ORIGINAL RESEARCH ARTICLE

Molecular Markers for Septoria Leaf Spot (*Septoria lycopersici* Speg.) Resistance in Tomato (*Solanum lycopersicum* L.)

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Abstract

Marker assisted selection (MAS) has not been initiated in tomato (*Solanum lycopersicum* L.) for septoria leaf spot (SLS) resistance caused by *Septoria lycopersici* Speg due to lack of molecular markers. We studied the inheritance of SLS resistance and identified molecular markers linked to SLS resistance using bulked segregant analysis (BSA) in a segregating F_2 population. Tomato inbred lines, NC 85L-1W (2007), susceptible to SLS and NC 839-2(2007)-1, resistant to SLS were used to develop the segregating population. A total of 250 F_2 plants, and 10 plants each of P_1, P_2 and F_1 were grown at the Mountain Horticultural Crops Research and Extension Center (MHCREC), Mills River NC in the summer of 2009. Disease severity was scored using a scale of 0 to 5, where 0 = no disease and 5 = complete development of disease. DNA was extracted from 2-3 week old plants and parental lines were screened with a total of 197 random amplified polymorphic DNA (RAPD) primers, of which 34 were polymorphic. Two DNA bulks, called resistant bulk (RB) and susceptible bulk (SB) were prepared from the F_2 individuals. The RB and SB consisted of 8 individuals each with disease scores of 0, and 4.0 or 4.5, respectively. The segregation ratio of resistant and susceptible plants in F_2 generation fit the expected Mendelian ratio of 3:1 for a single dominant gene. Five RAPD markers were linked to the SLS disease reaction, of which two were linked to susceptibility and three to the resistance. Subject to verification in independent populations, these markers may be useful for MAS of SLS resistance in tomato.

Key words: Bulked segregant analysis, resistant bulk, septoria leaf spot, *Solanum lycopersicum,* susceptible bulk

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Introduction

Tomato (*Solanum lycopersicum* L., 2n = 2x = 24) is one of the most important vegetable crops worldwide. Among the foliar diseases of tomato, septoria leaf spot (SLS) caused by *Septoria lycopersici* Speg is one of the most devastating diseases. It occurs worldwide including Canada and Northeast America. It can cause complete defoliation leading to a significant crop loss under favorable environmental conditions, particularly in humid regions during periods of heavy rainfall, frequent dew or over-head irrigation [1,2]. Although fungicides are effective to control this disease, breeding for resistance is preferred by tomato growers due to the costs involved in the management of the disease and their associated environmental hazards. However, because SLS is relatively easy to control with fungicides this disease has not been an important breeding priority in the past [3,4].

It has been reported that resistance to SLS is controlled by a single dominant gene [3]. While the majority of the source of resistance lines belongs to wild species including *S. peruvianum*, *S. glandulosum* and *S. pimpinellifolium*, the highest degree of resistance was found in *S. habrochaites* [1, 4]. In this study, 22 out of 700 accessions, mostly from *S. habrochaites* and *S. peruvianum,* had a score of 2.0 and 3.9 when scored on a scale of 0 to 9, where 0 = no disease and 9 = severe disease. The resistance was found to be associated with small fruit size and late maturity[5]. Useful levels of resistance have also been found in *S. pennelli, S. pimpinellifolium, S. chilense,* and *S. lycopersicum* var. *cerasiforme.* Breeding lines of interspecific crossing with *S. habrochaites* accessions have shown high level of resistance. However, these interspecific lines had one or more undesirable horticultural traits such as

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indeterminate growth habit, late maturity, small fruits or low yield.

Breeding for SLS resistance was not a priority for tomato breeders for a long time. However, SLS has become a major problem in Canada and Northeastern America [6,7,8] and North Carolina(NC) (Randy Gardner, *personal communication*). The level of intensity of the disease has become so high that it may be even more severe than early blight (Randy Gardner, *personal communication*). Because of the magnitude of the problem, breeders at Cornell University have begun to introgress SLS resistance into tomato breeding lines and NC State is following suit. As discussed above, sources of SLS resistance are available but resistance is linked with horticulturally unacceptable traits. One of the ways to mitigate this problem is to use molecular markers. Molecular markers linked to the gene(s) of interest can be used to select the plants that are genetically similar to the recurrent parent possessing the desired horticultural traits. However, due to lack of molecular markers linked to the SLS resistance in tomato, marker assisted selection (MAS) has not been initiated for SLS resistance.

Michelmore et al. (1991) developed a rapid and simple PCR based method, which was called bulked segregant analysis (BSA), to identify single genes linked to a trait [9]. Using this approach, they identified random amplified polymorphic DNA (RAPD) markers linked to the downy mildew resistance gene in lettuce. For BSA, any kind of mapping population (e.g. recombinant inbred lines (RIL), backcross (BC), F_2 or double haploid (DH) that are segregating for a trait of interest can be used.

Many disease resistance genes have been identified in tomato using RAPD following the BSA approach. For example, De Giovanni et al. (2004) identified RAPD marker linked to the *ol-2* gene conferring resistance to powdery mildew using BSA in F_2 population [10]. Stevens et al. (1995) and Chague et al. (1996) identified RAPD markers linked to the *Sw-5* gene, resistance to tomato spotted wilt virus (TSWV) [11,12]. Smiech et al. (2000) used BSA in an F_2 segregating population and found five primers that distinguished resistant and susceptible bulks. In this study, we used BSA technique to identify RAPD markers linked to SLS resistance in tomato using an F_2 population [13]. As explained by Michelmore et al. (1991), this is an appropriate starting point for molecular studies of disease like SLS in tomato [9].

Material and Methods
Plant materials

Two tomato inbred lines, NC 85L-1W (2007) (referred onward as NC 85L) and NC 839-2(2007)-1 (referred onward as NC 839) were used to produce an F_2 population in the greenhouse. NC 85L, was used as a female and is susceptible to SLS (susceptible parent, SP) and NC 839, was the male and is resistant to SLS (resistant parent, RP). The source of resistance in NC 839 traces back to LA3707, a *S. pimpinellifolium* line (Randy Gardner, *personal communication*). A total of 250 F_2 plants, and 10 plants each of SP, RP and F_1 were grown at the Mountain Horticultural Crops Research and Extension Center (MHCREC), Mills River, NC. Among F_2 plants, data could not be recorded from 16 plants, which were used as missing points. Therefore, we used observations from 234 F_2 plants for data analysis. The fruits of NC 85L were mini-roma type with dark red color whereas NC 839 was a grape tomato with light red fruit color (**Table 1**).

The NC 85L selection was made for late blight and early blight resistance in the disease nursery at Waynesville, NC and the NC 839 selection was made at Mills River for outstanding fruit and plant type along with SLS resistance.

Field evaluation

Seeding was done on June 1, 2009 in 30.5 x 45.5 cm trays containing peat moss and vermiculite. Trays were kept in the greenhouse at an average temperature of 21.1^{o}C. Twelve-day old seedlings were transplanted in a 12.7 x 24.4 cm 50-cell tray. Six-week old seedlings were transplanted in the field with silty-loam soil with a row-to-row and plant-to-plant spacing of 150 cm and 45 cm, respectively. The beds were raised and covered with black plastic. Other recommended cultural practices were followed as described in the Southern US 2009 Vegetable Crop Handbook [14]. A total of 280 plants consisting of 10 plants each of SP, RP and F_1, and 250 F_2 plants were planted in a hotspot for SLS at the MHCREC, Mills River, NC in summer of 2009.

Data scoring and analysis

Disease severity was scored at 60 days after transplanting (August 17, 2009). Individual disease rating scores were based on visual assessment of

Table 1. Parental description along with their partial pedigree and coefficient of parentage in the population used for tagging septoria leaf spot resistance gene in tomato.

Parent	Maturity	Fruit characters	Septoria leaf spot reaction	Pedigree	Common pedigree	COP
NC 85L-1W (2007)	Early	Mini roma type, dark red	Susceptible	NC051(x)-18//0463/9722(x)-18	NC0179(x)-1-18-4, NC215E-1(93), NC9722(x)-18, NC051, NC03220, LA3707	0.23
NC 839-2 (2007)-1	Average	Grape type, light red	Resistant	NC051(x)-18//CB25(x)-18-3/9722(x)-18/0464		

severity. The following scoring criteria were developed based on [15,16] and used in this study:

    0   = no disease symptoms
    0.5 = Less than 10% leaf area with symptoms
    1   = 10-20% leaf area with symptoms
    1.5 = 20-30% leaf area with symptoms
    2   = 30-40% leaf area with symptoms
    2.5 = 40-50% leaf area with symptoms
    3   = 50-60% leaf area with symptoms
    3.5 = 60-70% leaf area with symptoms
    4 = 70-80% leaf area with symptoms
    4.5 = 80-90% leaf area with symptoms
    5 = 90-100% leaf area with symptoms

For the inheritance study, we grouped the segregating plants into resistance groups with scores from 0 to 2, and susceptible groups with a score from 2 to 5. Scores of parental lines and F_1 were an average of individual plants. Frequency of different score categories was estimated for F_2 populations using SAS v.9.1 for segregation analysis and frequency distribution. Skewness was estimated using SAS v.9.1. Frequency data were analyzed by χ^2 to test the goodness of fit for a single dominant gene using SAS v.9.1 [17].

DNA extraction, quantification and dilution

DNA was extracted from 2-3 weeks old plants following the method of Fulton et al. (1995). Approximately 100 mg of young leaves from 2-3 week old tomato seedlings were collected from the greenhouse in 1.5 ml Eppendorf tubes.[18] The tubes were dipped into liquid nitrogen and the samples were ground by glass rod. After adding 200 µL microprep buffers, samples were incubated in a 65°C water bath for about 60 min and filled with chloroform/isoamyl (24:1) solution. Samples were then centrifuged at 10,000 rpm for 5 minutes. The aqueous phase was pipetted out into a new micro-centrifuge tube and 2/3 to 1 times the volume of cold isopropanol was added to precipitate the DNA. After centrifuging this sample at 10,000 rpm for 5 minutes, the DNA pellet remaining was separated and washed with 70% ethanol. The dry DNA pellet was re-suspended in 100 µL of TE buffer and stored at -20°C. The concentration of DNA was determined by Nanodrop (NanoDrop 1000, Thermo Scientific, DE, USA). Working solutions of DNA samples with a concentration of 20 ng/ µL were prepared from original DNA samples in TE buffer.

RAPD screening and Bulked Segregant Analysis

A total of 197 10-mer random amplified polymorphic DNA (RAPD) primers were used to screen parental lines using 20 ng DNA template. Primers polymorphic to parental lines were then used to screen resistant and susceptible bulks. Amplification reactions were performed in 10 µL reaction volume containing 1x buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂), 200 µM of each dNTP, 0.2 µM primer and 1 U Taq polymerase. About 15 µL mineral oil was overlaid on the reaction mixture. DNA amplifications were performed in thermal cycler (Eppendorf, NY) using the following cycling condition: one cycle of 92°C for 3 min; 45 cycles of 92°C for 30 seconds, 42°C for 1 min and 72°C for 30 seconds; one cycle of 72°C for 8 min followed by holding at 4°C.

Bulked segregant analysis (BSA) was performed following the method of Michelmore et al. (1991) [9]. Two DNA bulks, called resistant bulk (RB) and susceptible bulk (SB) were prepared from F_2 individuals. The RB consisted of 8 individuals with disease score of 0 and the SB contained 8 individuals with the score of 4 or 4.5 (**Figure 1**). DNA bulks were

prepared by pooling equal amounts of DNA of eight resistant and eight susceptible F_2 individual plants for RAPD analysis. PCR was run with polymorphic primers on the bulked samples using the same reaction conditions as described above. PCR was repeated for at least two times for those primers that were polymorphic between bulks.

Gel electrophoresis

All RAPD PCR products were analyzed in 2% agarose gels containing ethidium bromide in TBE buffer (40 mM Tris-borate, pH 8.0, 1 mM EDTA) with a 100 bp ladder. Electrophoresis was run at 135 V for 2 hr. Gels were rinsed with water to enhance contrast and photographed under UV light. RAPD fragments were scored as 1 for presence and 0 for absence. Bands size was estimated based on the 100 bp DNA ladder. Simple statistics based on the DNA bands were calculated using MS Excel 2007.

Figure 1. Frequency distribution of 234 F_2 individuals derived from NC 085L-1W(2007) x NC839-2(2007)-1 based on the score of infestation of septoria leaf spot in tomato at Mills River, NC in 2009. Figure shows the bulked segregant analysis method adopted in this study and schematic representation of RAPD band linked to resistance gene. The average phenotypic values of the parents and F_1 are shown by arrow. SP = Septoria leaf spot susceptible parent, NC 85L-1W(2007). RP = Septoria leaf spot resistant parent, NC 839-2(2007)-1. R = Resistant. S = Susceptible. P = Parent. B = Bulk.

Results
Segregation of resistance

Severity of SLS infestation was assessed in 234 individual plants at 60 days after transplanting, based on the percentage of total leaf area infected. Only 234 plants out of 250 were scored as some of the plants were dead or malformed. No symptoms were observed in the resistant parent, but the susceptible parent had intermediate levels of SLS infection (**Figure 1**). Comparing the SLS scores of F_2 individuals with their parents, it was clear that transgressive segregation was found towards susceptibility. The distribution of disease reaction was highly left-skewed (**Figure 1**). This suggests that the susceptible parent may also have contribution to resistance. Based on the distribution of F_2 individuals, we found two distinct groups of resistant and susceptible plants. This allowed us to readily perform BSA to identify the linked markers.

About fifty percent of the F_1 plants had a disease score of 0 indicating that resistance to SLS was incomplete dominant. Among 234 F_2 individuals, 164 were resistant (0-2 score) and 70 were susceptible (2-5 score). The segregation ratio of resistant and susceptible plants fit the expected ratio of 3:1 for a single dominant gene ($\chi^2 = 3.014$, $p > 0.05$) which indicated that the inheritance of resistance to SLS was based on a single dominant gene in the present study.

RAPD markers and Bulked Segregant Analysis

Out of the 197 RAPD primers used to screen parent lines, 34 (17.26%) were polymorphic (Data not shown). A total of 176 bands with a maximum fragment size of 1500 bp and minimum fragment size of 100 bp were amplified using 34 primers. Among these fragments, 84 were polymorphic between parents. The 34 polymorphic RAPD primers were used to screen the resistant and susceptible bulks and 11 exhibited polymorphisms between resistant and susceptible bulks (Data not shown). A total of 87 bands were amplified by 34 RAPD primers. Among these bands, 34 and 20 bands were polymorphic between the parents and bulks, respectively. The size of bands ranged from 150 to 2000 bp. Five primers were linked to SLS reaction (**Table 2**).

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One band of each of two primers, namely MRTOMR-121 and MRTOMR-031 (**Figure 2**) was found only in the susceptible parent NC 085L and the susceptible bulk. Similarly, one band of each of three RAPD primers (MRTOMR-022, MRTOMR-117 and MRTOMR-121) was amplified only in the resistant parent NC 839 and the resistant bulk. Amplified band sizes linked to susceptibility were 800 and 600 bp whereas those linked to resistance ranged from 600 to 1000 bp (**Figure 3**).

Six primers were not linked to any of the loci (**Figure 4**). These primers distinguished only the parents and not the bulks. Some of the amplified bands were only found in bulks but not in either parent (**Figure 4**). This may be due to recombination in F_2 population.

Table 2. Polymorphic bands of RAPD markers linked to either resistance or susceptible genes of tomato to septoria leaf spot.

Marker	Sequence	PBN	Size, bp	SP	RP	RB	SB
MRTOMR-022	AGGGC CAGC	1	**1000**	0	1	1	0
		2	800	0	1	0	0
		3	600	1	0	1	1
		7	250	1	0	1	1
		8	150	1	0	1	1
MRTOMR-031	GGGAC GTCGC	1	1100	0	1	1	1
		3	600	1	0	0	1
MRTOMR-117	CCGAA CAATC	2	**850**	0	1	1	0
MRTOMR-118	TGCTTG GGGG	3	800	1	0	0	0
		4	750	0	1	0	0
		5	650	1	1	1	1
		6	**600**	0	1	1	0
MRTOMR-121	GGCGTC GTAA	1	1100	0	0	0	1
		3	900	1	1	0	0
		4	850	1	1	0	1
		5	**800**	1	0	0	1
		6	650	1	1	1	0
		7	420	1	0	0	0
		8	380	1	0	0	1

Discussion

Resistance to SLS in tomato was found to be controlled by a single incomplete dominant gene in this study. Andrus and Reynard (1945) also reported that SLS resistance was dominant and named it the *Se* gene.[1] However, Wright and Lincoln. (1940) have reported recessive gene conferring resistance to the SLS in the field observation in the past studies.[19] The differences observed in the

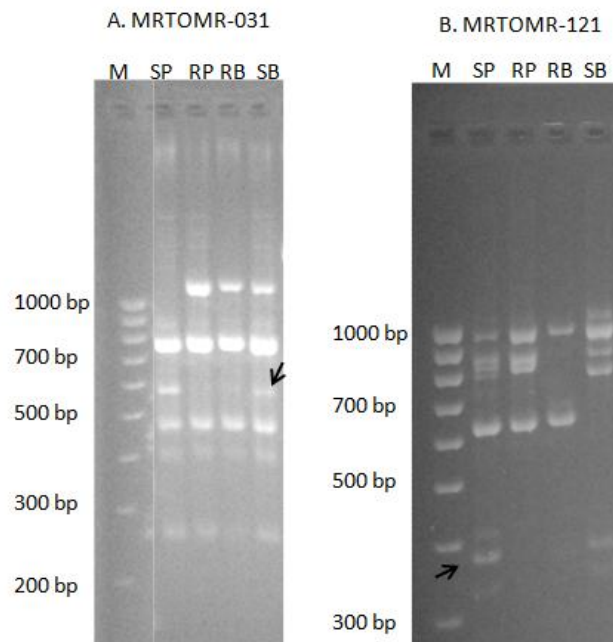inheritance of resistance in the present study from the past studies might be due to use of different

Figure 2. Electrophoresis pattern of DNA fragments generated by RAPD markers (A. MRTOMR-031, B. MRTOMR-121). Polymorphic band (i.e. linked to susceptible) between parents, and between resistant and susceptible bulks are indicated by arrow. SP = Susceptible parent, NC 085L. RP = Resistant parent, NC 839. RB = Resistant bulk. SB = Susceptible bulk. M = Marker.
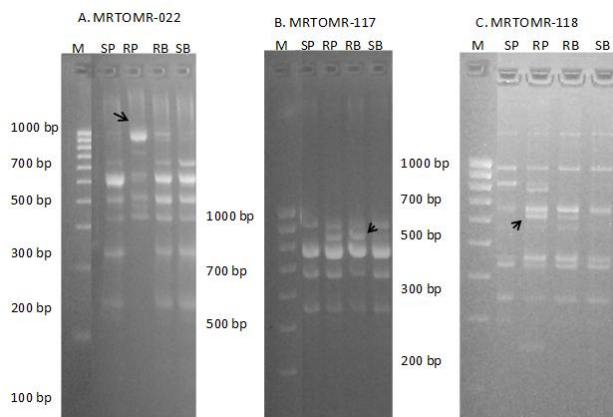

Figure 3. Electrophoresis pattern of DNA fragments generated by RAPD marker (A. MRTOMR-022, B. MRTOMR-117 and C. MRTOMR-118). Polymorphic band (i.e. linked to resistance) between parents and between resistant and susceptible bulks are indicated by arrow. SP = Susceptible parent, NC 085L. RP = Resistant parent, NC 839. RB = Resistant bulk. SB = Susceptible bulk. M = Marker.

sources of resistance. The susceptible parent used in this study did not appear completely susceptible suggesting that there may be its allelic difference in the expression of resistance. In fact both parents, NC
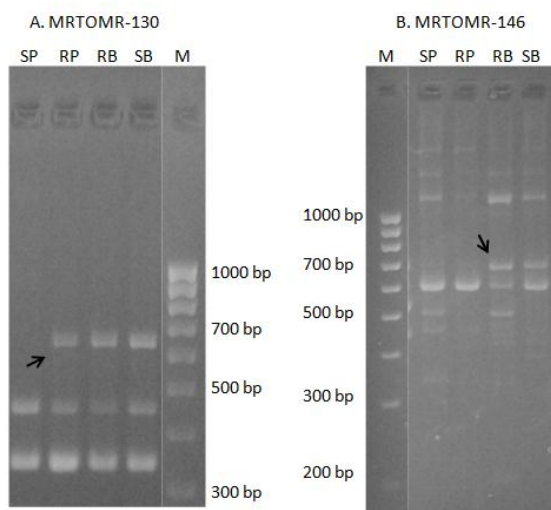
Figure 4. RAPD marker (A. MRTOMR-130) showing polymorphic band (indicated by arrow) only to parents, i.e. band with unlinked loci and RAPD marker (MRTOMR-146) showing band (indicated by arrow) only in two bulks. SP = Susceptible parent, NC 085L. RP = Resistant parent, NC 839. RB = Resistant bulk. SB = Susceptible bulk. M = Marker

85L and NC 839 have a coefficient of parentage (COP) of 0.23 (**Table 1**) indicating that they have common parentage. This fact has been confirmed based on their common pedigree (Randy Gardner, *personal communication*).

Based on the field screening of the F_2 population with 197 RAPD primers, we identified three RAPD markers linked to resistance alleles and two RAPD markers linked to susceptible alleles. Through the bulking of the extreme individuals segregating in the F_2 population we were able to rapidly tag the markers associated with chromosomal segment that has a role in reaction to SLS in tomato. For BSA consisting of eight individuals in each bulk, five primers yielded different banding patterns, which were useful markers in SLS screening in tomato. Bands of two of these markers were only present in susceptible parent and bulk, and bands of three markers were present only in resistant parent and bulk. Therefore, these bands were considered associated either susceptible allele or resistant allele. Tagging of resistance genes using BSA is very fast, which facilitates the screening of new alleles of resistance for a particular disease, especially for one that does not have background information available such as SLS in tomato. The two parental lines used in this study are closely related to each other (COP=0.23). However, we found RAPD to distinguish these parents at the molecular level.

RAPDs are multi locus-based markers. Therefore, the primers identified might be from the same regions of the chromosome. For example, MRTOMR-022 produced a 1000 bp band and MRTOMR-118 produced a 600 bp band. The band produced by MRTOMR-118 might be the part of the band produced by MRTOMR-022. The disadvantages associated with RAPDs include the fact that they anneal in multiple sites, and they are dominant in nature, and sensitive to reaction conditions, which may limit their use directly in MAS. Therefore, these RAPD markers need to be converted to sequence characterized amplified region (SCAR) or cleaved amplified polymorphic sequence (CAPS), which are much more useful for MAS.

Through BSA, marker development and MAS has been used for the selection of resistance to a number of diseases in tomato. For example, De Giovanni et al. (2004) identified RAPD marker linked to the *ol-2* gene conferring resistance to powdery mildew.[10] A single RAPD marker, OPU31500 with 1500 bp in size was detected in the susceptible bulk, which was converted into a CAPS marker. Stevens et al. (1995) and Chague et al. (1996) identified RAPD markers linked to the *Sw-5* gene conferring resistance to tomato spotted wilt virus (TSWV).[11][12] Among the four RAPD markers, two were tightly linked to *Sw-5* gene. Linkage analysis mapped these markers within a distance of 10.5 cM from *Sw-5*. Czech et al. (2003) have used MAS using a co-dominant marker through BSA for developing TSWV resistant tomato.[20] Smiech et al. (2000) used BSA in F_2 segregating population and found 5 primers that distinguished resistant and susceptible for TSWV. [13] A PCR-based co-dominant marker, tightly linked to *Mi* was developed using the information from BSA [21](Williamson et al. 1994). In light of these past reports, the five RAPD primers identified in the present study may be informative to develop co-dominant markers for SLS resistance breeding.

RAPD markers identified here needs to convert into SCAR or CAPS marker for MAS of resistance to SLS in tomato. The MAS is cost effective and more reliable for screening, because it does not need to have a pathological evaluation and can genotype at any growth stage. Molecular markers linked to the SLS resistance in tomato may also have a potential

role on gene pyramiding. To our knowledge, there are no any molecular markers reported associated with SLS resistance in tomato. Molecular markers identified in this study are novel, and provide enough background to develop different group of markers (SCAR or CAPS) which may be useful for speeding up the tomato breeding program aiming to improve SLS resistance.

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