MOLECULAR DIVERSITY OF nrDNA ITS REGION AFFECTED HETEROSIS IN SUNFLOWER (Helianthus annuus)

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Received:
Keywords: nrDNA; ITS; highest scoring genotypes; CMS; heterosis

ABSTRACT
A study was conducted to specify mutations of the internal transcribed spacer (ITS) in the nucleotides of nuclear ribosomal DNA (nrDNA) and their molecular relationship with heterosis exhibited by the six A- lines and three R-testers of sunflower. The trail was conducted at the fields of the College of Agriculture, University of Anbar, Abu Ghraib during two seasons (spring and fall 2016). Parental lines were crossed according to lineXtester crossing scheme to produce 18 single hybrids, genotypes were sown to assess their phenotypic performance in the fall season. Results of the molecular analysis of ITS sequencing showed that the rearrangement of single nucleotides had accumulated in a higher rate in the F1 hybrids compared with their ancestor inbreds. The total number of mutations was 268, and deletion mutations accounted for the largest proportion with 209 mutation (71 in lines and 138 in hybrids). In contrast, the transition mutations were 25 all occurred in the hybrids, however the number of transversions recorded 17only. Based on DNA sequence of the nrDNA ITS region the total genotypes were separated into two main groups in cluster analysis following the nearest neighbor method. There was a significant convergence between R2 and both A4XR2 and A6XR3 hybrids scoring higher values of similarity in context ITS sequence. To be specific, the hybrid A2XR3 exhibited the best desirable heterosis for days to 75% flowering and leaf area, while hybrid A5XR3 had the highest heterosis for head traits, height and area.

Keywords: nrDNA; ITS; sunflower; Helianthus annuus; CMS; heterosis

Part of Ph.D. dissertation of the 1st author

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INTRODUCTION

Sunflower (Helianthus annuus) is an important crop belongs to the largest plant family (Asteraceae), and mainly growing for premium quality oil production. The developing sunflower crop is highly restricted by the narrow genetic background due to limited gene pool. Thus, much attention has been directed to widen the genetic base of sunflower via either conventional or modern biotechnologies (14). Sunflower crop has undergone substantial genetic and phenotypic changes during domestication process. Therefore, the discovering of cytoplasmic male sterility (CMS) and exploring the restorer fertility (RF) considered a main pillars for the previously mentioned changes that lead to considerable alteration in sunflower production to hybrid breeding (5),(15). The revolutionary effect of heterosis in different plant species encourages specialists to pay considerable attention to this unique phenomenon. Consequently, there is always a strong demand to know the way of genes action and interaction that serve in tuning such actions via customizing breeding methods and developing heterotic groups (12). The genetic mechanism laying behind heterosis still not consistent, hence researchers continuing to push forward but not for sure new assumptions to solve the puzzle of heterosis (20). However, in the last two decades, significant progress was made in developing new molecular tools and/or updating previous strategies that facilitating the researchers mission. High priority has been given to study Internal Transcribed Spacer (ITS) as a prominence sequence data for phylogenic studies in plants (7),(18). Apparently, the detection of single nucleotide polymorphisms (SNPs) is an important addition to the molecular diagnosing of genetic diversity for many reasons. For instance, such domains are associated with key phenotypic and physiological variants in many plant species cv(8),(3),(1). Furthermore, the nucleotides diversity in the nuclear ribosomal DNA (nrDNA) of ITS region can provide an efficient tool for characterizing genotypes heterogeneity as well as the classification of different species (2).The study was proposed to evaluate the possible polymorphism in the nucleotides of nuclear ribosomal DNA (nrDNA) and their relationship with heterosis in different cms sources of sunflower

MATERIALS AND METHODS

Genomic DNA extraction

A field study was conducted during two growing seasons (spring and fall, 2016) using six male sterile A-lines (A1 to A6) and three fertility restorer R-lines (R1, R2 and R3) obtained from Seed Test and Certification Board (STCB), Ministry of Agriculture. The genotypes were crossed according to line X tester matting system in the first season to be evaluated in the second. DNA was extracted from seven leaf-stage seedlings in the STCB labs. using Genomic DNA Mini Kit-Plant (Geneaid Biotech Ltd., South Korea).

DNA Quantity and Quality

Nanodrop was used to check the DNA quality according to the following formula:

\[
Purity\text{ of } DNA = \frac{O.D_{260}}{O.D_{280}}\geq 1.8
\]

Reads ranged between 1.8-2, and D oNA concentration was adjusted to 50 ng/μl final concentration(18).

Amplification of ITS region

Polymerase chain reaction (PCR) was performed and the specific primer (F-5'-TCCGTAGGTGAACCTG-3) was used to amplify the ITS region in the targeted genomes (2). The mixture of PCR reaction was 12.5 μl of Green Master Mix, 1μl of primer, 3 μl of DNA template, finally volume was completed to 25 μl using nuclease-free water. The samples were submitted to the PCR machine for amplification step with the following thermal profile: Initial denaturation and denaturation at 95°C for 4 min, Denaturation step was at 55°C for 30 sec., extension and final extension was at 72°C for 1 and 7 min., respectively. The ITS region sequence of the pooled plant samples were identified by applying Sanger sequencing method in (Macrogen Inc., Seoul, Korea) with the aid of ABI 3730 Genetic Analyzer (Applied Biosystems, USA).

Electrophoresis

Agaros gel was prepared by adding 2 g of agarose to 100 ml of 1X TAE buffer (16). Ten microliters from each amplification product were loaded into the wells. Electrophoresis was performed at a voltage of 5 volt cm⁻¹ till DNA reached the edge of the gel. Agarose gel
was exposed to a UV transilluminator, pictured and documented at 340 nm cannon.

**Statistical analysis**

DNA sequencing of ITS region was analyzed using MEGA6 software (Molecular Evolutionary Genetics Analysis version 6.0) to identify SNP cases (single nucleotide polymorphism) within the targeted genomes. Cluster analysis of SNP results were estimated using Euclidean distance according to the nearest neighbor method (2).

**Heterosis**

Heterosis was calculated as stated by Laosuwan and Atkins (1997) using the following formula: Heterosis (H) % = \( \frac{\{F1-BP\}}{BP} \times 100\).

**RESULTS AND DISCUSSION**

Single nucleotide Polymorphism (SNP) of the nrDNA ITS sequence

The results of nrDNA ITS sequences (Figure 1) showed that alterations in the single nucleotides have occurred at a higher rate in hybrids compared to their parental inbred lines and testers. The counted total of mutations was about 268 (Table 1), deletion mutations took the lead as it scored a total of 209 detected within most studied genotypes (71 in the inbreds and 138 in hybrids). These kinds of mutations were residents in a specific loci 450, 549, 550, 570, 579, 580, 661 and 667, except for R2, A4×R2 and A6×R3 genotypes that showed addition mutation in loci 540, 549, 570, 579, 661 or adding guanine (G) in loci 550, 580 and 667. Mutation of transition occurred in A2×R3, A3×R1, A3×R2, A4×R1, A4×R2, A5×R2 and A6×R3 hybrids, most of which were G>A (guanine to adenin), while there were three mutations of A>G (adenine to guanine) in A3×R1, A5×R1 and A6×R3 hybrids and three mutations of T>C (thymine to cytosine) exhibited by the three hybrids (A5×R1, A5×R3, A6×R3). Only one mutation was found to be C>T (cytosine to thymine) in A6×R3 hybrid at the nucleotide loci of 576. Transversion mutations recorded a total of 17 mutations, substitution of adenine (A) with thymine (T) occurred in five hybrids (A2×R3, A3×R1, A3×R2, A5×R3 and A6×R3). The other type of substitution was T>A occurred at single locus in A4×R2 hybrid and at two loci (502 and 613) in A6×R3 hybrid. The A nucleotide substituted C (C>A) in A3×R1, A5×R1 and A6×R3 hybrids, however T>G was a single transversion mutation displayed by A5×R1 hybrid, meanwhile G>T transversion revealed by A6×R3 hybrid and A>C transversion by A2×R3 hybrid.
Table 1 (A). Single nucleotides polymorphism of the nrDNA ITS sequence in sunflower

| No. | Genotype | Type of substitution | Location | Nucleotide |
|-----|----------|----------------------|----------|------------|
| 1   | A1       | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667, 692 | ----- |
| 2   | A2       | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667 | ----- |
| 3   | A3       | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667 | ----- |
| 4   | A4       | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667, 687 | ----- |
| 5   | A5       | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667, 692 | ----- |
| 6   | A6       | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667, 692 | ----- |
| 7   | R1       | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667 | ----- |
| 8   | R2       | Deletion             | 10       | ----- |
| 9   | R3       | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667 | ----- |
| 10  | A1×R1    | Transition           | 697      | G>A       |
|     |          | Addition             | 692      | G         |
| 11  | A1×R2    | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667, 694, 695 | ----- |
| 12  | A1×R3    | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667, 682 | ----- |
|     |          | Addition             | 692      | A         |
| 13  | A2×R1    | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667, 692 | ----- |
| 14  | A2×R2    | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667 | ----- |
|     |          | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667 | ----- |
| 15  | A2×R3    | Transition           | 693, 696, 697 | G>A       |
|     |          | Transversion         | 685      | A>T       |
|     |          | Transversion         | 689      | A>C       |
| 16  | A3×R1    | Transition           | 1        | A>T       |
|     |          | Transversion         | 690      | C>A       |
|     |          | Transversion         | 693      | G>C       |
|     |          | Transition           | 694, 695 | A>G       |
|     |          | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667, 688 | ----- |
| 17  | A3×R2    | Transition           | 78       | G>A       |
|     |          | Transversion         | 686      | A>T       |
| 18  | A3×R3    | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667, 692 | ----- |
| 19  | A4×R1    | Transition           | 694, 698 | G>A       |
|     |          | Deletion             | 10       | -----     |
|     |          | Transition           | 456      | G>A       |
| 20  | A4×R2    | Transversion         | 502      | T>A       |
|     |          | Addition             | 540, 549, 570, 579, 661 | T |
|     |          | Addition             | 550, 667, 580 | G |
Table 1(B). Single nucleotides polymorphism of the nrDNA ITS sequence in sunflower

| No. | Genotype  | Type of substitution | Location                        | Nucleotide |
|-----|-----------|----------------------|---------------------------------|------------|
| 21  | A4×R3     | Deletion             | 540, 549, 550, 570, 579, 580, 661, 667 | -----      |
|     |           | Transition           | 683                              | T>C        |
| 22  | A5×R1     | Transition           | 696                              | A>G        |
|     |           | Transversion         | 684                              | C>A        |
|     |           | Transversion         | 688                              | T>G        |
|     |           | Deletion             | 540, 549, 550, 661, 667, 570, 579, 580 | -----      |
| 23  | A5×R2     | Transition           | 691                              | G>A        |
|     |           | Transition           | 693                              | G>C        |
|     |           | Deletion             | 540, 549, 550, 661, 667, 570, 579, 580 | -----      |
| 24  | A5×R3     | Transition           | 691                              | G>A        |
|     |           | Transition           | 693                              | T>C        |
|     |           | Transversion         | 682                              | A>T        |
| 25  | A6×R1     | Transition           | 78                               | G>A        |
|     |           | Transition           | 194                              | A>G        |
| 26  | A6×R2     | Deletion             | 540, 549, 550, 661, 667, 570, 579, 580, 692 | -----      |
|     |           | Transition           | 10                               | G>A        |
|     |           | Transition           | 657                              | A>G        |
|     |           | Transition           | 657                              | C>T        |
|     |           | Transition           | 577                              | T>C        |
|     |           | Transversion         | 502, 613                         | T>A        |
|     |           | Transversion         | 697                              | G>T        |
|     |           | Transversion         | 587, 688                         | A>T        |
|     |           | Transversion         | 678                              | G>C        |
|     |           | Transversion         | 623                              | C>A        |
| 27  | A6×R3     | Addition             | 540, 549, 570, 579, 661          | T          |
|     |           | Addition             | 550, 667, 580                    | G          |

Cluster analysis of nrDNA ITS sequence

Cluster analysis for single nucleotide polymorphism (SNP) of the ITS region in inbred lines, testers and their F1 hybrids was accomplished according to the nearest neighbor method (Figure 2). Results indicated that the studied genotypes distributed into two main groups, the first consisted of three genotypes (R2, A4×R2 and A6×R3), meanwhile the second group divided into many sub-groups. It can be noticed that most of the studied genotypes tended to organize in pairs according to its ITS sequence showing high level of genetic similarity. Some other genotypes revealed unique pattern of ITS sequence, hence genetic distinctness by occupying single sub-clusters (A2, A6, R2, A1×R3 and A5×R3). In the same context, hybridization process had an effective role in shaping unique DNA sequence in hybrids, like A5×R2 that was the most divergent genotype in term of nrDNA sequence. The estimated Euclidean distance among the studied cms genotypes of sunflower (Table 2) approved the existence of common relationship between the R2 tester and its descended hybrid A4×R2 and A6×R3 hybrids resulting in too high values with all other genotypes not less than 0.243. This is in part of it genotypes expressed almost the mutation types (Table 1). Meanwhile, the highest value (0.305) was against the inbred line A3. In addition, A4×R2 and A6×R3 hybrids showed the highest value of the Euclidean distance with all other hybrids. The highest value was 0.0336 for A4×R2 hybrid against A1×R2 hybrid. The A6×R3 single hybrid gave the highest value (0.0352) with A6×R1 hybrid. where they showed the lowest value of the Euclidean distance (0.0015) in A4×R3, A3×R3, A6×R2 and A4×R2 hybrids.
Figure 1. Single nucleotide Polymorphism (SNP) of the nrDNA ITS sequence in inbreds (1-6), testers (7-9) and lineXtester hybrids (10-27) of sunflower.
Heterosis

**Number of days to 75% flowering**
Heterosis for flowering time was calculated according to the deviation of the first generation over the earliest parent (Table 3). The observed heterosis was in significant negative values for some hybrids and ranging from -6.01% in A2×R3 hybrid to -1.27% in A1×R2 hybrid, which approved the overdominance effect of the early parents genes implying that these hybrids could mature earlier and avoid the undesirable environmental conditions. The other hybrids acted in different way as it exhibited a significant positive effect of partial dominance genes of the early parent when it had positive values ranging from 4.62% for A3×R3 hybrid to 1.24% for A2×R1 hybrid. These findings may due to the high frequency of different mutations (deletion, transition and transversion) within ITS region of hybrids genomes (9),(19), (10).

**Plant height (cm)**
Although negative heterosis for plant height is desirable, most of the tested hybrids tended to be taller than their inbred parents, hence they had a positive heterosis degrees (Table 3). Among the 18 experienced F1 hybrids, ten exhibited significant positive values of heterosis ranging from 13.29% in A5×R2 hybrid to 7.47% in A3×R1 hybrid. Such estimates indicated the effect of overdominance gene action of the taller parent for plant height trait. The different polymorphism rate of ITS region may play a key role in magnifying the total diversity, which in turn improved the chances of getting heterosis in the desired direction (13), (6),(17).

**Leaf area (cm²)**
Heterosis expressed by the single hybrids for leaf area show in Table 3. The magnitude of heterosis ranged from significant positive to negative values for this trait. However, the majority of the studied hybrids (17 hybrid) expressed significant heterosis values. The highest positive estimate was 63.51% in A2×R3 hybrid against the lowest (3.38%) showed by A6×R3 hybrid. Whereas, others hybrids gave significant negative values of heterosis ranged between -67.79% in A6×R2 hybrid to -3.59% in A3×R2 hybrid. The positive values of heterosis indicated the presence of over-dominance genes action, meanwhile the negative values pointed to partial-dominance gene of action of the best parent (17).
| 1 | 2 | 0.0030 |
|---|---|---|
| 3 | 0.0105 | 0.0105 |
| 4 | 0.0060 | 0.0090 | 0.0105 |
| 5 | 0.0030 | 0.0030 | 0.0075 | 0.0080 |
| 6 | 0.0030 | 0.0030 | 0.0075 | 0.0080 | 0.0080 |
| 7 | 0.0045 | 0.0045 | 0.0080 | 0.0075 | 0.0045 | 0.0015 |
| 8 | 0.0274 | 0.0305 | 0.0305 | 0.0243 | 0.0274 | 0.0274 | 0.0290 |
| 9 | 0.0045 | 0.0045 | 0.0060 | 0.0075 | 0.0015 | 0.0045 | 0.0060 | 0.0290 |
| 10 | 0.0120 | 0.0120 | 0.0120 | 0.0135 | 0.0090 | 0.0120 | 0.0135 | 0.0336 | 0.0090 |
| 11 | 0.0030 | 0.0030 | 0.0105 | 0.0075 | 0.0030 | 0.0030 | 0.0045 | 0.0289 | 0.0045 | 0.0090 |
| 12 | 0.0075 | 0.0075 | 0.0030 | 0.0105 | 0.0045 | 0.0075 | 0.0060 | 0.0305 | 0.0030 | 0.0090 | 0.0075 |
| 13 | 0.0000 | 0.0000 | 0.0105 | 0.0080 | 0.0030 | 0.0030 | 0.045 | 0.0274 | 0.0045 | 0.0120 | 0.0030 | 0.0075 |
| 14 | 0.0030 | 0.0000 | 0.0105 | 0.0090 | 0.0030 | 0.0045 | 0.0305 | 0.0045 | 0.0120 | 0.0030 | 0.0075 | 0.0030 |
| 15 | 0.0105 | 0.0105 | 0.0000 | 0.0105 | 0.0075 | 0.0075 | 0.0060 | 0.0305 | 0.0060 | 0.0120 | 0.0105 | 0.0030 | 0.0105 |
| 16 | 0.0080 | 0.0080 | 0.0105 | 0.0000 | 0.0060 | 0.0060 | 0.0075 | 0.0243 | 0.0075 | 0.0135 | 0.0075 | 0.0105 | 0.0050 | 0.0090 | 0.0105 |
| 17 | 0.0030 | 0.0030 | 0.0075 | 0.0080 | 0.0000 | 0.0030 | 0.0045 | 0.0274 | 0.0015 | 0.0090 | 0.0030 | 0.0045 | 0.0030 | 0.0030 | 0.0075 | 0.0060 |
| 18 | 0.0030 | 0.0030 | 0.0075 | 0.0080 | 0.0030 | 0.0030 | 0.0015 | 0.0274 | 0.0045 | 0.0120 | 0.0030 | 0.0075 | 0.0030 | 0.0030 | 0.0075 | 0.0060 |
| 19 | 0.0045 | 0.0045 | 0.0060 | 0.0075 | 0.0048 | 0.0015 | 0.0000 | 0.0290 | 0.0060 | 0.0135 | 0.0045 | 0.0060 | 0.0045 | 0.0045 | 0.0060 | 0.0075 | 0.0045 | 0.0015 |
| 20 | 0.0274 | 0.0305 | 0.0305 | 0.0243 | 0.0274 | 0.0274 | 0.0290 | 0.0000 | 0.0290 | 0.0336 | 0.0289 | 0.0305 | 0.0274 | 0.0305 | 0.0243 | 0.0274 | 0.0274 | 0.0290 |
| 21 | 0.0045 | 0.0045 | 0.0060 | 0.0075 | 0.0031 | 0.0045 | 0.0060 | 0.0290 | 0.0000 | 0.0090 | 0.0045 | 0.0030 | 0.0045 | 0.0045 | 0.0060 | 0.0075 | 0.0015 | 0.0045 | 0.0060 | 0.0290 |
| 22 | 0.0120 | 0.0120 | 0.0120 | 0.0135 | 0.0090 | 0.0120 | 0.0135 | 0.0336 | 0.0090 | 0.0000 | 0.0090 | 0.0090 | 0.0120 | 0.0120 | 0.0120 | 0.0135 | 0.0090 | 0.0120 | 0.0135 | 0.0336 | 0.0090 |
| 23 | 0.0045 | 0.0075 | 0.0120 | 0.0080 | 0.0060 | 0.0075 | 0.0090 | 0.0274 | 0.0060 | 0.0120 | 0.0060 | 0.0000 | 0.0045 | 0.0075 | 0.0120 | 0.0060 | 0.0060 | 0.0075 | 0.0000 | 0.0274 | 0.0060 | 0.0120 |
| 24 | 0.0120 | 0.0120 | 0.0090 | 0.0150 | 0.0105 | 0.0120 | 0.0105 | 0.0352 | 0.0090 | 0.0050 | 0.0090 | 0.0060 | 0.0120 | 0.0120 | 0.0030 | 0.0150 | 0.0105 | 0.0120 | 0.0105 | 0.0352 | 0.0090 | 0.0060 | 0.0105 |
| 25 | 0.0030 | 0.0000 | 0.0105 | 0.0000 | 0.0030 | 0.0030 | 0.0045 | 0.0305 | 0.0045 | 0.0120 | 0.0030 | 0.0075 | 0.0030 | 0.0030 | 0.0075 | 0.0060 | 0.0030 | 0.0000 | 0.0015 | 0.0274 | 0.0045 | 0.0120 | 0.0075 | 0.0120 | 0.0030 |
| 26 | 0.0030 | 0.0030 | 0.0075 | 0.0060 | 0.0030 | 0.0000 | 0.0015 | 0.0274 | 0.0045 | 0.0120 | 0.0030 | 0.0075 | 0.0030 | 0.0030 | 0.0075 | 0.0060 | 0.0030 | 0.0000 | 0.0015 | 0.0274 | 0.0045 | 0.0120 | 0.0075 | 0.0120 | 0.0030 |
| 27 | 0.0274 | 0.0305 | 0.0305 | 0.0243 | 0.0274 | 0.0274 | 0.0290 | 0.0000 | 0.0290 | 0.0336 | 0.0289 | 0.0305 | 0.0274 | 0.0305 | 0.0243 | 0.0274 | 0.0274 | 0.0290 | 0.0000 | 0.0290 | 0.0336 | 0.0274 | 0.0352 | 0.0305 | 0.0274 |
Head area (cm²)
Head area represents a key secondary component of yield trait simply because it accommodates many and/or large seeds. Heterosis percentage for head area varied in response to the genetic and epigenetic differences between the crossed parental lines. Heterosis percentage was positive and significant for this trait in two hybrids only, nevertheless A5×R3 was in the lead scoring 44.06%. These results were further supported by ITS sequencing, especially as this hybrid has two genetically distinct parents (A5 and R3) raising a great single nucleotide polymorphism in ITS domain. The negative values ranged between the minimum (-66.45%) in hybrid A6×R2 and the highest (-5.94%) in A6×R1. These results indicate a clear effect of the over-dominance gene action in the hybrids that gave positive heterosis, while the effect of partial-dominance marked the hybrids with negative heterosis (4), (10).

Table 3. Percentage of heterosis of line×tester matting sachem in sunflower

| Hybrids | Days to 75% flowering | Plant height (cm) | Leaf area (cm²) | Head area (cm²) |
|---------|-----------------------|------------------|----------------|----------------|
| A1×R1  | 1.88                  | 14.98            | -29.54         | -53.81         |
| A1×R2  | -1.27                 | 0.16             | -25            | -57.11         |
| A1×R3  | -3.03                 | 12.06            | 45.45          | 23.65          |
| A2×R1  | 1.24                  | 1.87             | -18.42         | -50.51         |
| A2×R2  | -0.63                 | 8.66             | 44.73          | 16.31          |
| A2×R3  | -6.01                 | 8.10             | 63.15          | 11.39          |
| A3×R1  | -1.98                 | 7.47             | 0.00           | -9.88          |
| A3×R2  | -1.33                 | -2.83            | -56.89         | -61.97         |
| A3×R3  | 4.62                  | 9.08             | -8.62          | -10.53         |
| A6×R1  | -1.92                 | 5.45             | 15.38          | 12.45          |
| A6×R2  | -1.93                 | 4.85             | -53.84         | -52.14         |
| A6×R3  | 1.26                  | 9.14             | 3.84           | 2.87           |
| A3×R1  | -4.45                 | 4.29             | -9.80          | 1.52           |
| A5×R2  | -4.51                 | 13.29            | -50.98         | -52.10         |
| A5×R3  | -0.63                 | 16.18            | 41.17          | 44.06          |
| A6×R1  | -4.41                 | -5.98            | 3.38           | -5.94          |
| A6×R2  | -3.21                 | -2.22            | -67.79         | -66.45         |
| A6×R3  | -1.79                 | -4.63            | 3.38           | 7.48           |
| S.E (sij) | 1.19                  | 6.10             | 0.016          | 23.33          |

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