional breeding methods. Some genes, notably those from *H. argophyllus*, allow sparse sporulation on the cotyledons, which does not progress further and is known as cotyledon-limited sporulation (CLS) (Bert et al., 2001). Only a few genes are available that provide resistance to all known races in North

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Abbreviations: CLS, cotyledon-limited sporulation.

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America. The Pl_6 gene, derived from *H. annuus* and allelic to Pl_7 , derived from *H. praecox*, is used worldwide to provide resistance and was recently defeated by at least eight new races in France, of which race 304 is one (Bert et al., 2001). Pl_8 (allelic to *H. tuberosus*-derived Pl_5 found in Russian cultivars 'Novinka' and 'Progress') and Pl_{ARG} are both derived from *H. argophyllus* but located on different linkage groups in sunflower (Bert et al., 2001; Dušle et al., 2004; Vear et al., 2008). Pl_5 , Pl_8 , and Pl_{ARG} provide resistance to all known races of downy mildew worldwide, but their commercial use is limited due to potential fears on the part of breeders concerning CLS. Additional sources of vertical resistance are needed to provide long-term durability of resistance to downy mildew, and the lines described herein will partially fill this need.

Methods

Description of Parent Stocks

PI 435434 is a wild *Helianthus annuus* L. accession collected near Riviera, TX, in 1976. PI 468435 is a wild *H. annuus* L. accession collected near Caldwell, ID, in 1979. RHA 340 (PI 518778) is a downy mildew resistant germplasm restorer line released by the USDA-ARS and the North Dakota Agricultural Experiment Station in 1987. It is derived from the population HA 89*3/*H. argophyllus* 415 (Miller and Gulya, 1988). HA 434 (PI 633744) is a high-oleic maintainer germplasm released by the USDA-ARS and the North Dakota Agricultural Experiment Station in 2001. It is a high-oleic acid selection from the mid-oleic germplasm HA 424 (Miller et al., 2004). Experimental line 00 3046 was the pre-release designation of HA 434. Seeds of the plant introductions were obtained from the North Central Regional Plant Introduction Station, Ames, IA, and seeds of the HA and RHA lines were obtained from internal collections.

Downy Mildew Screening

We evaluated 20 to 30 seeds from each experimental line for downy mildew resistance in greenhouse trials. Seeds were germinated for 3 d at 22°C and inoculated by the whole-seedling immersion technique, which involves immersing the seedlings in a zoosporangia suspension (20,000 spores mL⁻¹) for 3 h at 18°C (Cohen and Sackston, 1973). The resistance genes under selection in this study may truly be single gene resistances, or they may be the result of several, very closely linked loci. Clustering of disease resistance genes is common in sunflower, and plants in general (Radwan et al., 2008). For breeding purposes, it is important to maintain simplicity in the selection procedure while attempting to maintain the resistance genes for all the targeted races. For this reason, the zoosporangia solution contained a mixture of races 304, 730, 733, and 770 for testing all populations and finished lines except the population in which PI 435434 is a parent. For this population and its resulting lines, only races 730, 733, and 770 were included in the zoosporangia composite solution. By this method, we were able to screen populations quickly while eliminating any possibility that a rare recombinant could be carried through the process. This could occur if

only a single race were used and the resistance was due to a gene cluster instead of a single gene. A set of nine race differentials were included both to verify the virulence of the composite spore samples and to serve as controls. The inoculated seedlings were then planted in flats containing a sand-perlite mixture (3:2 v/v) and grown in the greenhouse maintained at approximately 24°C with a 16-h photoperiod provided by high-pressure sodium lamps. After 11 d, the flats were transferred to a chamber maintained at 18°C, misted, and kept in the dark overnight. In the morning, susceptible seedlings displayed profuse sporulation on the cotyledons and portions of the first true leaves, while resistant seedlings had no sporulation. Seedlings and the planting medium were autoclaved before disposal.

Gas Chromatography Screening

A sample of 20 seeds from each experimental line was pulverized with a hammer in an uncontaminated space, and approximately 0.2 g of the pulverized seed was transferred to a 13- by 100-mm test tube. The sample was derivatized by vortexing in 3 mL of hexane-chloroform-0.5 M sodium methoxide in methanol (75:20:5, v/v) to produce the fatty acid methyl esters. After the sample tube was vortexed for 10 s, about 1 mL of the sample solution was transferred to a 2-mL gas chromatograph autosampler vial and the vial was capped. The sample was injected into a Hewlett-Packard 5890 gas chromatograph (now Agilent Technologies, Santa Clara, CA) containing a DB-23 capillary column (30 m by 0.25 mm; J&W Scientific [now Agilent Technologies], Santa Clara, CA), which was held at 190°C for 4 min, then increased to 220°C at a rate of 15° min⁻¹, held at 220°C for 1 min, then increased to 240°C at a rate of 25° min⁻¹ and held at 240°C for 1 min, for a total run time of 9 min. Results were output to a computer text file as the quantity in grams of oleic fatty acid per kilogram of total fatty acids.

Line Development

Population and line development followed backcross and pedigree selection methodology. Hybridizations were made between experimental line 00 3046 and three sources of downy mildew resistance, PI 468435, PI 435434, and RHA 340, in summer 2000. The progeny of each cross were grown in the greenhouse in spring 2001, subjected to a downy mildew test with race 730 only, and uninfected individuals were transplanted to the field in summer 2001. The F₁ plants from each of the three populations were crossed with HA 434 in the field. The BC₁F₁ progeny of each cross were screened in the greenhouse during spring 2002 for resistance to the downy mildew race composites described earlier. Uninfected progenies were transplanted and backcrossed to HA 434 in the field in 2002. The BC₂F₁ progeny were grown in the greenhouse in winter 2002–2003, screened for downy mildew resistance with the appropriate race composite, and backcrossed to HA 434 in the greenhouse. The BC₃F₁ progeny were germinated in the greenhouse, screened against the appropriate race composite of downy mildew, and transplanted to the field in 2003 for self-pollination. Seeds

from each head harvested from the field were subjected to gas chromatography analysis to determine oleic acid content. Seeds from three single heads, each possessing oleic acid content above 800 g kg⁻¹ and each from a different source population, were germinated in the greenhouse in spring 2004 and subjected to downy mildew screening with the appropriate composite race sample. In the case of all three sources, the BC₃F₂ population segregated in a 3:1 (resistant:susceptible) ratio, indicative of a single, dominant gene conferring resistance (Table 1). Five to 10 resistant BC₃F₂ plants of each population were transplanted to the field in 2004 and self-pollinated. The gas chromatography screening, downy mildew screening, and self-pollination steps were repeated during the next three seasons: greenhouse, 2004–2005; field, 2005; and greenhouse, 2005–2006. After the greenhouse 2005–2006 season, the self-pollinated heads were threshed, seeds from each head were tested for oleic acid content using gas chromatography, and two of the resulting lines were chosen for release. The progeny of plant 06GH 116-9 was named HA 458 and 06GH 113-4 was named HA 459. Seed of the released genetic stocks was increased in summer 2006. Line 05GH 138-4 from the 2004–2005 greenhouse season was grown in the field during sum-

mer 2006, self-pollinated, purified by excluding seed from plants with <800 g kg⁻¹ oleic acid, and released as HA 460.

Statistical Analysis

The segregation ratios within the three BC₃F₂ families under selection were tested using chi-square tests to confirm that the resistance alleles were segregating in the populations as single, dominant alleles (Table 1). Progeny tests of the three finished genetic stocks for downy mildew resistance provided no segregation, as would be expected in genetically pure lines (Table 2). To ensure that the lack of segregation was not due to a sample size issue, we calculated the probability that a heterozygous line would produce at least one individual in a progeny test with the recessive phenotype, given the number of plants sampled from the population (Method III in Sedcole, 1977; also cited in Fehr, 1987). This method is based on a one-tailed test of a normally distributed (*z*) variable. Gas chromatography data was calculated from an average of at least three plants per location, year, and genetic stock (range 3–14), and corresponding standard errors calculated for each statistical mean (Table 3).

Table 1. Results of downy mildew [caused by *Plasmopara halstedii* (Farl.) Berl. & De Toni] screening of the BC₃F₂ progeny of three independent sunflower populations from which genetic stocks HA 458, HA 459, and HA 460 were derived, with tests of deviation from a 3:1 phenotypic ratio.

Line/population	Pedigree	Races tested [†]	Segregation distribution [‡]		$\chi^2_{df=1}$	$P > \chi^2$
			Resistant	Susceptible		
— no. of plants —						
HA 458	HA 434*4/PI 468435	304, 730, 733, 770	15 (14.25)	4 (4.75)	0.1579	0.6911
HA 459	HA 434*4/PI 435434	730, 733, 770	23 (21.75)	6 (7.25)	0.2874	0.5919
HA 460	HA 434*4/RHA 340	304, 730, 733, 770	14 (13.5)	4 (4.5)	0.0741	0.7855
HA 434	High oleic selection from HA 424	304, 730, 733, 770	0	19		
RHA 340	HA 89*3/ <i>Helianthus annuus</i> 415	304, 730, 733, 770	20	0		

[†]Races listed were included in the mildew spore bulk used to test the lines for resistance. Susceptibility to at least one of the races will lead to infection and a susceptible response.

[‡]All tests consist of a random sample of 20 seeds from a single BC₃F₁ plant. Not all seeds germinated. HA 459 shows the pooled results of two samples from two different BC₃F₁ plants. Numbers in parenthesis are the expected number of resistant and susceptible plants assuming a 3:1 phenotypic ratio.

Table 2. Downy mildew reaction of sunflower genetic stocks HA 458, HA 459, and HA 460, with their parents and checks.

Line	Pedigree	<i>Pl</i> gene [†]	Races tested	Segregation distribution [‡]		Probability [§]	
				Resistant	Susceptible	z_{α}	$P < z_{\alpha}$
— no. of plants —							
HA 458	HA 434*4/PI 468435	unknown	304, 730, 733, 770	30 (30)	0 (0)	2.95	0.998
HA 459	HA 434*4/PI 435434	unknown	730, 733, 770	27 (27)	0 (0)	2.78	0.997
HA 460	HA 434*4/RHA 340	<i>Pl_g</i>	304, 730, 733, 770	18 (18)	0 (0)	2.18	0.985
RHA 340	HA 89*3/ <i>Helianthus annuus</i> 415	<i>Pl_g</i>	304, 730, 733, 770	19 (19)	0 (0)	2.25	0.988
PI 435434	Wild collection from Texas	unknown	304, 730, 733, 770	18	2		
PI 468435	Wild collection from Idaho	unknown	304, 730, 733, 770	16	3		
Cargill 270	Susceptible commercial hybrid	—	304, 730, 733, 770	0 (0)	20 (20)		

[†]The *Pl* gene indicates the genetics of the resistance to downy mildew, if known.

[‡]Resistant = resistance to the downy mildew race (indicated by few or no spores on cotyledon tissues and no spores on true leaves), Susceptible = susceptibility to the downy mildew race (indicated by profuse sporulation on cotyledons and true leaves, as well as stunting of the seedling). Plant introductions may contain both susceptible and resistant types because they are wild collections. Parentheses indicate expected numbers of each category.

[§]Probability of finding at least one susceptible individual after self-pollinating a heterozygote given a 3:1 (resistant:susceptible) expected phenotypic ratio and a sample size equal to the sum of the resistant plants observed.

Table 3. Oleic acid composition of sunflower genetic stocks HA 458, HA 459, and HA 460, with HA 467 as a distantly related check line.

Line	Pedigree	Oleic acid composition \pm SE		
		2005–2006 greenhouse	2006 Fargo, ND, field	2007 Fargo, ND, field
HA 458	HA 434*4/PI 468435	865 \pm 8	843 \pm 6	826 \pm 7
HA 459	HA 434*4/PI 435434	873 \pm 10	865 \pm 5	834 \pm 10
HA 460	HA 434*4/RHA 340	888 \pm 2	835 \pm 15	843 \pm 3
HA 467	HA 411/ROM PH//IMI/87CAEB/3/HA 434/HA 412	881 \pm 2	886 \pm 2	863 \pm 3

Results

HA 458

HA 458 is a BC₃F_{5,6} maintainer genetic stock selected from the cross HA 434*4/PI 468435. This genetic stock is resistant to the North American races 730, 733, and 770 and to the French race 304 (Table 2). Inheritance studies indicated that the resistance was controlled by a single, dominant gene or a set of tightly linked genes that is independent of the *Pl₆* locus (Table 1; F. Vear, personal communication, 2005). Preliminary molecular marker comparisons also indicated that this resistance gene may be different from the *Pl₈* gene (Hu et al., 2004). In that study, resistance cosegregated with a single TRAP marker, but when the same marker was screened in genetic backgrounds containing *Pl₆* or *Pl₈*, the marker appeared as the susceptible form. HA 458 averaged 826 to 865 g kg⁻¹ oleic fatty acid in seed from each of three different environments (Table 3). None of the environments yielded oil of this genetic stock that were below or within two standard errors of the Codex Standard for high oleic sunflower oil of 750 g kg⁻¹ (Codex Alimentarius Commission, 2001).

HA 459

HA 459 is a BC₃F_{5,6} maintainer genetic stock selected from the cross HA 434*4/PI 435434. The downy mildew resistance in HA 459 is conferred by a single dominant gene or a tightly linked set of genes (Table 1), but it is currently unknown if this resistance is allelic or independent of other known resistance genes. This genetic stock is resistant to the North American races 730, 733, and 770 but is susceptible to the French race 304 (Table 2). HA 459 averaged 834 to 873 g kg⁻¹ oleic fatty acid in seed from each of three different environments (Table 3). None of the environments yielded oil of this genetic stock that were below or within two standard errors of the international CODEX standard for high oleic sunflower oil of 750 g kg⁻¹ (Codex Alimentarius Commission, 2001).

HA 460

HA 460 is a BC₃F_{3,4} maintainer genetic stock selected from the cross HA 434*4/RHA 340. This genetic stock carries the *Pl₈* gene, which confers resistance to the North American races 730, 733, and 770 and to the French race 304 (Table 2). Resistance is controlled by a single dominant gene or a tightly linked set of genes (Table 1). Recent studies have found that this resistance gene is at or near the *Pl₅* locus for downy mildew resistance and is on a different link-

age group than *Pl₆* and *Pl₇* (Bert et al., 2001). Polymerase chain reaction–based sequence tagged site markers have been discovered that are specific to the *Pl₈* locus in other genetic backgrounds (Radwan et al., 2004). HA 460 averaged 835 to 888 g kg⁻¹ oleic fatty acid in seed from plants from each of three different environments (Table 3). None of the environments yielded seed of this genetic stock that were below or within two standard errors of the international CODEX standard for high oleic sunflower oil of 750 g kg⁻¹ (Codex Alimentarius Commission, 2001). HA 460 represents the first maintainer genetic stock released to combine the high-oleic fatty acid characteristic with the *Pl₈* downy mildew resistance.

Availability

Small quantities of seed of each genetic stock will be available from the NDSU Seedstocks Project, Department of Plant Sciences, North Dakota State University Dep. 7670, P.O. Box 6050, Fargo, ND 58108-6050. Seed from the 2006 and 2007 increases will be deposited in the National Plant Germplasm System, where it will be available for research purposes. No limit will be imposed on the number of generations of seed increase. All seed increases will be conducted using cages or individually bagged heads for isolation. U.S. Plant Variety Protection will not be requested for HA 458, HA 459, and HA 460. It is requested that appropriate recognition be made if these genetic stocks contribute to the development of a new germplasm, breeding line, or cultivar.

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