Progress on the transferring Sclerotinia resistance genes from wild perennial *Helianthus* species into cultivated sunflower

Zhao Liu[1], Fang Wei[1], Xiwen Cai[1], Gerald J. Seiler[2], Thomas J. Gulya[2], Khalid Y. Rashid[3], Chao-Chien Jan[2]

[1]Department of Plant Sciences, North Dakota State University, Fargo, ND 58102 USA

[2]USDA, Agricultural Research Service, Northern Crop Science Laboratory, Fargo, ND 58102 USA

[3]Agriculture and Agri-Food Canada, Morden, Manitoba, Canada R6M 1Y5

Abstract

Cultivated sunflower lacks sufficient genes for Sclerotinia resistance, but wild perennial *Helianthus* species are highly resistant. Replicated field trials tested 163 progeny entries for head rot resistance in 2009 and identified entries that had moderate to good resistance indicating successful gene introgression. Eighty-seven entries were retested in 2011 as well as 120 new entries from seed increased in 2009 and 2010. According to the two-year field test data, entries with good to poor resistance were identified. Further evaluation will be conducted on only the lightly infected families in 2012. In order to provide more diverse resistance genes for developing Sclerotinia resistant germplasms, new crosses were started in 2010, with 11 accessions from five perennial and one annual species in the greenhouse using HA 410 and HA 451 as recurrent parents. Embryo rescue was applied to both F1 and BC1 crosses in order to obtain more BC1 seedlings. One chromosome addition line was identified using SSR markers from the progenies derived from former crosses involving interspecific tetraploid amphiploids. The materials derived from other crosses will be analyzed, and the alien chromosome or segments will be verified by genomic *in situ* hybridization (GISH) in future experiments.

Introduction

The necrotrophic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary attacks sunflower (*Helianthus annuus* L.) causing root, stalk, and head rot, and is one of the most damaging and difficult-to-control sunflower diseases (Gulya, 2004). Some wild perennial *Helianthus* species have been identified to be resistant to this fungus (Block et al., 2011; Feng et al., 2007a, b; Feng et al., 2008; Feng et al., 2009). Genetic analysis has indicated that Sclerotinia resistance is multigenic (Gentzbittel et al., 1998), and that resistance to basal stalk and head rot are not related. Crosses and backcrosses have been made to introgress the resistance genes from hexaploid, tetraploid, diploid wild species and interspecific amphiploids into cultivated sunflower, using HA 441 or HA 410 as the recurrent parent. Progenies derived from different crosses have been evaluated in the field since 2009. In order to diversify the resistance sources, 11 accessions from five wild perennial and one annual *Helianthus* species were established in the greenhouse in 2010.

The objectives of this study were to: (1) transfer Sclerotinia stalk and head rot resistance from resistant wild perennial *Helianthus* accessions, and interspecific amphiploids into cultivated sunflower via the traditional backcross method; (2) evaluate stalk and head rot-resistance via field test to screen progenies with higher levels of resistance; and (3) genetic study of resistance and QTL mapping.

Materials and Methods

Field test

Seed of the progenies obtained from earlier crosses were increased for field testing for stalk and head rots, or chromosome addition line identification, including four diploid species (*H. giganteus*, *H. grosseserratus*, *H. nuttallii*, and *H. maximiliani*), five interspecific amphiploids (Amp *H. strumosus* × P21, Amp *H. grosseserratus* × P21, Amp *H. maximiliani* × P21, Amp *H. nuttallii* 730 × P21, and Amp (*H. divaricatus* ×P21) × (*H. grosseserratus* × P21)), and one hexaploid species (*H. californicus*). One hundred and sixty-three progeny entries were tested in the field for head rot resistance in 2009. Eighty-seven entries evaluated in 2009 and 120 new progeny entries in various BC generations were screened for head rot resistance in replicated field trials at Staples, MN in 2011. Head rot was rated on a 0 to 5 scale, with 0=no infection, and 5=complete destruction of the head. Eleven accessions from five perennial wild species (*H. hirsutus*, *H. salicifolius*, *H. occidentalis* subsp. *plantagineus*, *H. silphioides*, *H. resinosus*) and one annual species (*H. agrestis*) were used to initiate new crosses in 2011.

New crosses

The 11 accessions were crossed with NMS HA89, HA 410, HA 451, or *H. nuttallii* 102, respectively, followed by embryo rescue. Backcrossing and embryo rescue were used to produce BC1 seedlings. Alexander's procedure (Alexander, 1969) was employed to check pollen stainability. Chromosome counting was conducted using the standard Feulgen staining method.

Addition line identification

Ninety-three plants with 2n=34-36 chromosomes derived from the crosses involving the five interspecific tetraploid amphiploids were analyzed using polymorphic linkage-specific SSR markers of the sunflower map (Tang et al. 2003). Four primers from each linkage group were screened. The plants that amplified the same band patterns as the wild parents were expected to be alien addition lines.

Results and Discussion

Field evaluations for head rot resistance in 2009 and 2011

Since 2008, the seeds of the progeny families derived from different diploid, hexaploid wild *Helianthus* species and tetraploid interspecific amphiploids have been increased in order to provide sufficient seeds for field evaluation. A three-year field test for Sclerotinia head or stalk

rot has been conducted for some of the progenies. However, due to the high midge damage in Carrington, ND in 2010, and hail damage in Carrington in 2011, we only report the results of field evaluations for head rot resistance in 2009 and 2011 here.

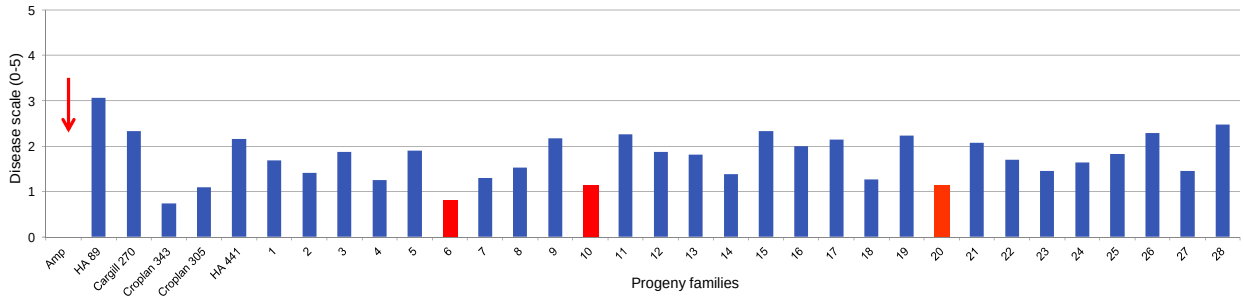

Figure 1. Average field Sclerotinia head rot disease rating at Carrington, ND in 2009 for 28 families from 163 entries. Susceptible checks: Cargill 270, HA 89; resistant checks: Croplan 343, 305. The arrow indicates the Amp bulk with disease rating of "0". The red columns indicate the families showing better or close to the resistant checks

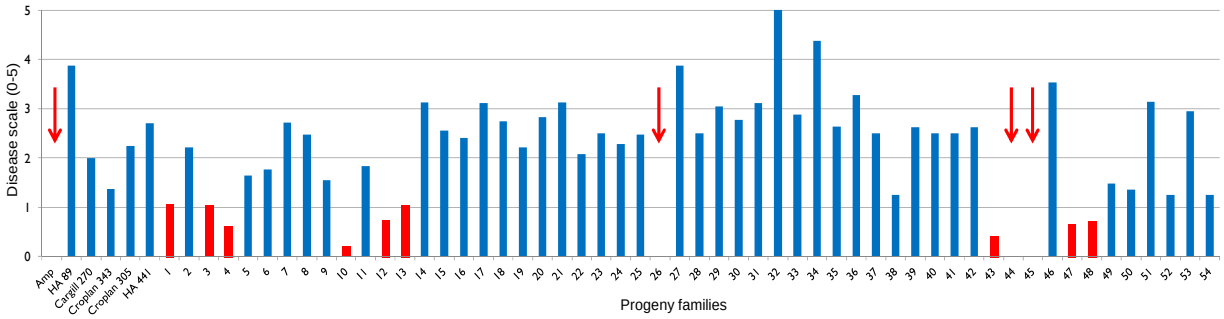

Figure 2. Average field Sclerotinia head rot disease rating at Staples, MN in 2011 for 54 families from 207 entries. The arrows indicate the Amp bulk and progeny families with disease rating of "0". The red columns indicate the families showing good resistance compared to the resistant checks

Figure 1 shows the average Sclerotinia head rot disease ratings of different progeny families at Carrington, ND in 2009 with 163 entries, and Figure 2 shows those at Staples, MN in 2011 with 207 entries. Every entry in each family was replicated twice, plus two susceptible checks, HA 89 and Cargill 270, two resistant checks Croplan 343 and Croplan 305, and the recurrent parent HA 441 each year. For the checks and HA 441, higher disease ratings were detected in 2011 than in 2009. Various disease ratings were observed in both years. The families showing good resistance in 2009 and 2011 are indicated in the red columns. The families or entries that

showed moderate to good resistance compared to the recurrent parent or susceptible checks will be evaluated further in 2012. Notably, the Amp bulk showed a disease rating of "0" in the both years. Three progeny families (No. 26, 44 and 45) also showed a disease rating of "0" in 2011. These results suggest the successful introgression of resistance genes. On the other hand, some families were also identified with very poor resistance, such as the No. 27, 32 and 34 families in 2011, which will be eliminated for further field testing in 2012.

New crosses in 2011

In order to increase the diversity of the resistance sources, 11 accessions from five perennial and one annual *Helianthus* species were crossed with cultivated sunflower (NMS HA 89, HA 410 and HA 451), or *H. nuttallii* 102, respectively. The results of embryo rescue for different crosses were shown in Table 1. In total, 3,480 florets were pollinated out of 55,300 (6.3%), 2,048 embryos were rescued (3.7%), and 329 F1 plants were obtained (0.59%). The most F1s obtained were from the crosses involving *H. hirsutus* (142). Many more F1s were obtained from the cross of *H. silphioides* x *H. nuttallii* 102 (96) than from the No. 12, 13 and 15 crosses involving *H. silphioides*. No matter which cultivated sunflower or *H. nuttallii* 102 was used, no F1s were obtained from the crosses involving *H. agrestis*. The difficulty of crossing *H. agrestis* with other *Helianthus* species maybe due to the distant relationship between them.

The F1 plants (Column b) showed an intermediate phenotypes between the wild (Column a) and cultivated parents (representative pictures were shown in Figure 3). The flowers of F1s produced a large amount of pollen (Column c), however, the pollen stainability of F1s were quite low (Column d). Higher pollen stainability was observed for the F1s derived from the cross between *H. hirsutus* and cultivated sunflower than those from other crosses. Also, various sizes of the pollens were observed for different F1s. In order to obtain as many BC1 seeds as possible, we also used embryo rescue during the backcrossing (Table 2). Pollination of 27,180 florets produced 675 seeds (2.5%), 571 embryos were rescued (2.1%) with 253 embryos transferred to test tubes (0.93%). Those surviving this stage were transferred to Jiffy-7 pellets and then to soil. Developmental stages of the embryos rescued were also analyzed for different F1 crosses (Fig. 4a) and backcrosses (Fig. 4b). The dominant stages varied among different crosses with most embryos at the globular stage failing to develop F1 plants.

Table 1. Embryo rescue results of the new crosses involving five perennial and one annual *Helianthus* species.

No.	Female	Male	Seeds	Florets	Embryos	F1
1	*H. hirsutus*	HA 410	173	1715	140	27
2	*H. hirsutus*	HA 451	69	446	63	34
3	NMS HA 89	*H. hirsutus*	1492	6044	470	81
4	*H. salicifolius*	HA 410	280	4700	241	26
5	*H. salicifolius*	HA 451	23	795	15	0
6	NMS HA 89	*H. salicifolius*	41	11300	31	5
7	*H. occidentalis*	HA 410	285	1210	217	15
8	*H. occidentalis*	HA 451	269	1060	182	27
9	*H. occidentalis*	*H. nuttallii* 102	10	180	10	4
10	NMS HA 89	*H. occidentalis*	10	3650	4	3
11	*H. resinosus*	HA 451	63	1875	8	8
12	*H. silphioides*	HA 410	277	2540	234	2
13	*H. silphioides*	HA 451	123	2415	110	1
14	*H. silphioides*	*H. nuttallii* 102	161	540	155	96
15	NMS HA 89	*H. silphioides*	88	10960	54	0
16	*H. agrestis*	HA 410, HA451, and *H. nuttallii* 102	112	1170	111	0
17	NMS HA 89	*H. agrestis*	4	4700	3	0
	Total		3480	55300	2048	329

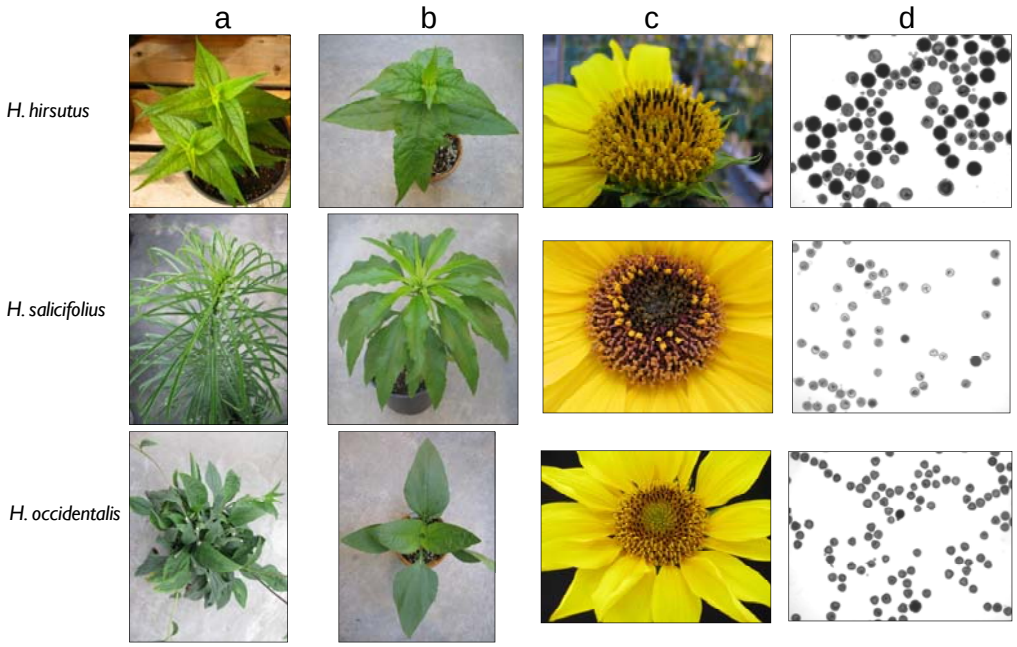

Figure 3. F1 plants derived from the crosses of three wild perennial *Helianthus* species and cultivated sunflower. a) wild parents; b) F1 plants; c) flowers of F1s; d) pollen stainability of F1s

Table 2. Embryo rescue results of the backcrosses involving five perennial *Helianthus* species.

No.	Female	Male	Recurrent	Seeds	Florets	Embryos	Test tubes
1	*H. hirsutus*	HA 410	HA 410	67	3790	55	26
2	*H. hirsutus*	HA 451	HA 451	19	2450	16	9
3	NMS HA 89	*H. hirsutus*	HA 410	32	3525	30	4
4	NMS HA 89	*H. hirsutus*	HA 451	13	740	8	1
5	*H. salicifolius*	HA 410	HA 410/ HA 451	137	6698	88	36
6	NMS HA 89	*H. salicifolius*	HA 410	5	350	5	3
7	*H. occidentalis*	HA 410	HA 410	30	3732	23	6
8	*H. occidentalis*	HA 451	HA 410/ HA 451	22	2580	20	9
9	*H. occidentalis*	*H. nuttallii* 102	HA 451	49	700	46	9
10	NMS HA 89	*H. occidentalis*	HA 410	3	350	4	3
11	NMS HA 89	*H. occidentalis*	HA 451	3	250	3	0
12	*H. silphioides*	HA 410	HA 410/ HA 451	8	640	5	0
13	HA 89	*H. nuttallii* 102	HA 410	75	470	63	45
14	HA 89	*H. nuttallii* 102	HA 451	212	905	205	102
	Total			675	27180	571	253

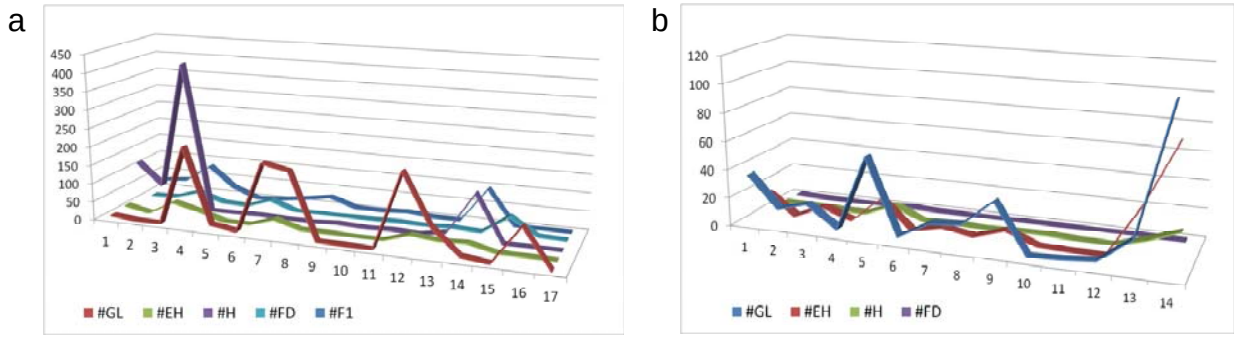

Figure 4. Developmental stages of embryos rescued from 17 F1 cross combinations (a), and 14 BC1 cross combinations (b). GL= globular, EH= early heart, H= heart, FD = full development

Chromosome addition line identification

Plants with 2n=34, 35 and 36 were identified by counting the chromosome number for each plant derived from different backcrosses derived from five interspecific tetraploid amphiploids. Representative chromosome squashes with 2n=34 and 2n=35 are shown in Figure 5a and 5b, respectively. After analysis with four chromosome specific marker from each linkage group of the sunflower SSR map, one chromosome addition line was identified using a marker specific to linkage group (LG) 5, which showed the same band pattern as the wild species (Figure 5c). The

alien chromosome was expected as this linkage group. More markers and progenies will be analyzed in the future.

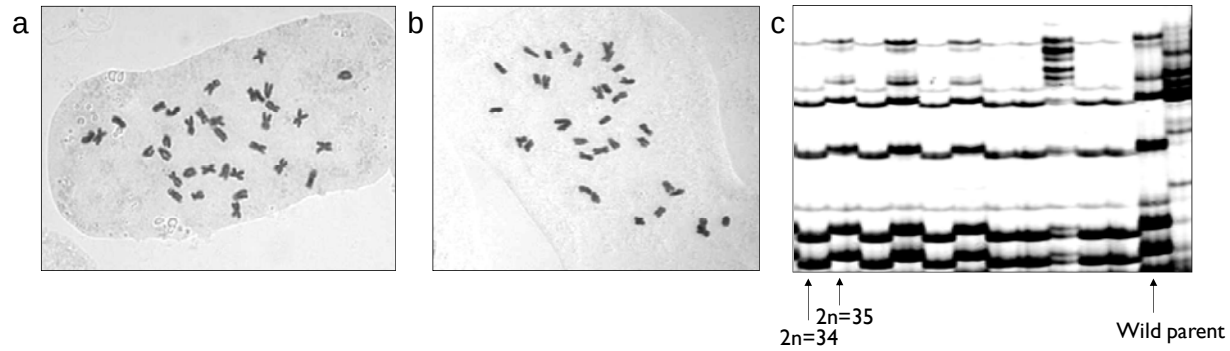

Figure 5. Chromosome addition line identification using polymorphic linkage group-specific SSR markers for the backcross progenies derived from five interspecific tetraploid amphiploids. a) 2n=34; b) 2n=35; c) a non-denaturing polyacrylamide gel showed that the alien chromosome belonged to LG 5 of the sunflower SSR map

Summary

Seed increased in 2008-2011 provided sufficient seeds for field evaluation. Replicated field tests in 2009 and 2011 for head rot resistance showed that progeny families derived from both head and stalk rot resistance sources were performing well. The families with moderate to good resistance will be evaluated further in 2012, while the families showing low levels of resistance will be eliminated. The results showed successful gene introgression from wild *Helianthus* species to cultivated sunflower. New progenies with 2n=34 chromosomes obtained from the crosses will be field evaluated for both head and stalk rot resistance in 2012.

For the new crosses, extensive efforts were made to obtain F1 plants and BC1 plants via embryo rescue. More than 300 F1 plants were obtained from the crosses between the cultivated sunflower and wild perennial *Helianthus* species, except annual *H. agrestis*. *Helianthus silphioides* was difficult to cross with cultivated sunflower, but was easier to cross with *H. nuttallii* 102. *Helianthus nuttallii* 102 could be considered as a bridge parent for the cross. BC seed set was lower than that of the F1s for these new crosses due to the low fertility of the F1 plants. The production of BC1 seedlings are in progress.

The identification of additional chromosome addition lines are also in progress. The progenies derived from other crosses will also be investigated using cytogenetic analysis and polymorphic SSR markers. GISH will be used to verify the alien chromosomes or fragments in cultivated background in the future.

Acknowledgement

Technical assistance of Lisa Brown, Alicia Garcia, Marjorie Olson, Leonard Cook, Megan Ramsett and Dr. Nikolay Balbyshev is greatly appreciated.

References

Alexander, P. 1969. Differential staining of aborted and non-aborted pollen. Stain Technol. 44:117-122.

Block, C., L. F. Marek, and T. J. Gulya. 2011. Evaluation of wild *Helianthus* species for resistance to Sclerotinia stalk rot. Sclerotinia Initiative Annual Meeting, Bloomington, MN. January 19-21, 2011. Abstract p.13

Feng, J., G. J. Seiler, T. J. Gulya, and C. C. Jan. 2007a. Advancement of pyramiding new *Sclerotinia* stem rot resistant genes from *H. californicus* and *H. schweinitzii* into cultivated sunflower. Proc. 29th Sunflower Research Workshop, January 10-11, 2007, Fargo, ND. Available: http://www.sunflowernsa.com/research/research-workshop/documents/Feng_etal_Pyramid_2007.pdf

Feng, J., G. J. Seiler, T. J. Gulya, C. Li, and C. C. Jan. 2007b. *Sclerotinia* stem and head rot resistant germplasm development utilizing interspecific amphiploids. Proc. 29th Sunflower Research Workshop, January 10-11, 2007, Fargo, ND. Available: http://www.sunflowernsa.com/research/research-workshop/documents/ Feng_etal_Amphiploids_ 2007.pdf

Feng, J., G. J. Seiler, T. J. Gulya, X. Cai, and C. C. Jan. 2008. Incorporating Sclerotinia stalk rot resistance from diverse perennial wild *Helianthus* species into cultivated sunflower. Proc. 30th Sunflower Research Workshop, National Sunflower Association, January 10-11, 2008, Fargo, ND. Available: http://www.sunflowernsa.com/research/research-workshop/documents/Feng_etal_ StalkRot_08.pdf.

Feng, J., Z. Liu, X. Cai, G. J. Seiler, T. J. Gulya, K. Y. Rashid, and C. C. Jan. 2009. Transferring Sclerotinia resistance genes from wild *Helianthus* into cultivated sunflower. Proc. 31st Sunflower Research Workshop, National Sunflower Association, January 13-14, 2009, Fargo, ND. Available: http://www.sunflowernsa.com/research/research-workshop/documents/Feng _ Genes_09.pdf

Gentzbittel, L., S. Mouzeyar, S. Badaoui, E. Mestries, F. Vear, D. Tourvieille de Labrouhe, and P. Nicolas. 1998. Cloning of molecular markers for disease resistance in sunflower, *Helianthus annuus* L. Theor Appl Genet 96:519-525.

Gulya, T. J. 2004. Sunflower disease incidence and distribution in midwestern U.S. in 2003. Proc. 26th Sunflower Research Workshop, January 14-15, Fargo, ND. Available: http://www.sunflowernsa/com/reserach-workshop/document/ Gulya_Disease_ midwest_ 2003_ 04.pdf

Tang, S., V.K. Kishore, and S.J. Knapp. 2003. PCR-multiplexes for a genome-wide framework of simple sequence repeat marker loci in cultivated sunflower. Theor Appl Genet 107:6-19