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Broomrape (*Orobanche Cumana* Wallr.) Resistance Breeding Utilizing Wild *Helianthus* Species

Abstract: Wild *Helianthus* species possess valuable resistance genes for sunflower broomrape (*Orobanche cumana* Wallr.), especially the 39 largely underutilized perennial species. Resistance to race F has been transferred into a cultivated background via bridging of interspecific amphiploids. More recently, a single dominant gene resistant to race G was identified in annual *H. debilis* ssp. *tardiflorus* and transferred into cultivated HA 89. Interspecific crosses between wild annual *Helianthus* species and cultivated lines are relatively easy compared to those involving wild perennial species, which were made easier only after the development of embryo rescue techniques. Interspecific amphiploids resulting from colchicine treatment of F₁ hybrids provide bridging materials for transferring genes without relying on embryo rescue. Among the diploid, tetraploid, and hexaploid perennial species, the speed of gene utilization follows the ploidy level of diploids, tetraploids, and hexaploids due to the time-consuming backcrosses required to eliminate the extra chromosomes in the latter two groups. In the development of pre-breeding materials, the retention rate of genetic material of the wild species is another concern with each additional backcross. For crosses involving tetraploid and hexaploid wild perennials, the use of $2n = 51$ chromosome F₁ or BC₁F₁ generation, as pollen source, could accelerate chromosome reduction to $2n = 34$ in BC₁F₁ or BC₂F₁, resulting in useful materials with fewer backcrosses for trait selection.

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Introduction

Sunflower broomrape (*Orobanche cumana* Wallr.) is a holoparasitic plant, which parasitizes sunflower roots and constrains sunflower production worldwide (Skoric *et al.*, 2010). Vranceanu *et al.* (1980) identified five broomrape races, A to E with a set of differential lines carrying corresponding major dominant resistance genes Or_1 to Or_5 . *Orobanche* was not a major problem in sunflower production, being controlled by the Or_5 gene for over 20 years until the appearance of race F in Spain in the mid-1990s, making the gene ineffective (Alonso *et al.*, 1996). Shortly afterward, race G was reported by Skoric *et al.* (2010). More recently germplasm resistant to race G was overcome in Romania (Pacureanu-Joita *et al.*, 2009) and Turkey (Kaya *et al.*, 2009), suggesting the appearance of a new race H. These rapid race shifts of *Orobanche* since the mid-1990s sent an alarming message to the sunflower community and all sunflower breeders who were in a tight race with the ever-evolving broomrape races, with the majority of the sunflower hybrids lacking the critically needed resistance genes to the new races.

Fortunately, resistance to *Orobanche* race F was identified in both cultivated and wild *Helianthus* sources with exceptional resistance in wild perennials. Race F resistance genes from four interspecific amphiploids (Amps) were transferred into a cultivated background with the resistance genes from one source suggesting a digenic model of resistance (Jan *et al.*, 2002; Velasco *et al.*, 2007). Subsequently, a single dominant race G resistance gene was transferred from *H. debilis* ssp. *tardiflorus* into a cultivated background (Velasco *et al.*, 2012).

Wild *Helianthus* species have been shown to possess an abundance of valuable genes for improving cultivated sunflower, including broomrape resistance, but have long been neglected and underutilized, especially the perennials, due to the difficulties of crossing with cultivated sunflower. Major problems include cross incompatibility, F_1 sterility, and the time-consuming backcrossing to eliminate extra chromosomes when polyploid perennial species are used, as well as the concern of the loss of wild species genetic materials. However, embryo rescue techniques developed in the 1980s (Chandler and Beard, 1983) have made interspecific hybridization a routine process, coupled

with improved greenhouse evaluation procedures that have enabled effective selection with speed and accuracy.

However, it took 3 years to obtain resistant BC_1F_2 and BC_2F_1 plants for the race G resistance, and even longer for the race F resistance utilizing interspecific Amps that required several cycles of backcrossing to reduce the chromosome numbers from $2n = 68$ to $2n = 34$, while selecting for resistant progenies (Jan *et al.*, 2002; Velasco *et al.*, 2012). As a general rule, diploid wild \times diploid cultivated crosses produce adequate progenies for selection in BC_1F_1 , BC_1F_2 , or BC_2F_1 , requiring the least amount of time of 2 to 3 years. Tetraploid wild \times cultivated crosses require more time to reduce the $2n$ chromosomes from 68 to 34, with $2n = 34$ progenies starting to appear in BC_3F_1 generation at the earliest. Hexaploid wild \times cultivated crosses take even longer, beginning to observe $2n = 34$ plants in approximately the BC_4F_1 generation.

Since wild perennials are known to be highly resistant to broomrape, proactive pre-breeding efforts should be considered before race shifts by focusing on the production of a large number of progeny families, since the wild species accessions can be screened for resistance and the corresponding progeny families can be quickly screened. Research is in progress to compare the effectiveness of producing progenies with maximum wild species genetic diversity with $2n = 34$ chromosomes for breeders' use utilizing different wild species sources. It is generally believed that progenies with fewer backcrosses retain more wild species genetic information, and therefore the diploid wilds would be the best candidates for pre-breeding. However, the tetraploids, hexaploids, and the interspecific Amps all have merit and value for interspecific gene transfer due to their large genome size with multiple genomes, as well as the improvement in backcrossing allowing for faster utilization in breeding for broomrape resistance.

During the past 10 years, we have conducted interspecific gene transfer for *Sclerotinia* stalk and head rot resistance from wild *Helianthus* species, with a focus on the utilization of the difficult-to-cross perennial species, which are mostly immune to *Sclerotinia* stalk rot and have high levels of resistance to head rot. Since the resistance to *Sclerotinia* is controlled by quantitative genes, the strategy has been to develop early generation backcross progenies with maximum genetic diversity from the wild parent(s) for selection in replicated field trials using artificial inoculation. Families with significantly higher resistance than the recurrent parents have been identified verifying the usefulness of this approach (Liu *et al.*, 2014). A high level of resistance to broomrape in wild *Helianthus*, particularly the perennials, has been reported by Fernandez-Martinez *et al.* (2008). To stay ahead of the expected evolution of new broomrape races, a similar approach to that used for the *Sclerotinia* resistance could be

used by breeders to save time by using the already developed pre-breeding segregating families.

Previously, we successfully transferred resistance to broomrape race F from interspecific Amps involving perennial species, and race G resistance from annual *H. debilis* ssp. *tardiflorus*, while monitoring resistance in early generations. While utilizing tetraploid Amps, plants with $2n = 34$ to 51 chromosomes were observed in the BC₂F₁ generation, resistant plants with $2n = 34$ were self-pollinated, and BC₂F₂ plants were used to produce seeds for germplasm release (Jan et al., 2002). A resistance gene for race G was identified in *H. debilis* ssp. *tardiflorus* plants, crossed by HA 89, and BC₁F₁, BC₁F₂, BC₂F₁, and BC₂F₂ were used to demonstrate the monogenic dominant gene control of the resistance (Velasco et al., 2012).

Materials and methods

Plant materials

The following representative wild species groups will be used to demonstrate the germplasm development, including embryo rescue, the F₁s, backcrosses, and resulting lines for resistance selection; (1) wild diploid perennial species *H. salicifolius* and *H. occidentalis*, (2) wild tetraploid perennial *H. hirsutus*, (3) wild hexaploid perennial *H. californicus*, and (4) tetraploid interspecific Amps of *H. grosseserratus* × P21 and *H. nuttallii* × P21, and hexaploid interspecific Amps with *H. strumosus* × P21. Traditional crossing and backcrossing methods were used to transfer the genes from wild species, with the aid of embryo rescue technique for some crosses. Two oilseed maintainer lines, HA 410, HA 451, and a nuclear male-sterile line NMS HA 89 were used for backcrossing. Selected F₁ or BC₁F₁ progenies were used both as female and as the pollen parent for backcrosses in order to compare the efficiency of germplasm development, as well as for chromosome reduction to $2n = 34$ when polyploid wild *Helianthus* species were involved.

Embryo rescue and mitotic chromosome counts

Embryos from crosses were rescued six to eight days after pollination following Jan and Chandler (1989). Root tips were collected from 2- to 3-week old seedlings for chromosome counts for each plant in different generations following Liu

et al. (2013). Slides were analyzed using a Zeiss Axioplan-2 imaging fluorescence microscope (Jena, Germany).

Results

Representative crosses involving wild species with different ploidy levels are shown in Table 1. Crosses involving hexaploid *Helianthus* are represented by *H. californicus* × HA 410. Initial crosses were obtained relatively easily, with a high percentage of hybrid embryo formed (32.39%), resulting in a large number of F₁ seedlings (388) after embryo rescue. The process of chromosome reduction from the $2n = 68$ to $2n = 34$ required repeated backcrosses, with $2n = 34$ chromosome progeny first observed in BC₄F₁, with sufficient progeny families with $2n = 34$ selected for trait selection with good seed set of 35.3%.

Crosses involving tetraploid *Helianthus* were represented by *H. hirsutus* × HA 451. It was moderately easy to produce F₁s via embryo rescue, with 14.13% embryo formation. However, the backcrosses using the F₁s as the female parent resulted in extremely low embryo formation (0.65%). The same low seed set problem continued for the subsequent backcrosses with no plants having $2n = 34$ in BC₁F₁. Three $2n = 34$ plants out of 27 BC₃F₁/BC₂F₂ plants were obtained with a range of $2n = 34$ –37 chromosomes, and an average seed set of 28.3%. The long time needed to reduce the chromosome number cannot be ignored since it increased the probability of losing valuable genes. Alternatively, crosses of NMS HA89 × F₁ resulted in all $2n = 34$ BC₁F₁ progeny, with an average seed set percentage of 73.2%, which drastically shortened the breeding time when utilizing this tetraploid species.

Crosses involving diploid *Helianthus* were represented by *H. salicifolius* and *H. occidentalis* × HA 410. Interspecific crosses of this group were the most difficult to obtain, with low embryo formation and fewer F₁ plants. BC₁F₁ embryo formation was low, 1.31 and 0.62%, respectively producing only a small number of plants having $2n = 35$ or 36 chromosomes. However, $2n = 34$ BC₁F₁ plants could be selected providing an adequate number of families for selection, with low, but acceptable backcross seed set of 7 to 10%. Alternatively, BC₁F₁s resulting from NMS HA89 × F₁s of this group resulted in all $2n = 34$ plants, with an average seed set of 66.2 and 68.3%, respectively. Therefore, the BC₁F₃ families can be equally utilized without the complication of having to select for $2n = 34$ plants in BC₁F₁.

Crosses involving tetraploid Amps were represented by Amps *H. grosseserratus*/P21 and *H. nuttallii*/P21 crossed with HA 410. Plants with $2n = 34$ were first

Table 1: The chromosome number and seed set percentage of F_1 and backcross progeny for selected crosses during development

| Parentage | F_1 | | The first backcross generation with $2n = 34$ plants observed | | The backcross generation with the most $2n = 34$ plants observed ^a | | | | |
|---|----------------|------|---|---------------------|---|------------|-------------------|--------|------------|
| | F_1 embryo % | $2n$ | BC embryo/seed % | Generation | $2n$ | Seed set % | Generation | $2n$ | Seed set % |
| <i>H. salicifolius</i> × HA 410 | 5.13 | 34 | 1.31 ^b | $BC_1F_1^c$ | 34–36 | 9.6 | BC_1F_1 | 34–36 | 9.6 |
| <i>H. salicifolius</i> × HA 410 ^d | – | 34 | 3.53 ^b | BC_1F_1 | 34 | 66.2 | BC_1F_1 | 34 | 66.2 |
| <i>H. occidentalis</i> × HA 410 | 17.93 | 34 | 0.62 ^b | $BC_1F_1^c$ | 34, 35 | 7.1 | BC_1F_1 | 34, 35 | 7.1 |
| <i>H. occidentalis</i> × HA 410 ^d | – | 34 | 11.09 ^b | BC_1F_1 | 34 | 68.3 | BC_1F_1 | 34 | 68.3 |
| <i>H. hirsutus</i> × HA 451 | 14.13 | 51 | 0.65 ^b | $BC_3F_1/BC_2F_2^c$ | 34–37 | 28.3 | – | – | – |
| <i>H. hirsutus</i> × HA 451 ^d | – | 51 | 0.59 ^b | BC_1F_1 | 34 | 73.2 | BC_1F_1 | 34 | 73.2 |
| <i>H. californicus</i> × HA 410 | 32.39 | 51 | 2.7 | BC_4F_1 | 34–40 | 35.3 | BC_4F_3 | 34–37 | 39.6 |
| Amp <i>H. grosseserratus</i> /P21 ($2n = 68$) × HA 410 | – | 51 | 9.1 | BC_2F_1 | 34–38 | 59.1 | BC_2F_2/BC_3F_1 | 34–35 | 67.9 |
| Amp <i>H. nuttallii</i> 730/P21 ($2n = 68$) × HA 410 | – | 51 | 1.1 | BC_2F_1 | 34–37 | 50.7 | BC_2F_2/BC_3F_1 | 34–36 | 35.9 |
| Amp <i>H. strumosus</i> /P 21 ($2n = 102$) × HA 410 | – | 68 | 19.7 | BC_2F_1 | 34–41 | 78.2 | BC_2F_2/BC_3F_1 | 34–36 | 62.1 |

Notes: ^aThe plants with $2n = 34$ chromosomes were selected for seed increase for field evaluation for Sclerotinia stalk or head rot. ^bThe percentage of embryos/florets in the BC_1 generation. ^cThe first generation with $2n = 34$ plants observed when the F_1 s were used as the female parents in backcrossing with HA 410 or HA 451. ^dThe generation with the most $2n = 34$ plants observed when the F_1 s were used as the male parents in backcrossing with NMS HA 89.

observed in the BC_2F_1 generation, and the majority of families with $2n = 34$ observed in the BC_2F_2 and BC_3F_1 generations. Useful families adequate for breeding would require one or two more generations of self-pollination when reaching BC_2F_3 or BC_3F_3 . Seed set of this group does not present any problem from either further backcrosses or selfing.

Crosses involving hexaploid Amps were represented by Amp *H. strumosus*/P21 \times HA 410. The speed of advancement of this group is similar to that of the tetraploid Amps, with usable materials available at BC_2F_3 or BC_3F_3 . As for the tetraploid Amps, plants of this group are expected to have adequate backcross and selfing seed set after identification of $2n = 34$ plants.

Discussion

Even though the *Or*₅ gene was effective against broomrape race E for up to 20 years, the fast pace of new and more virulent races being identified since the mid-1990s and the lack of sufficient resistance in sunflower hybrids presents a major challenge to the international sunflower community threatening its survival as a major global oil seed crop. Wild *Helianthus* species possess valuable genes to increase the genetic diversity of cultivated sunflower by continually providing genes resistant to diseases and pests. In our ultimate war against broomrape, the high resistance in perennial wild *Helianthus* species has been established, with occasional discovery of resistance in cultivated germplasm and in wild annual species. The main question today is how to transfer the resistance genes into cultivated lines for easy access and utilization by sunflower breeders.

A special project “Interspecific gene transfer for resistance to sunflower Sclerotinia utilizing wild *Helianthus* species” funded by the National Sclerotinia Initiatives has demonstrated good progress utilizing the various perennial *Helianthus* species and interspecific Amps since 2005. Since the nature of Sclerotinia resistance involves quantitative genes with mostly additive effects, and screening for resistance in the F_1 and BC_1F_1 generations is impossible, early backcross progeny families were produced and the screening conducted in the field trials with artificial inoculation. For a proactive breeding program for broomrape resistance, similar progeny families could be produced and screened against the new broomrape races, after resistance confirmation of the contributing wild species. In addition, early generation families produced from the Sclerotinia project could also be used for the broomrape resistance, as well as for resistance genes for numerous sunflower diseases, and other agronomic characteristics.

However, interspecific hybridization between perennial *Helianthus* species and cultivated sunflower has always been difficult or nearly impossible. This problem was solved with the development of embryo rescue techniques in the 1980s. Embryo rescue can also be used for the backcrosses whenever needed. For the crosses involving the tetraploid and hexaploid *Helianthus* species, and the interspecific Amps, the $2n = 51$ generation has been the most problematic for backcross seed set with additional backcrosses required to reduce the chromosome number to $2n = 34$, the same as cultivated sunflower. Valuable genetic variability from the wild species parents will be further reduced after each additional backcross. Efforts to shorten this process by using the $2n = 51$ plants as the pollen parents were shown to be effective, which resulted in all $2n = 34$ progeny plants in BC_1F_1 for diploids *H. salicifolius* and *H. occidentalis* and tetraploid *H. hirsutus*. Similar rapid reduction of extra chromosomes is expected for other wild tetraploids and hexaploids by utilizing $2n = 51$ generation plants as the pollinator in further backcrosses. The same rule should apply when using tetraploid interspecific amphiploids and the hexaploid interspecific Amps. This is a time savings of two backcross generations when using the polyploid wild species parents and the interspecific amphiploids. This rapid reduction of chromosome number when using $2n = 51$ plants as the pollen parents in backcrossing is expected to have a much stronger selection pressure against male gametes with extra chromosomes other than the normal $n = 17$, since this selection pressure is generally known to be less for the female gametes.

The recent spread of broomrape in China is seen as a good example of broomrape race evolution from continuous planting of sunflower without rotation, the importation of contaminated hybrid seeds, and the gradual introduction of resistant hybrids. Highly virulent broomrape biotypes have started to appear and rapidly spread over areas of the main sunflower production regions. The migration of highly virulent broomrape biotypes to areas with less virulent biotypes is of great concern and needs to be closely monitored, and breeding strategies need to be considered. The appearance of the virulent broomrape races in China could be the result of selection or from the imported seeds from broomrape-infected areas. The breeding strategy is critical for areas like China where a mixture of all the races exists. A single gene resistance to all races is likely to lead to even more virulent races, and the competition between the broomrape race shift and resistance hybrids will never end. We were lucky that the Or_5 gene was able to keep broomrape under control for over 20 years, but looking at the rapid race shifts in recent years, this may not be the case in the future.

In conclusion, optimistically we consider broomrape is just one of the major sunflower pests or diseases that can be controlled by major genes. The resistance genes exist in abundance in wild species, especially the perennials. Utilization of valuable perennial *Helianthus* species for resistance genes has been greatly improved with the development of embryo rescue and the subsequent improvement in backcross seed set. The breeding cycle for producing selectable progeny families has been shortened by reciprocal backcrossing using $2n=51$ plants as the pollen parents when starting from polyploid *Helianthus* species. Interspecific Amps have been produced for immediate selection and use in the following generations while monitoring broomrape resistance. Progeny families from the Sclerotinia project could also be used for immediate selection for resistance after the resistance confirmation of their wild species parents. With our new approach, in most cases when starting with resistant wild *Helianthus* species, the time required to identify resistant backcross progeny with reasonable self-compatibility will be at BC_1F_1 for the diploid wild and the tetraploid wild and interspecific Amps, and BC_2F_1 for the wild hexaploids and the interspecific Amps. Breeding for broomrape resistance is a continual challenge. Since we have a large pool of resistance genes and good tools to engage the battle against broomrape, the only other tool needed will be a good breeding strategy and international cooperation.

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