Triploid Production from Interspecific Crosses of Two Diploid Perennial Helianthus with Diploid Cultivated Sunflower (Helianthus annuus L.)

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ABSTRACT Wild Helianthus species are a valuable genetic resource for the improvement of cultivated sunflower. We report the discovery and characterization of a unique high frequency production of triploids when cultivated sunflower was pollinated by specific accessions of diploid Helianthus nuttallii T. & G. and H. maximiliani Schr. Genomic in situ hybridization (GISH) analyses indicated that the triploid F1s had two genomes from the wild pollen sources and one from the cultivated line. Mitotic chromosome analyses indicated that the frequency of triploid progenies from the crosses of cultivated lines × H. nuttallii accession 102 (N102) was significantly higher than those of unexpected polyploid progenies from the crosses of wild perennial species × N102, and no unexpected polyploids were obtained from the reverse crosses. Pollen stainability analysis suggested the existence of a low percentage of unreduced (2n) male gametes in some accessions, especially N102 and H. maximiliani accession 1113 (M1113), which were generated at the telophase II and tetrad stages of meiosis. The triploid F1s could be the results of preferred fertilization of the low frequency of 2n male gametes with the female gametes of the cultivated sunflower, due to the dosage factors related to recognition and rejection of foreign pollen during fertilization. The triploids have been used to produce amphiploids and aneuploids. Future studies of the male gametes’ fate from pollination through fertilization will further uncover the mechanism of this whole genome transmission. Studies of the genetic control of this trait will facilitate research on sunflower polyploidy speciation and evolution, and the utilization of this trait in sunflower breeding.

KEYWORDS Helianthus triploids genomic in situ hybridization (GISH) mitotic analysis meiotic analysis

Polyploidy is the presence of two or more complete sets of chromosomes from a single species (autopolyploidy) or two or more species (allopolyploidy) in a single organism (Matsushita et al. 2012; Mason and Pires 2015). Hybridization and polyploidization are important mechanisms for diversification and speciation during evolution (Storme and Mason 2014). Phylogenetic studies and comparative genome analyses confirmed that most flowering plants have undergone one or more rounds of ancient polyploidy early in their evolutionary history, such as two recent whole genome duplications (named α and β) within the crucifer (Brassicaceae) lineage and one common genome triplication event (γ) within all core eudicots (Jaillon et al. 2007; Jiao et al. 2011). Up to 70% of angiosperm species are secondary polyploids, which are believed to arise commonly through the production of unreduced gametes (2n gametes) resulting from meiotic and premeiotic defects (Brownfield and Köhler 2011; Storme and Mason 2014).

Unreduced gametes are commonly produced by interspecific hybrids, and can also be induced by abiotic and biotic stresses (Mason and Pires 2015). In a comparison of 11 hybrid to 11 nonhybrid angiosperm
species, diploid F1 hybrids produced 27.52% 2n gametes, and the non-hybrids only 0.56%. It was also noted that higher frequencies of polyploidy occurred in perennial taxa capable of vegetative reproduction, and plant families lacking endosperm in mature seeds, such as the Asteraceae, Crassulaceae, Onagraceae, Rosaceae, and Salicaceae (Ramsey and Schemske 1998). Triploidy can result either from the fusion of a 2n gamete to a regular reduced gamete (n), with both produced by diploid individuals, or from crosses between diploid and tetraploid individuals. The evolution of polyploids was more likely through a triploid bridge than through other mechanisms (Mason et al. 2011). Triploids also could act as vectors for gene flow between diploid and tetraploid populations (Henry et al. 2005).

The genus Helianthus includes 53 species, i.e., 39 perennials and 14 annuals maintained at the USDA-ARS, North Central Regional Plant Introduction Station (NCRPIS) in Ames, IA (Seiler and Jan 2014). The 14 annual species are diploid (2n = 2x = 34), and the 39 perennial species include 26 diploid, three tetraploid (2n = 4x = 68), seven hexaploid (2n = 6x = 102), and one mix-ploid of either diploid or tetraploid, and two mix-ploids of tetraploid or hexaploid. A large number of sunflower interspecific hybrids have been evaluated since the pioneering work of Heiser and his students in the 1940s (Jan 1997). Initial interspecific hybridizations were among wild annual Helianthus species and their crosses with cultivated sunflower. Interspecific hybrids involving perennial species followed the establishment of embryo rescue techniques (Chandler and Beard 1983). No unusually abnormal cytological observations have been reported in wild Helianthus species, but abnormal meiosis is the norm in almost all the interspecific F1 progenies, especially between wild species and the cultivated sunflower. However, a limited number of unreduced gametes can frequently be observed, which could be the driving force of sunflower polyploidization, speciation, and evolution.

Several tetraploid interspecific amphiploids were produced by the authors via colchicine treatment of the F1 hybrids followed by intercrossing of heads containing a high frequency of larger pollen grains, assumed to be chromosomally doubled, compared to the smaller pollen grains associated with a haploid set of chromosomes. As a general rule, large pollen grains of interspecific hybrids indicated unreduced gametes which have the potential of producing a low frequency of interspecific amphiploids via self-pollination without colchicine treatment. This has been confirmed in our lab by backcrossing several interspecific hybrids with cultivated sunflower pollen without emasculation, resulting in progenies having chromosome numbers of 2n = 4x = 68 or 2n = 2x + x = 51 for

| Accession No. | Plant ID | Species | Ploidy | Collection Place | Year |
|---------------|----------|---------|--------|------------------|------|
| 102           | N102     | H. nuttallii | 2x     | Morden, MB, Canada | 2003 |
| 314           | N314     | H. nuttallii | 2x     | Morden, MB, Canada | 2005 |
| 622           | N622     | H. nuttallii | 2x     | Morden, MB, Canada | 2001 |
| PI 650025     | NUT-RYD-2 | H. nuttallii | 2x     | Kindred, ND       | 1993 |
| 1116          | N1116    | H. nuttallii | 2x     | Morden, MB, Canada | 2005 |
| 1113          | M1113    | H. maximiliani | 2x | Morden, MB, Canada | 2005 |
| PI 435753     | MOL-2    | H. mollis    | 2x     | Okmulgee, OK      | 1984 |
| PI 650013     | MOL A 3201 | H. mollis    | 2x     | Chadds Ford, PA   | 1984 |
| Ames 30356    | G10/1163 | H. silphioides | 2x | Brandsville, MO   | 2009 |
| PI 503209     | G11/1301-10 | H. divaricatus | 2x | New Castle, VA    | 1985 |
| PI 503216     | G11/1320-39 | H. divaricatus | 2x | Kingston, NY      | 1985 |
| Ames 30340    | G10/1120-30 | H. salicifolius | 2x | Garnett, KS       | 2009 |
| Ames 30348    | G10/1131-37 | H. salicifolius | 2x | Ponca City, OK    | 2009 |
| PI 547177    | G07/15   | H. giganteus | 2x     | Odanah, WI        | 1989 |
| PI 613793    | G07/25-27 | H. grosseserratus | 2x | Onawa, IA        | 1999 |
| PI 547174    | G10/1138-46 | H. hirsutus   | 4x     | Beeche City, IL   | 1989 |
| PI 435648    | CIL29-3  | H. ciliaris    | 6x     | Adrian, TX        | 1976 |
| PI 650079    | RES28382 | H. resinosus  | 6x     | Statesville, AL   | 2006 |
| PI 650082    | RES28386 | H. resinosus  | 6x     | Summit, MS        | 2006 |
| PI 435707    | LAC28-2  | H. lacinatus   | 2x     | San Lorenzo, TX   | 1977 |
| 405          | N405     | H. nuttallii  | 2x     | Morden, MB, Canada | 2005 |
| 187          | N817     | H. nuttallii  | 2x     | Morden, MB, Canada | 2005 |
| 1408         | N1408    | H. nuttallii  | 2x     | Morden, MB, Canada | 2005 |
| 902          | N902     | H. nuttallii  | 2x     | Morden, MB, Canada | 2005 |
| 903          | N903     | H. nuttallii  | 2x     | Morden, MB, Canada | 2005 |
| 412          | N412     | H. nuttallii  | 2x     | Morden, MB, Canada | 2005 |
| PI 435860    | PUM24-1  | H. pumilus    | 2x     | Boulder, CO       | 1977 |
| PI 435860    | PUM24B   | H. pumilus    | 2x     | Boulder, CO       | 1977 |
| 424          | N424     | H. nuttallii  | 2x     | Morden, MB, Canada | 2005 |
| 609          | M609     | H. maximiliani | 2x | Morden, MB, Canada | 2005 |
| 1008         | N1008    | H. nuttallii  | 2x     | Morden, MB, Canada | 2005 |
| 1324         | N1324    | H. nuttallii  | 2x     | Morden, MB, Canada | 2005 |
| 214          | M214     | H. maximiliani | 2x | Morden, MB, Canada | 2005 |
| 513          | M513     | H. maximiliani | 2x | Morden, MB, Canada | 2005 |
| 1018         | M1018    | H. maximiliani | 2x | Morden, MB, Canada | 2001 |
| 1314         | M1314    | H. maximiliani | 2x | Morden, MB, Canada | 2005 |
| 1323         | M1323    | H. maximiliani | 2x | Morden, MB, Canada | 2005 |
| 1418         | M1418    | H. maximiliani | 2x | Morden, MB, Canada | 2005 |

ID, identifier.
diploid hybrids, $2n = 6x = 102$ or $2n = 4x = 68$ for triploid hybrids, or $2n = 8x = 136$ or $2n = 5x = 85$ for hexaploid hybrids, suggesting self-pollination of the unreduced male to female gametes or unreduced female gametes pollinated by the haploid cultivated pollen.

A group of accessions of diploid perennial *Helianthus nuttallii* and *H. maximiliani* collected in the vicinity of Morden, Manitoba, Canada with resistance to *Sclerotinia sclerotiorum* (Lib.) de Bary head rot were pollinated to a cultivated sunflower line to transfer the resistance genes (Jan et al. 2007). An unusually high frequency of triploids in the F1 progeny was observed. This phenomenon had never been observed in diploid·diploid crosses involving wild perennial *Helianthus* and cultivated lines. These wild accessions were further confirmed to be diploid and had $2n = 2x = 34$ chromosomes. However, as expected for diploid·diploid crosses, all F1 progeny were diploid when the wild accessions were used as the female parents. The clear-cut reciprocal differences of producing F1 progeny at $2n = 3x$ and $2n = 2x$ pointed to the normal megasporogenesis of the wild accessions and the likely abnormal microsporogenesis of the male gametes and/or abnormal fertilization process. This study focuses on the confirmation of this novel discovery with additional interspecific crosses, GISH verification of F1 chromosome constituents, and an examination of the microsporogenesis of the wild accession that was most effective in producing triploid F1 progenies, N102.

### MATERIALS AND METHODS

#### Plant materials

The wild species used in this study included 10 diploid species ($2n = 2x = 34$); *H. nuttallii* T. & G., *H. maximiliani* Schr., *H. mollis* Lam., *H. silphioides* Nutt., *H. divaricatus* L., *H. salicifolius* Dietr., *H. giganteus* L., *H. grosseserratus* Mar, *H. laciniatus* A. Gray, and *H. pumilus* Nutt; one tetraploid species ($2n = 4x = 68$) *H. hirsutus* Raf.; and two hexaploid species ($2n = 6x = 102$) *H. ciliaris* DC., and *H. resinosus* Small (Table 1). The cultivated sunflower parents included nuclear male-sterile (NMS), cytoplasmic male-sterile (CMS), and male-fertile (MF) maintainer and restoration lines; NMS HA 89-552, NMS P21, CMS HA 821, HA 89, P21, HA 821, HA 410, and RHA 274. All were publicly released by USDA. HA 89, HA 821, and HA 410 are oilseed maintainer lines, and

### Table 2 Number of diploid and triploid F1 progenies from *H. nuttallii* (N) and *H. maximiliani* (M) crossed with cultivated sunflower line NMS HA 89-552

| Crosses       | 2x (2n = 34) | 3x (2n = 51) | Triploids % | Total |
|---------------|--------------|--------------|-------------|-------|
| NMS HA 89-552/N102 | 1            | 28           | 96.55       | 29    |
| NMS HA 89-552/N314 | 0            | 2            | 100.00      | 2     |
| NMS HA 89-552/N412 | 0            | 1            | 100.00      | 1     |
| NMS HA 89-552/N1324 | 4            | 10           | 71.43       | 14    |
| NMS HA 89-552/M1113 | 2            | 7            | 77.78       | 9     |
| NMS HA 89-552/M1323 | 2            | 1            | 33.33       | 3     |
| NMS HA 89-552/M1418 | 19           | 2            | 9.52        | 21    |
| Subtotal      | 28           | 51           | 64.56       | 79    |
| NMS HA 89-552/N1008 | 5            | 0            | 0.00        | 5     |
| NMS HA 89-552/M1018 | 2            | 0            | 0.00        | 2     |
| NMS HA 89-552/M513 | 1            | 0            | 0.00        | 1     |
| NMS HA 89-552/M1314 | 5            | 0            | 0.00        | 5     |
| NMS HA 89-552/M214 | 5            | 0            | 0.00        | 5     |
| NMS HA 89-552/N405 | 2            | 0            | 0.00        | 2     |
| NMS HA 89-552/N817 | 1            | 0            | 0.00        | 1     |
| NMS HA 89-552/N1408 | 5            | 0            | 0.00        | 5     |
| Subtotal      | 26           | 0            | 0.00        | 26    |
| Total         | 54           | 51           | 48.57       | 105   |

NMS, nuclear male-sterile.

### Table 3 Numbers of diploid or triploid F1 progenies from the crosses between *H. nuttallii* acc. 102 (N102) and cultivated sunflower lines

| Crosses         | Floret Number | 2x (2n = 34) | 3x (2n = 51) | Triploids % | Total Plants with 2n | Success %* |
|-----------------|---------------|--------------|--------------|-------------|----------------------|------------|
| NMS HA 89-552/N102 | 11,830       | 4            | 94           | 95.92       | 98                   | 0.83       |
| NMS P21/N102     | 8,450         | 0            | 22           | 100.00      | 22                   | 0.26       |
| CMS HA 821/N102  | 7,750         | 0            | 5            | 100.00      | 5                    | 0.06       |
| Subtotal         | 28,030        | 4            | 121          | 96.80       | 125                  | 0.45       |
| HA 89/N102       | 11,560        | 9            | 28           | 75.68       | 37                   | 0.32       |
| RHA 274/N102     | 9,150         | 0            | 16           | 100.00      | 16                   | 0.17       |
| HA 821/N102      | 5,900         | 0            | 2            | 100.00      | 2                    | 0.03       |
| Subtotal         | 26,610        | 9            | 46           | 83.64       | 55                   | 0.21       |
| Total            | 54,640        | 13           | 167          | 92.78       | 180                  | 0.33       |
| N102/HA 89       | 8,140         | 17           | 0            | 0.00        | 17                   | 0.21       |
| N102/P21         | 2,060         | 28           | 0            | 0.00        | 28                   | 1.36       |
| N102/HA 821      | 5,020         | 7            | 0            | 0.00        | 7                    | 0.00       |
| N102/HA 410      | 8,090         | 18           | 0            | 0.00        | 18                   | 0.22       |
| Subtotal         | 23,310        | 70           | 0            | 0.00        | 70                   | 0.30       |

NMS, nuclear male-sterile; CMS, cytoplasmic male-sterile.

*Success % = total plants with 2n / floret number x 100.
Interspecific crossing and embryo rescue

Interspecific crosses of 15 accessions of *H. nuttallii* and *H. maximiliani* with cultivated sunflower were made in the greenhouse in 2006–2013 with the primary goal of introgressing *Sclerotinia* resistance (Table 2). The expanded reciprocal crosses between *H. nuttallii* N102 and other cultivated lines were made in the greenhouse in 2014 (Table 3). The reciprocal crosses of N102 with additional wild species accessions were made in the greenhouse in 2014 using N102 as male or female parents (Table 4 and Table 5). The crosses between perennial *H. divaricatus*, *H. salicifolius*, *H. giganteus*, *H. grosseserratus*, and *H. hirsutus*, and cultivated sunflower lines NMS HA 89-552 and HA 410, were made in the greenhouse in 2006–2012 (Table 6). MF female parents were emasculated 2–3 days before pollination, with embryos of all the crosses rescued 6–8 d after pollination. The apical meristems of the F1 progeny were treated with 0.15% colchicine at pH 5.4 for 5 hr in the dark for chromosome doubling following Jan and Chandler (1989).

Mitotic chromosome counts and GISH

Chromosome numbers in root tip cells were determined for individual plants using the standard Feulgen staining method. Chromosome squashes were made following the method of Liu et al. (2007) with minor modifications. F1 plants with $2n = 2x = 34$ and $2n = 3x = 51$ derived from different crosses were used for GISH analysis, according to Liu et al. (2013). Briefly, the root tips were digested at 37°C for 2.5 hr in an enzyme mixture consisting of 2% cellulase (Sigma, St. Louis, MO) and 24% pectinase (Sigma) in 10 mM sodium citrate buffer (4 mM citric acid and 6 mM sodium citrate). The treated root tips were squashed in 45% acetic acid. Cover slips were removed from the slides after being frozen over liquid nitrogen for 5 min.

Genomic DNA of wild *Helianthus* species was used as a probe after being sheared in boiling water for 10 min and labeled with digoxigenin-11-dUTP using the nick translation method according to the manufacturer’s instructions (Roche Applied Science, Nutley, NJ). Genomic DNA of HA 89 was used as a blocking DNA after shearing in boiling water for 20 min and placed on ice for 5 min, with the ratio of blocking DNA to probe DNA of 50:1. Probe detection, image capture, and analysis followed Liu et al. (2013).

Meiotic and mitotic division and pollen fertility analysis

Heads used for meiosis were collected and fixed in Carnoy’s fixative with 95% ethanol: chloroform: glacial acetic acid (6:3:1) at 4°C for 24 hr, then rinsed several times with 70% ethanol and stored in 70% ethanol at 4°C for analysis. The anthers at the appropriate developmental stage were placed on slides; pollen mother cells were squeezed out of the anther tissue and stained with 1% aceto-carmine. The pollen mother cells at different stages were analyzed using an Axioplan2 Imaging microscope (Zeiss, Germany). The pollen from undehisced anthers and open florets were stained with 1% aceto-carmine for pollen mitotic analysis at different stages of development.

| Table 4: Mitotic chromosome numbers of F1 progenies from the crosses of nine wild *Helianthus* accessions × *H. nuttallii* N102 |
|------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|------------------|-------------------|
| Crosses                | Floret Number | $2n = 32$ | $2n = 33$ | $2n = 34$ | $2n = 36$ | $2n = 51$ | $2n = 68$ | $2n = 85$ | Unexpected Polyploids % | Total Plants with $2n$ | Success % |
| N314/N102              | 440        | 0         | 133       | 0         | 1         | 0         | 133       | 0.00          | 30.23             |
| N622/N102              | 785        | 0         | 111       | 1         | 0         | 0         | 112       | 0.89          | 14.27             |
| PI 650025/N102         | 595        | 0         | 85        | 1         | 0         | 0         | 86        | 1.16          | 14.45             |
| M1113/N102             | 4,585      | 3         | 119       | 0         | 0         | 0         | 122       | 0.00          | 18.21             |
| PI 435753/N102         | 565        | 0         | 90        | 0         | 0         | 0         | 9         | 1.59          |                   |
| N1116/N102             | 540        | 0         | 88        | 1         | 0         | 0         | 89        | 1.12          | 16.48             |
| Ames 30356/N102        | 1,100      | 3         | 656       | 3         | 2         | 1         | 662       | 0.45          | 8.09              |
| PI 547174/N102         | 3,070      | 86        | 2         | 32        | 1         | 0         | 33        | 2.27          | 8.27              |
| PI 435648/N102         | 1,520      | 1         | 2         | 149       | 2         | 0         | 154       | 0.77          | 3.17              |
| Overall                | 12,770     | 3         | 656       | 89        | 34        | 1         | 783       | 6.13          |                   |

Success % = total plants with $2n /$ floret number × 100.

| Table 5: Mitotic chromosome numbers of F1 progenies from the crosses of *H. nuttallii* N102 × seven wild *Helianthus* accessions, with N314/M1113 as a control |
|------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|------------------|-------------------|
| Crosses                | Floret Number | $2n = 32$ | $2n = 33$ | $2n = 34$ | $2n = 36$ | $2n = 51$ | $2n = 68$ | $2n = 85$ | Normal Plants % | Total Plants with $2n$ | Success % |
| N102/N314              | 290        | 1         | 2         | 149       | 2         | 0         | 96.75     | 154            | 53.10             |
| N102/N622              | 450        | 0         | 20        | 34        | 0         | 0         | 100.00    | 20             | 4.44              |
| N102/PI 650025         | 150        | 0         | 34        | 0         | 0         | 0         | 100.00    | 34             | 22.67             |
| N102/M1113             | 310        | 0         | 127       | 0         | 0         | 0         | 100.00    | 127            | 40.97             |
| N102/PI 435753         | 1050       | 0         | 42        | 0         | 0         | 0         | 97.67     | 43             | 4.10              |
| Total                  | 2250       | 1         | 3         | 372       | 2         | 0         | 98.41     | 378            | 16.80             |
| N102/PI 547174         | 980        | 0         | 8         | 0         | 100.00    | 8         | 0.82      | 3              | 1.58              |
| N102/PI 435648         | 190        | 0         | 3         | 0         | 100.00    | 3         | 0.94      | 11             | 0.94              |
| Total                  | 1170       | 0         | 8         | 3         | 100.00    | 11        | 0.94      | 112            | 32.94             |

Success % = total plants with $2n /$ floret number × 100.

RHA 274 is an oilseed restorer line. NMS HA 89-552 is an induced NMS mutant of inbred maintainer line HA 89 (Jan and Rutger 1988), and NMS P21 is a NMS mutant selected from open-pollinated variety Peredovik.
The pollen fertility of N102, M1113, and 20 other perennial *Helianthus* accessions, cultivated HA 89, and the F1 plants was determined by pollen stainability using an Axioplan2 Imaging microscope. Pollen staining followed Alexander’s method (Alexander 1969). About 20 fields with a total of ~1500–2000 pollen grains were observed for the *Helianthus* accessions and HA 89. Pollen grains were divided into four categories: large fertile (LF), large sterile (LS), small fertile (SF), and small sterile (SS). The SF pollen had a reduced chromosome number. For the F1s, five fields with a total of 300–500 pollen grains were analyzed. The percentage of each category was used for analysis. The pollen diameter was measured using Axiovision 4.8 software after the images were captured by a charge-coupled device camera (Zeiss Axiocam HRM).

DNA extraction and PCR analysis

Genomic DNA was extracted according to the protocol of the QIAGEN DNAeasy 96 Plant Kit (QIAGEN, Valencia, CA). To identify the F1 plants \(2n = 4x = 68\) derived from *H. hirsutus* and *H. nuttallii* N102, six selected SSR markers mapped to the sunflower linkage groups from the Compositae database (http://compositdb.ucdavis.edu) were used for polymorphism screening between the parents, and the polymorphic primers were used for F1 genotyping. The PCR amplification and genotyping followed Liu et al. (2012).

Backcrossing of diploid F1s and the production of amphiploids and aneuploids

Diploid F1s of NMS HA 89-552 crossed with *H. nuttallii* N102, N1008, and N1323, and *H. maximiliani* M1018, M1113, M1314, M1323, and M1418 were pollinated by cultivated sunflower HA 441 in 2006. The chromosomally-doubled heads of diploid F1s derived from *H. nuttallii* N412 and N1324, and *H. maximiliani* M1113, M1314, and M1323, were crossed with the triploid F1s derived from *H. nuttallii* N102 and N1324, and *H. maximiliani* M1113 and M1323 within the same species in 2006. The triploids from *H. nuttallii* N102, N314, and N1324 and *H. maximiliani* M1018, M1113, M1323, and M1418 were also used as the female parents crossed with HA 441 in 2006.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

**RESULTS**

Triплоид hybrids obtained from diploid cultivated sunflower pollinated by diploid wild perennial species

*H. nuttallii* N102, N314, N405, N412, N817, N1008, N1324, and N1408, and *H. maximiliani* M214, M513, M1018, M1113, M1314, M1323, and M1418 \(2n = 2x = 34\) were crossed to cultivated NMS HA 89-552 \(2n = 2x = 34\) with the aim of producing amphiploids for improving the backcross seed set of the F1s (Table 2). The embryos were rescued and the F1 progeny seedlings were treated with colchicine. Then, the pollen stainability of 10 heads of each F1 plant was examined. LF pollen was predominant on heads of 28 of the 29 F1s derived from NMS HA 89-552/N102 (96.55%, Figure 1). This nearly 100% chromosome doubling is in contrast to the small number normally observed for colchicine-treated F1s in our prior experiments. In addition, all the heads of seven out of nine F1s derived from the cross of NMS HA 89-552/*H. maximiliani* M1113 also had a high percentage of LF pollen. High frequencies of large pollen grains were also observed in some F1s derived from other materials tested without colchicine treatment, which suggested that the high percentage of LF pollen was not the result of colchicine treatment. The subsequent mitotic chromosome analysis for the F1 progenies demonstrated that 51 plants had \(2n = 3x = 51\) (48.57%) and 54 plants had \(2n = 2x = 34\) (51.43%). Triploid F1s were detected from the crosses involving seven *H. nuttallii* and *H. maximiliani* accessions, with a high frequency of 64.56% (plant number \(n = 79\), but not detected for 26 F1 plants from eight other accessions (Table 2). Among the accessions, *H. nuttallii* N102 and *H. maximiliani* M1113 produced the highest frequency of triploid F1s. Since the corresponding wild accessions were confirmed to be diploid with \(2n = 2x = 34\) (Figure 2A for N102), we used GISH analysis to confirm the chromosome constituents of the \(2n = 3x = 51\) F1s.

**GISH analyses of F1s derived from interspecific crosses involving *H. nuttallii* and *H. maximiliani***

Using the genomic DNA of N102 and M1113 as the probe DNA, respectively, and HA 89 as a blocking DNA, the F1 individuals with \(2n = 3x = 51\) were GISH analyzed. The F1s derived from *H. nuttallii* N102 and M1113, and *H. maximiliani* M1018, M1113, and M1323 were rescued and the F1 progeny seedlings were treated with colchicine. Then, the pollen stainability of 10 heads of each F1 plant was examined. LF pollen was predominant on heads of 28 of the 29 F1s derived from NMS HA 89-552/N102 (96.55%, Figure 1). This nearly 100% chromosome doubling is in contrast to the small number normally observed for colchicine-treated F1s in our prior experiments. In addition, all the heads of seven out of nine F1s derived from the cross of NMS HA 89-552/*H. maximiliani* M1113 also had a high percentage of LF pollen. High frequencies of large pollen grains were also observed in some F1s derived from other materials tested without colchicine treatment, which suggested that the high percentage of LF pollen was not the result of colchicine treatment. The subsequent mitotic chromosome analysis for the F1 progenies demonstrated that 51 plants had \(2n = 3x = 51\) (48.57%) and 54 plants had \(2n = 2x = 34\) (51.43%). Triploid F1s were detected from the crosses involving seven *H. nuttallii* and *H. maximiliani* accessions, with a high frequency of 64.56% (plant number \(n = 79\), but not detected for 26 F1 plants from eight other accessions (Table 2). Among the accessions, *H. nuttallii* N102 and *H. maximiliani* M1113 produced the highest frequency of triploid F1s. Since the corresponding wild accessions were confirmed to be diploid with \(2n = 2x = 34\) (Figure 2A for N102), we used GISH analysis to confirm the chromosome constituents of the \(2n = 3x = 51\) F1s.

**Figure 1** Pollen stainability of F1s with \(2n = 3x = 51\) (A) and \(2n = 2x = 34\) (B) derived from the cross of nuclear male-sterile (NMS) HA 89-552/*H. nuttallii* N102. The dark or black pollen grains are fertile, and the light or gray ones are sterile. Bars = 50 μm.
3x = 51 and 2n = 2x = 34 were analyzed by the GISH technique for genome compositions (Figure 3). The results clearly showed that, among the 51 chromosomes of the triploid F1s, 34 chromosomes had signals of the two wild *Helianthus* species (red) and the remaining 17 chromosomes had no probe signals (blue) (Figure 3, B and D). In comparison, only 17 chromosomes were stained by the two probes in the diploid F1 individuals with 2n = 34 (Figure 3, A and C). Therefore, GISH analysis indicated that the triploids arose from an excess paternal genome contribution.

**Triploid F1 hybrids not related to the male sterility of female parents**

To determine if the abnormally high frequency of triploids was due to the use of the male-sterile line as the female parent in the interspecific hybridizations, N102 pollen was applied to two NMS lines (NMS HA 89-552 and NMS P21), one CMS line (CMS HA 821), and three MF lines (HA 89, RHA 274, and HA 821) (Table 3). A total of 180 F1 plants were obtained from 54,640 florets (0.33%) using embryo rescue. For NMS HA 89-552/N102, 95.92% of F1 plants were triploids (Figure 2B) (n = 98), with only four normal diploids (Figure 2C). The triploid and diploid plants looked similar, except that the triploids appeared more vigorous at the seedling stage, were more branched as adults, produced higher amounts of pollen, and had higher pollen stainability (Figure 2, D–G).

In addition, all 27 F1s derived from NMS P21/N102 and CMS HA 821/N102 were triploids. The overall percentage of triploids obtained from the MS female parents × N102 was 96.80% (n = 125). For the MF female parents × N102, a lower percentage of triploids was obtained from the cross HA 89/N102 (75.68%, n = 37), and all 18F1s derived from RHA 274/N102 and HA 821/N102 were triploids. The overall number of triploids obtained from the MF female parents × N102 was 83.64% (n = 55). In summary, 92.78% of the F1s derived from the both MF and MS female parents were triploids (n = 180), and triploid F1 hybrid production was not related to the male sterility of female parents.

Only normal ploidy levels were observed when N102 was pollinated with cultivated sunflower

To examine whether abnormal triploids would be obtained from crosses when N102 was used as the female parent, N102 was pollinated with pollen from four cultivated sunflower lines (HA 89, P21, HA 821, and HA 410). Seventy F1 plants from 23,310 florets were obtained for mitotic chromosome counts after embryo rescue (0.30%). However, all the F1s had 2n = 2x = 34, which was in stark contrast to the results obtained when N102 was used as the male parent. The results suggested that the female megasporogenesis of N102 was normal, which led us to suspect the possible abnormal pollen meiosis, pollen mitotic division, pollen fertility, or fertilization process of N102.

**Pollen fertility and grain size of wild *Helianthus* accessions**

Pollen fertility was analyzed for 22 wild *Helianthus* accessions including N102 and M1113, with HA 89 as a control (Figure 4 and Supplemental Material, File S1, Table S1). For N102, 95.18% of the pollen was SF, with 2.80% SS (n = 1640, Figure 5A). The total large pollen was 2.01%. The total of LF and LS grains for M1113 was 0.85%, slightly lower than that of N102, with 90.89% SF, 0.52% LF, 0.33% LS, and 8.26% SS (n = 1537). Nine other *Helianthus* accessions had low frequencies of LF grains (range = 0.05–0.46%), with or without LS grains. However, four other *Helianthus* accessions only contained a
low percentage of LS grains (range = 0.04–0.13%). The average LF and LS grains for the 22 wild *Helianthus* accessions were 0.15 and 0.10%, respectively. In comparison, N102 and M1113 combined produce over 10 times more large pollen grains than the other *Helianthus* accessions. For comparison, HA 89 did not produce any large grains, with 95.98% SF and 4.02% SS (n = 1715, Figure 5B). In addition, the total percentage of sterile male gametes (LS + SS) in N102 and M1113 was 3.60 and 8.59%, respectively. In comparison, except for five accessions having very high male sterility (above 20%), the average percentage of sterile male gametes of the 15 other *Helianthus* accessions was 2.23% (Table S1). The high sterility of the five accessions was mainly caused by pollen size differentiation and partial staining of the pollen grains.

Measurements of normal and LF pollen grains indicated that the average diameter of normal and large pollen of N102 was 26.19 μm (n = 150, range = 23.99–28.23 μm) and 33.82 μm (n = 35, range = 31.42–35.74 μm), respectively (Figure 5A). The ratio of normal vs. “large” fertile pollen was 1:1.29 for N102. The preliminary analysis of the diameter of normal and “large” pollen for six other *Helianthus* accessions indicated that the average size ratio of normal vs. “large” pollen was 1:1.27 (range = 1.122–1:1.37). Therefore, the diameter of “large” fertile pollen was nearly 30% larger than that of normal fertile pollen for these *Helianthus* accessions.

**Meiotic and pollen mitotic analyses of N102**

Meiosis in N102 progressed similarly to the cultivated line HA 89 (Figure 6, E–I) until the end of telophase I (Figure 6, A–D). No preduplication was observed before meiosis at interphase. At diakinesis, the 17 bivalents could be identified. At metaphase I, the bivalents were aligned on the metaphase plate, with a few cells having one, two, or even four chromosomes separated early. At telophase I, the homologous chromosomes were separated, with a few cells having one or two chromosomes lagging behind. HA 89 had a normal meiosis I, with no lagging chromosomes (Figure 6, E–H).

At metaphase II, two well-separated metaphase II plates were observed (indicated by arrows in Figure 6I), with 8.57% showing one or two lagging chromosomes (n = 210) (Figure 6I). However, some cells appeared to contain two plates at the same angle, which were spread parallel as two plates (Figure 6, K and L) or squashed as one cluster (Figure 6, M and N) (with the latter type excluded from calculation to avoid confusion with metaphase I), suggesting the possible existence of parallel spindles (compare Figure 6, K–N with Figure 6I). In comparison, HA 89 had two well-separated metaphase II plates (Figure 6O). At telophase II, 96.98% of the cells had four normal nuclei (Figure 6P), with 0.79% of cells containing one large nucleus (two merged nuclei) and two normal nuclei (Figure 6Q), 0.22% containing two large nuclei (Figure 6R), and 2.01% having other unbalanced meiotic products (such as three or four normal nuclei and one or two tiny nuclei, or with lagging chromosomes, Figure 6S) (n = 1392). In total, we observed 94.55% normal tetrads (four sets of 17 chromosomes, Figure 6T), 4.61% triads (two sets of 17 chromosomes and one set of 34, Figure 6U), 0.82% dyads (two sets of 34 chromosomes, Figure 6V), and 0.03% other unbalanced types at the tetrad stage (Figure 6W) (n = 3431). Large daughter cells with two nuclei were also observed. HA 89 had a normal meiosis II, with 100% tetrad being observed at the tetrad stage (Figure 6X) (n = 2490), except for a few cells having nuclei of slightly different sizes.

Correspondingly, 1.62% large microspores were observed in N102 (n = 1544). These results suggest that a low percentage of chromosome nonreduction for N102 occurred during meiosis II, especially at the telophase II and tetrad stages, leading to the different sizes of pollen grains. Therefore, pollen size could be used to infer ploidy level of pollen grains, with normal or small pollen size corresponding to reduced microspores, and “large” pollen corresponding to unreduced 2n microspores, respectively.

Pollen mitotic analysis indicated a normal process for N102 (Figure S1). Of the nearly 1400 mature pollen grains analyzed, all contained one vegetative nucleus and two spermatids, including the large pollen (1.07%). With only ~1% unreduced pollen compared to 99% normal pollen in N102, differential fertilization favoring unreduced pollen certainly played an important role in the production of more triploids than the normal diploid F1 hybrids, when N102 was used as the pollen source.

**Rare abnormal chromosome numbers observed in the progenies derived from the crosses between N102 and wild perennial *Helianthus* species**

To determine whether the triploids were obtained specifically from the interspecific crosses between N102 and cultivated sunflower, interspecific and interspecific crosses were made between N102 and diploid *H. nuttallii* N314, N622, PI 650025, N1116, *H. maximiliani* M1113, *H. mollis* (PI 435753), and *H. silphioides* (Ames 30356); tetraploid *H. hispatus* (PI 547174); and hexaploid *H. ciliaris* (PI 435648); with N102 as the male (Table 4) or female parent (Table 5). Noticeably, N314 and M1113 were also observed to produce triploids when they were crossed to NMS HA 89-552. A total of 662 F1 plants (8.09%) were obtained from 8180 florets from the crosses involving seven diploid perennials with N102 as the male parent after embryo rescue (success rate = 1.59–30.23%). However, only three F1 plants from the crosses involving *H. nuttallii* N622, PI 650025, and *H. silphioides* (Ames 30356) (Figure S2, A–F) were triploids (0.45%). The percentages of triploids from the three crosses were 0.89, 1.16, and 1.12%, respectively (Table 4).
Crosses, the ratio of F1 hybrids appears to re-
and L) from the cross of M1113/N102 (2.46%,
N102. In addition, three plants had 2
plants (2.27%, 2n = 4x = 68) were detected, which were con-
using SSR marker ORS505 from linkage group 5 of the
flower SSR map (Figure S2G for 2n = 3x = 51, Figure S2H for
2n = 4x = 68, and Figure S3). For the cross H. ciliaris (PI 435648,
2n = 6x = 102)/N102, 33 F1 plants (2.17%) were obtained from 1520
florets. Except for one with 2n = 5x = 85 (3.03%), all F1 plants had 2n =
4x = 68 (Figure S2I for 2n = 4x = 68 and Figure S2J for 2n = 5x = 85).
The overall percentage of abnormal F1 plants was 0.77% of the
783 progenies from the crosses of nine wild Helianthus accessions.
In addition, all 112 F1s derived from N314/
M1113 were normal diploids, i.e., 2n = 2x = 34, with a success rate of
32.94%.

Low frequency of abnormal chromosome numbers in
progenies of interspecific crosses involving other
perennial species

H. salicifolius, H. diversicatus, H. hirsutus, H. giganteus, and H. grosse-
serratus were also crossed with NMS HA 89-552 and HA 410, with the
aim of transferring useful agronomic genes into cultivated sunflower.
The F1 seedlings were obtained through embryo rescue, and mitotic
chromosome counts taken (Table 6). Only one plant with 2n = 3x =
51 and one with 2n = 3x = 2 = 49 were detected (6.25%) in the 32 F1
progenies derived from the cross NMS HA 89-552/H. diversicatus (Fig-
ure S2, P–R). Additionally, the pollen stainability analysis indicated that
one H. diversicatus accession had 0.85% LF and 0.09% LS pollen grains.

The overall percentage of plants with abnormal chromosome numbers
was 0.64% (n = 314). Compared to the > 75% of triploids obtained
from the crosses with N102 as the male parent and cultivated sunflower
as the female parent, the frequency of plants with abnormal chro-
osome numbers in these interspecific crosses was very low, likely corre-
seeding to a low frequency of unreduced pollen in these species.

Diploid and triploid progenies used to introgress genes
from wild into cultivated sunflower by backcrossing and
producing amphiploids/aneuploids

Backcrosses of triploid F1s of NMS HA 89-552/H. nuttallii N102,
N1008, or N1323, and NMS HA 89-552/H. maximiliani M1018,
M1113, M1314, M1323, or M1418 with HA 441 produced no seed,
and the diploid F1s crossed with HA 441 had very low seed set. How-
ever, most of the backcross progenies of the diploid F1s with HA 441 as
the pollen source had 2n = 2x = 34, with 37.14% of the progeny having
2n = 2x + 1 = 35 (n = 32). The average selfed seed set was 36.38%
(range = 0.17–95%), and the backcrossed seed set was 18.84% (range =
2.00–60%). These results would be consistent with unbalanced chro-
osome pairing in the interspecific F1s. However, 41.38% of progeny
had 20% or greater backcross seed set, demonstrating the practicality of
utilizing these 2n = 2x = 34 hybrids to introgress genes from wild
species into cultivated sunflower.

One tetraploid amphiploid with 2n = 67–69 was produced by cross-
ingsomatically doubled heads of colchicine-treated diploid F1
plants of NMS HA 89-552/N412 with the untreated pollen of triploid

For the cross H. hirsutus (PI 547174, 2n = 4x = 68)/N102, 88 F1
plants (2.87%) were obtained from 3070 florets (Table 4). Two tetra-
plody F1 plants (2.27%, 2n = 4x = 68) were detected, which were con-
firmed using SSR marker ORS505 from linkage group 5 of the
flower SSR map (Figure S2G for 2n = 3x = 51, Figure S2H for
2n = 4x = 68, and Figure S3). For the cross H. ciliaris (PI 435648,
2n = 6x = 102)/N102, 33 F1 plants (2.17%) were obtained from 1520
florets. Except for one with 2n = 5x = 85 (3.03%), all F1 plants had 2n =
4x = 68 (Figure S2I for 2n = 4x = 68 and Figure S2J for 2n = 5x = 85).
The overall percentage of abnormal F1 plants was 0.77% of the
783 progenies from the crosses of nine wild Helianthus accessions.
In addition, all 112 F1s derived from N314/
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progenies of interspecific crosses involving other
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H. salicifolius, H. diversicatus, H. hirsutus, H. giganteus, and H. grosse-
serratus were also crossed with NMS HA 89-552 and HA 410, with the
aim of transferring useful agronomic genes into cultivated sunflower.
The F1 seedlings were obtained through embryo rescue, and mitotic
chromosome counts taken (Table 6). Only one plant with 2n = 3x =
51 and one with 2n = 3x = 2 = 49 were detected (6.25%) in the 32 F1
progenies derived from the cross NMS HA 89-552/H. diversicatus (Fig-
ure S2, P–R). Additionally, the pollen stainability analysis indicated that
one H. diversicatus accession had 0.85% LF and 0.09% LS pollen grains.

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Diploid and triploid progenies used to introgress genes
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ingsomatically doubled heads of colchicine-treated diploid F1
plants of NMS HA 89-552/N412 with the untreated pollen of triploid

![Image](76x549 to 527x735)
F₁ plants of NMS HA 89-552/N102. Similarly, another tetraploid amphiploid with 2n = 66–68 was produced by crossing chromosomally doubled heads of colchicine-treated diploid F₁ plants of NMS HA 89-552/M1323 with the untreated pollen of triploid NMS HA 89-552/M1113. Mitotic GISH analysis of two individuals of one amphiploid derived from NMS HA 89-552/M1323/NMS HA 89-552/M1113 indicated that about half of the chromosomes were from *H. maximiliani* with the others from cultivated sunflower (Figure 7). This is only possible when the unreduced female gametes were fertilized by balanced unreduced gametes having 2n = 2x = 34, with half from the wild parent and half from cultivated line. The seed set for sib-crosses and selfs of the *H. nuttallii* amphiploid were 47.19 and 50.47%, respectively.

**Figure 6** Meiotic analysis of *H. nuttallii* N102 and HA 89. (A–D) Meiosis I of N102. (E–H) Meiosis I of the cultivated line HA 89. (A and E) Pachytene. (B and F) Diakinesis. (C and G) Metaphase I. (D and H) Telophase I. (I–N) Meiosis II of N102. (I) Two well-separated metaphase II plates, indicated by arrows. (J) Two well-separated metaphase II plates, with one lagging chromosome. (K and L) Two metaphase II plates at the same angle, which were spread paralleled. (M and N) Two metaphase II plates were squashed as one cluster. (O) Meiosis II of HA 89, showing two well-separated plates. (P–S) Telophase II of N102. (P) Cell with four normal nuclei. (Q) Cell with one large nucleus (two merged nuclei) and two normal nuclei. (R) Cell with two large nuclei. (S) Cell with four nuclei and two lagging chromosomes. (T–W) Tetrads of N102. (T) Normal tetrad. (U) Triad. (V) Dyad. (W) Unbalanced type at the tetrad stage. (X) Tetrad of HA 89. The meiosis I was normal for both materials. Abnormality was noticed at meiosis II for N102. Big nucleus resulted from the merging of two normal nucleuses were observed at telophase II. Low percentages of triads and dyads were noticed at meiosis II. Bars = 5 μm.
respectively. For the *H. maximiliani* amphiploid, the seed set for sib-crosses was 25%. Embryo rescue was not needed to produce seeds. Therefore, these amphiploids provide useful bridge materials for introgression of important agronomic traits into cultivated sunflower.

A series of aneuploids from crosses of the triploid *F*₁s of NMS HA 89-552/*H. nuttallii* N102, N314, or N1324, and NMS HA 89-552/*H. maximiliani* M1018, M1113, M1323, or M1418 with HA 441 were obtained utilizing embryo rescue. Chromosome numbers of the BC₁ *F*₁ progeny ranged from 2n = 37 to 51. However, the average seed set was very low (0.39%) with additional backcrosses with HA 441 due to the unbalanced chromosome numbers. These materials could be used for further backcrossing with the aim of reducing the chromosome number and improving fertility.

**DISCUSSION**

**Triploids provided evidence of abnormal polyploidization of sunflower**

In almost all plant species, 2n gametes were frequently observed in interspecific or intercrosses in groups, and could be triggered by abiotic and biotic stresses, such as heat and cold shocks, water stress, nutrition, and disease (Storme and Mason 2014; Mason and Pires 2015). It has been assumed that 2n gametes occur only rarely and the importance of 2n gametes in the origin of polyploids has been argued (Harlan and deWet 1975; Ramanna and Jacobsen 2003; reviewed by Dewitte et al. (2012)). However, accumulating evidence suggests that unreduced gametes may have had a major role in the evolution of polyploids (Tayalé and Parisod 2013).

In this study, a high frequency of triploids was observed in interspecific crosses of two diploid perennial *Helianthus* species, specifically N102, pollinated to cultivated sunflower. Mitotic analysis of nearly 1800 progenies indicated that the frequency of triploid *F*₁ progenies was 92.78% for the crosses of cultivated lines/N102, which was significantly higher than the 0.77% of unexpected polyploidy plants obtained from the crosses of other wild perennial *Helianthus* species/ N102. No unexpected polyploids were observed in crosses of N102/ cultivated sunflower, or N102/other perennial *Helianthus* species. By comparison, the percentages of progenies with abnormal chromosome numbers derived from five other perennial *Helianthus* species pollinated onto the cultivated NMS HA 89-552 was only 0.64%. A very low frequency of 2n pollen was observed in N102 (1.22%) and M1113 (0.52%). However, they still had the highest percentage of 2n pollen among the 22 wild *Helianthus* accessions studied, especially N102, but closely agree with the estimate of 2n gametes production in nonhybrid flowering plants (0.56%) (Ramsey and Schemske 1998). The results indicated that the female gametes of N102 are normal. The analysis of the pollen fertility of 22 wild *Helianthus* accessions and HA 89 suggested that the sterile male gametes normally exist in wild *Helianthus* species and cultivated sunflower, and that N102 and M1113 had a similar or higher male sterility than most of the other wild *Helianthus* accessions studied. On the other hand, the low frequency of 2n male gametes of N102 suggested that the high frequencies of triploids were caused by a higher compatibility of 2n gametes, relative to the reduced male gametes of N102, to the female cultivated sunflower. However, the difference between the 2n and n gametes was not as evident when N102 was pollinated to other perennial *Helianthus* species. In the latter case, low frequencies of unexpected polyploids were produced.

Therefore, the high frequency of triploid *F*₁s derived from diploid × diploid crosses provided evidence of extreme abnormal polyploidization of sunflower. Since the cross-incompatibility between perennial wild *Helianthus* and cultivated lines is high, these triploids are likely the results of the low frequency of 2n male gametes under specific crossing pressure, *i.e.*, due to the dosage balance of factors related to recognition and rejection of foreign pollen during fertilization (Mason and Pires 2015). This unique triploid *F*₁ production will expedite our understanding of sunflower evolution and speciation, and the effect of unreduced gametes on crossing compatibility between *Helianthus* species.

**Triploids and amphiploids are valuable for genetic study and plant breeding**

The semifertile triploid *F*₁ progenies derived from interspecific crosses of cultivated sunflower pollinated by the two wild *Helianthus* species have been used for developing amphiploids and aneuploids, and for interspecific gene transfer. Therefore, these triploids could act as an important bridge during the evolution and speciation of sunflower. Furthermore, these triploids could be a valuable tool for producing alien addition lines or monosomic lines and chromosome number tracking to reveal the effects of individual chromosomes. Under specific circumstances, alien addition lines will facilitate molecular gene mapping when the gene is linked with the alien chromosome, similar to the strategy for the mapping of the male fertility restoration gene *Rf₆* (Liu et al. 2013).

Amphiploids with improved seed set can be maintained by sib-pollination or produce backcross seed without embryo rescue, and reduce the effort required to introduce the genetic diversity in the perennial species into cultivated sunflower. Amphiploids are a useful “bridge” for transferring important agronomic traits into cultivated sunflower. For example, a broomrape resistance gene for race F in Spain has been successfully transferred from a wild *Helianthus* species into cultivated sunflower using amphiploids (Pérez-Vich et al. 2002), and two male fertility restoration genes, *Rf₆* and *Rf₆*, were identified in amphiploids and introgressed into cultivated sunflower (Feng and Jan 2008; Liu et al. 2013). Amphiploids could also enable crosses between species of different ploidy levels, further increasing the genetic diversity of cultivated sunflower.

**Reasons for the unexpected triploids derived from the two wild *Helianthus* species**

Triploids can form by fusion of a 2n gamete with a normal haploid gamete or by polypolyploidy (Ravi et al. 2008). Unreduced gametes most commonly arise through meiotic defects, including the omission of the first or second meiotic division, abnormal spindle morphology in the
second division, or disturbed cytokinesis (Brownfield and Köhler 2011). Several plant mutants producing triploid offsprings have been molecularly identified and characterized, such as DYAD/SWITCH1, RBR, and AGO104 (Ravi et al. 2008; Chen et al. 2009; Singh et al. 2011). A high frequency of triploids (80%) has been associated with the dyad allele of SW1 of Arabidopsis, which is expressed as an absence of meiosis in the female gamete formation caused by mutation of DYAD/SWITCH1 (Ravi et al. 2008). The Arabidopsis triploid rbr/+ offsprings presumably resulted from the fusion of a haploid egg with a diploid rbr sperm that originated from a postmeiotic genome duplication event (Chen et al. 2009). A maize mutant, Dnr 4 at locus AGO104, also results in the formation of functional unreduced gametes because of the defects in chromatin condensation during meiosis (> 80% of the meiocytes at metaphase I) (Singh et al. 2011). Triploid progeny could also be the result of 2x × 2x sexual hybridization in Citrus as a consequence of the formation of unreduced gametes at a low frequency (Aleza et al. 2010).

In this study, the low percentage of chromosome nonreduction in N102 was caused by meiosis II nonreduction. At the telophase II stage, we observed cells containing three spores (two haploid spores and one diploid one) and two diploid spores. We also observed a low percentage of triploids at dyads at the tetrad stage. It appeared to be the consequence of tripolar or paralleled spindles at meiosis II for chromosome nonreduction, which may be caused by defects in the interzonal microtubule array (derFurth et al. 2008; Storme and Geelen 2013). Since the triploid production using H. nuttallii N102 pollen has been observed over a long period of time, and under a range of environmental conditions, it is likely that the consistency of the frequent unreduced male gametes that result in the relatively high frequency of F1 triploids is due to unique genetic alterations.

**Future study**

The molecular mechanism for the occurrence of the high frequency of triploid production in the interspecific crosses involving the two diploid wild Helianthus species and cultivated sunflower lines remains to be further evaluated. Among the questions awaiting answers are: (1) the relative competitiveness of large and small pollen on fertilization, using diploid and tetraploid P21 pollens, and their mixed pollen to N102, or using N102 and chromosome-doubled N102 to cultivated sunflower; (2) examine pollen germination on the stigmas of NMS HA 89-552 and using N102 and chromosome-doubled N102 to cultivated sunflower hybrids; (3) examine pollen germination on the stigmas of NMS HA 89-552 and using N102 and chromosome-doubled N102 to cultivated sunflower to cultivated sunflower hybrids. We also thank Larry G. Campbell and Steven S. Xu for critical review of the manuscript. We thank the National Sclerotinia Initiative for funding to C.-C. J. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture (USDA). USDA is an equal opportunity provider and employer.

**ACKNOWLEDGMENTS**

We thank Lisa A. Brown and Marjorie A. Olson for technical assistance in this study, and Hongxia Wang and Jichong Zhang for their help in the collection of root tips and leaf tissue, and chromosome counts. We appreciate Yunnming Long (North Dakota State University), Qiuin Zhang (North Dakota State University), and Wentao Li (Nuseed America) for valuable discussion during the study and writing of the manuscript. We also thank Larry G. Campbell and Steven S. Xu for critical review of the manuscript. We thank the National Sclerotinia Initiative for funding to C.-C. J. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture (USDA). USDA is an equal opportunity provider and employer.

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Communicating editor: K. M. Devos