



OPEN Evaluation of soybean germplasms for resistance to stay-green syndrome

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Stay-green syndrome (SGS) resistant germplasms serve as the cornerstone for soybean improvement. A comprehensive assessment was conducted on a panel of 1553 germplasms to evaluate their resistance to SGS through natural inoculation. Over a three-year period, one landrace, ZaoShuHeiDou, emerged as resilient to SGS, displaying a significantly reduced risk of SGS ($p < 0.05$) compared to its counterparts. The broad-sense heritability of SGS resistance was 0.86. Notably, landraces demonstrated a substantially lower incidence of SGS compared to improved cultivars. Furthermore, the severity of SGS correlated with several key traits pertinent to soybean improvement efforts. In field trials, the application of insecticides effectively mitigated SGS severity, whereas the use of organic fertilizers and soil sterilization had no discernible impact. A total of 83 DNA samples were successfully amplified from SGS-afflicted plants using specific primers for soybean stay-green associated virus (SoSGV), and a SoSGV LCU-1 strain was isolated. The qPCR analysis confirmed a positive correlation between SGS severity and the relative SoSGV copy number in afflicted plants. The findings of this study lay a solid foundation for the development of SGS-resistant cultivars and the exploration of underlying resistance mechanisms.

Keywords Soybean stay-green syndrome, Germplasm, Soybean stay-green-associated virus, Huang-Huai region

Abbreviations

ANOVA	Analysis of variance
BS	Biological status
CC	Cotyledon color
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
EBN	Effective branch number
ER	Eco-region
FC	Flower color
FD	Flowering days
GH	Growth habit
GP	Growth period
H ²	Broad-sense heritability
HC	Hilum color
LCU	Liaocheng University
Lodg	Lodging
LS	Leaf shape
MG	Maturity group
NCBI	The National Center for Biotechnology Information
NNMS	Nod number on main stem
OC	Oil content
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction

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PH	Plant height
HBP	Height of bottom pod
PNPP	Pod number per plant
PodL	Pod length
PodW	Pod width
PodC	Pod color
ProC	Protein content
PubC	Pubescence color
PubD	pubescence density
p	Probability value
qPCR	Quantitative polymerase chain reaction
R1	Soybean developmental stage of beginning bloom
R8	Soybean developmental stage of full maturity
r	Pearson Correlation Coefficient
SAS	Statistics analysis system
SCC	Seed coat color
SGS	Stay-green syndrome
SL	Seed length
SNPP	Seed number per pod
SoSGV	Soybean stay-green associated virus
SS	Seed size (100 seeds weight)
ST	Stem termination
SW	seed width
SWPP	Seed weight per plant

Stay-green syndrome (SGS) in soybeans (*Glycine max* L. [Merr.]), also known as Zhengqing in Chinese, is a devastating disease characterized by green stems, leaves, and flat pods at the mature stage. SGS poses a significant threat to soybean production in the Huang–Huai region of China, leading to 20 to 84% yield losses or even crop failure depending on variety genotypes or environments^{1–3}. The earliest report of SGS dates back to 1977 in Shangqiu County, Henan Province, China⁴. Since then, extensive research has been conducted to investigate the causes of SGS. Initially environmental factors, such as drought and high temperatures⁵, boron deficiency^{4,6}, and cropping patterns⁷, were considered as primary contributors. Otherwise, phytohormones especially cytokinins play important roles for stay-green phenotype in tobacco⁸, *Arabidopsis*⁹ and ryegrass¹⁰. Wei et al.¹¹ also reported the expression levels of the cytokinin biosynthesis-/signaling pathway-related genes in stay-green soybean leaves were significantly higher than those in control leaves, implying that the cytokinin pathway is involved in SGS.

In order to eliminate the influence of SGS on soybean production, Guo et al.¹ found that insecticides could effectively alleviate SGS severity, suggesting the involvement of insects in its spread. Subsequently, feeding by the bean bug (*Riptortus pedestris*) was demonstrated to be the primary cause of SGS¹². Nonetheless, a widespread SGS outbreak in Yingshang County, Henan Province, China, in 2019 occurred despite low density of *R. pedestris* (0.4 per 100 plants) indicating the presence of other factors¹³. In 2022, three independent research groups^{14–16} identified a novel geminivirus, soybean stay-green-associated virus (SoSGV), as the causative agent of SGS. Similar geminiviruses have also been found in the northeast of China¹⁷ and South Korea¹⁸, suggesting that the SGS has a potential to expand to other soybean growing areas. Additionally, recent studies have demonstrated that the common brown leafhopper (*Orosius orientalis*), rather than *R. pedestris*¹¹, serves as the vector of geminiviruses^{19,20}.

Insecticides remain the primary strategy for controlling SGS, given that insect pests are either the direct or indirect agents of the disease. However, the widespread application of insecticides can lead to issues such as increased costs, environmental pollution, and the elimination of beneficial insect populations. Therefore, breeding cultivars that are resistant to SGS is a more viable and sustainable solution. Previous studies have revealed substantial variability in SGS resistance among improved cultivars^{1,21}, highlighting the potential for breeding SGS-resistant cultivars. However, the breeding process has been hampered by the scarcity of resistant germplasms. The shortage may be attributed to two primary factors: the limited availability of soybean accessions and the lack of genetic diversity in germplasm resources. For instance, Yan et al.²² evaluated the SGS resistance of only 16 improved cultivars in the Huang–Huai region and found no significant differences among them. Another challenge is the lack of precise SGS resistance assessments. Currently, most assessments rely on a single year of natural inoculation in the field, but the conditions for natural inoculation can vary from year to year³. Therefore, to identify stable and resistant germplasms from a diverse array of accessions, repeated assessments over multiple years under natural conditions are necessary.

Until the causative pathogen of SGS is identified, establishing a stable natural pathogenic environment is crucial for the identification of SGS-resistant germplasm. A scientific experiment field situated on the East Campus of Liaocheng University in the central Huang–Huai region of China, has served this purpose. Soybeans were first plant there in 2008. Initially, and for several subsequent years, there were only a few SGS-afflicted plants in the field. In recent years, however, the severity of SGS in this field has progressively increased each year (Supplementary Fig. S1 A–E). Because this field could provide a stable pathogenic environment and SoSGV was identified as the causative agent of SGS in 2022^{14–16}, we utilized this field between 2020 and 2022 to screen for SGS-resistant germplasms and to investigate the underlying causes of SGS (Fig. 1). This study will lay a solid foundation for breeding SGS-resistant cultivars, thereby safeguarding soybeans in the Huang–Huai region from the detrimental effects of SGS.

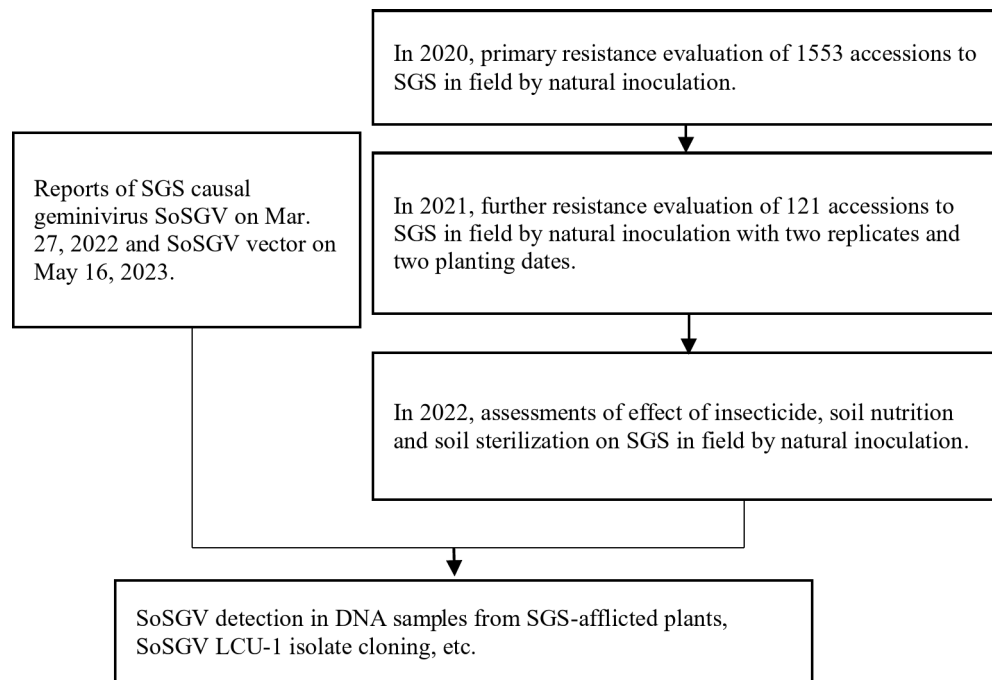


Fig. 1. Simplified flow chart of evaluation of soybean germplasms for resistance to SGS.

Results

Preliminary screening for SGS-resistant germplasm

On June 30, 2020, 1553 soybean germplasms (Supplementary Table S1) were randomly planted in the experimental field. Standard field management practices were followed, except for insect protection between the developmental stages R1 and R8²³. The primary objective of this experiment was to conduct an initial screening to identify SGS-resistant germplasm for further investigation in the following year. Therefore, only the accessions with no symptoms and those with very severe symptoms were recorded using a visual assessment scale ranging from 1 to 7. As a result, in 2020, we successfully screened out 140 accessions with no SGS symptoms (level 1) and 13 accessions with the most severe SGS symptoms (level 7) (Supplementary Table S1).

The assessments of Stay-Green Syndrome resistance for 121 germplasms

Based on seed quantity and quality, 113 out of 140 accessions at SGS resistance level 1, and 8 out of 13 accessions at level 7 were selected for further evaluation. The list of accessions can be found in Supplementary Table S2. The selected accessions were planted in a randomized complete block design experiment in the field, with each accession replicated twice. The planting dates were June 15 and July 1, 2021. The SGS levels of these accessions were observed to range from 1.75 to 6.50, with an average SGS severity score of 5.67. As shown in Fig. 2, the majority of the accessions evaluated in 2021 exhibited SGS severity levels close to 6, indicating that they had severe SGS symptoms with only a few normal pods on the plants. However, despite the overall high severity, a few accessions were identified as being resistant to SGS (Fig. 3).

The analysis of variance (ANOVA) results indicated that the SGS levels were significantly affected by the genotype (germplasm) ($p < 0.0001$), planting date ($p = 0.001$), and interactions between genotype and planting date ($p = 0.04$), but not by the replicates ($p = 0.99$) (Supplementary Table S3). The F-test results showed that both genotype ($p < 0.0001$) and planting date ($p = 0.005$) had significantly larger mean squares than the interaction between genotype and planting date, when considering the mean square of the interaction between genotype and planting date as the denominator (Supplementary Table S3). This indicates that genotype and planting date are more important factors influencing SGS severity than their interaction. According to the results of the ANOVA, the broad-sense heritability of SGS was determined to be 0.86, suggesting that the SGS levels were primarily controlled by genotype. Breeding could, therefore, effectively improve SGS resistance if SGS-resistant germplasms are identified. According to the multiple comparison results, two landraces, LaoBaiDou and ZaoShuHeiDou, had significantly lower SGS levels than the other accessions, at a significance level of $p = 0.05$ (Table 1).

In terms of the relationships between SGS levels and other characteristics, the landrace accessions exhibited lower SGS levels than the improved cultivars (Fig. 4A). Furthermore, the rampant accessions displayed reduced SGS levels in contrast to both erect and semi-erect varieties (Fig. 4B). Similarly, the prostrate accessions also showed lower SGS levels when compared to the other accessions (Fig. 4C). Overall, accessions belonging to the early maturity group had higher SGS levels than those in the late maturity group (see Fig. 4D). Notably, accessions from the Huang-Huai eco-region (HH) had significantly lower SGS levels than those from other eco-regions (see Fig. 4E). However, there were no differences between the different types in terms of stem

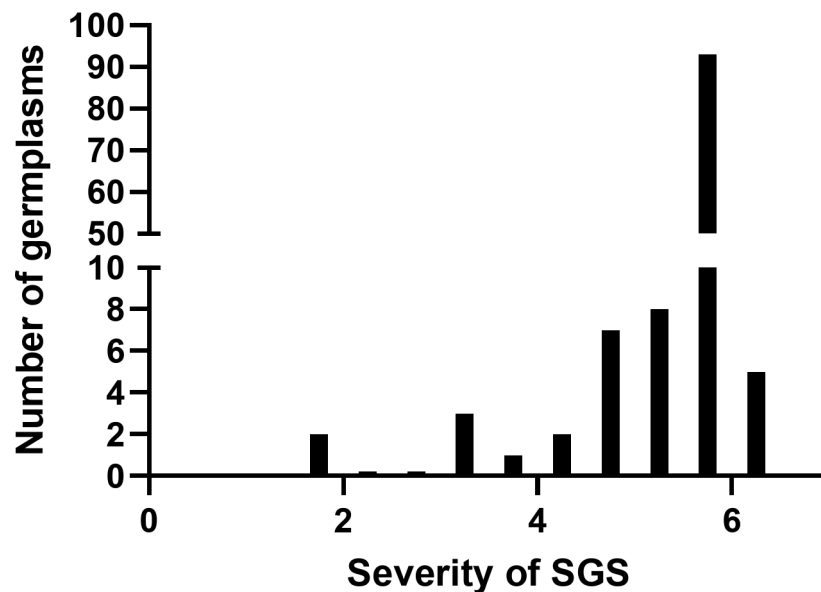


Fig. 2. Distribution of germplasms by severity of SGS in 2021.



Fig. 3. SGS evaluation for some accessions in 2021. (A) BaiJiaZi, mean SGS level of 5.88; (B) ZaoShuHeiDou, mean SGS level of 1.88; (C) LaoBaiDou, mean SGS level of 1.75.

termination, leaf shape, flower color, pod color, pubescence color, pubescence density, seed coat color, cotyledon color, and hilum color (Fig. 4F–N).

In terms of other quantitative traits, SGS levels showed significant positive correlations with soybean improvement traits, such as oil content ($r=0.32$, $p<0.01$), seed width ($r=0.22$, $p<0.05$), pod width ($r=0.27$, $p<0.05$), pod length ($r=0.24$, $p<0.05$), and seed size ($r=0.20$, $p<0.05$). Conversely, SGS levels showed significant negative correlations with soybean attenuation traits during domestication, such as plant height ($r=-0.52$, $p<0.001$), pod number per plant ($r=-0.43$, $p<0.001$), growth period ($r=-0.37$, $p<0.001$), effective branch number ($r=-0.31$, $p<0.01$), flowering date ($r=-0.33$, $p<0.01$), and seed weight per plant ($r=-0.22$, $p<0.05$). However, there were no significant relationships between SGS levels and other quantitative traits, such as seed number per pod, seed length, protein content, and pod height at bottom (Supplementary Table S4). As regards planting date, the average SGS level for early planting was 5.75, which was significantly higher than that of 5.61 for late planting.

Finally, seven germplasms with the lowest SGS levels and seven with the highest SGS levels (Table 1) were selected to study the effects of insecticide, soil sterilization, and soil nutrition on the severity of SGS in the following year.

Principal Component Analysis (PCA)

The eight principal components (PC1 to 8) with eigenvalues greater than 1.00 explained 75.59% of the total variation for all characteristics (Table 2). The first principal component (PC1) made up 31.07% of total variation and had an eigenvalue of 9.32. The main contributors for PC1 included some domestication-related traits, such as pod number per plant (0.29), plant height (0.27), effective branch number (0.26), nod number on main stem (0.25), lodging (0.23), flowering days (0.26), and oil content (-0.25). The eigenvalue of PC2 was 4.04, and it

Germplasms	Biological status	SGS level in 2021
LaoBaiDou	Landrace	1.75a
ZaoShuHeiDou	Landrace	1.88a
ZheChengXiaoHongDou	Landrace	3.13b
XiXianPingDingShiDaDou	Landrace	3.38bc
YanYaoDou	Landrace	3.38bc
XiChuanJiWoHuang	Landrace	3.88 cd
ZhongPin11091	Improved cultivar	4.25d
KeFeng 1	Improved cultivar	5.88e
JiDou17	Improved cultivar	6.00e
Weij127	Improved cultivar	6.00e
ZhongHuang35	Improved cultivar	6.00e
ZhongHuang57	Improved cultivar	6.00e
Er-Huan-Yan	Improved cultivar	6.25e
MN0201	Improved cultivar	6.25e

Table 1. The accessions with the lowest or the highest SGS levels in 2021. The SGS levels with the same lowercase letter did not vary significantly at $p=0.05$ level.

accounted for 13.47% of the total variation. Three seed quality related traits (seed width (0.36), seed size (0.35) and seed length (0.33)), two maturity related traits (maturity group (0.33) and growth period (0.30)), and one yield related trait (seed weight per plant (0.30)) mainly contributed to this PC. The third PC accounted for 7.97% of total variation and had an eigenvalue of 2.39. The traits that mainly contributed to this variation were seed quality related traits, seed coat color (0.38), protein content (-0.35), seed length (-0.29) and oil content (-0.27). PC4 accounted for 6.34% of total variation and had an eigenvalue of 1.90. Pubescence color (0.48), hilum color (0.33), pod color (0.31), biological status of accessions (-0.32), protein content (-0.33) and seed number per pod (0.34) were the main contributor to this PC. The fifth PC accounted for 5.07% variance and took an eigenvalue of 1.52 with stem termination (0.64) and height of bottom pod (0.39) being the major contributor. PC6 took a proportion of 4.64% variance and possessed an eigenvalue of 1.39. Flower color (0.64) and height of bottom pod (0.40) mainly contributed to this PC. PC7 occupied amount of 3.67% variance and bore an eigenvalue of 1.10. This PC depended mainly on cotyledon color (0.54), pubescence density (0.38) and eco-region (-0.38). The final PC8 took a share of 3.36% variance and owned an eigenvalue of 1.01 with two yield important traits, seed number per pod (0.43) and seed weight per plant (0.36), as the main contributors.

The relationships between the different characteristics and the SGS levels of accessions with respective principal components were further illustrated by the loading plot in Fig. 5A and the score plot in Fig. 5B. In Fig. 5A, SS and SW almost located at the same position indicating they played almost the same role for PC1 and PC2. MG, GP and SWPP also played similar roles for these two PCs. Some domestication-related traits that were usually strengthened for landrace or wild soybean, such as PNPP, NNMS, PH, EBN, GH, and Lodg, contributed positively for PC1. On the contrary, the traits that were strengthened for improved cultivars, such as OC, SS, SW, PodW, and PodL, contributed negatively for PC1. In Fig. 5B all the accessions that bore SGS levels < 4 located on the right part of PC1, indicating an accession that grew more like landraces or wild soybeans was more likely to have better SGS resistance.

Effect of insecticide, soil sterilization, and soil nutrition on Stay-Green Syndrome severity in the field

The ANOVA results indicated the insecticide ($p<0.0001$) and the germplasm ($p<0.0001$) had conclusively effects on SGS severity; however, the organic fertilizer and the soil sterilization had no significant effects (Supplementary Table S5). The flat pod rate of the insecticide-sprayed treatments (0.14) was significantly lower than that of the control treatments (0.33). The flat pod rates of the fourteen accessions ranged from 0.06 to 0.31, with an average of 0.17, respectively. In the field, the SGS-resistant accessions outperformed the SGS-sensitive accessions (Supplementary Fig. S2). ZaoShuHeiDou, the most SGS-resistant accession, had the lowest flat pod rate (0.06), which was significantly lower than that of the SGS-sensitive accessions, at a significance level of $p<0.05$ (Supplementary Table S6).

Furthermore, the SGS levels in 2021 were significantly and positively correlated with the flat pod rates in 2022 ($r^2=0.68$, $p=0.0003$), suggesting that the ocular estimate results in 2021 were reasonably consistent with those in 2022 (see Supplementary Fig. S3).

Detection of Soybean Stay-Green-associated Virus in Stay-Green Syndrome-afflicted plants and the cloning of the Soybean Stay-Green-associated Virus LCU-1 isolate

A set of 83 DNA samples collected from the SGS-afflicted plants in the field were evaluated with five pairs of SoSGV-specific primers (P1–P10) (Supplementary Table S7). The amplification results showed that all samples were successfully amplified by at least one of the five primer pairs, suggesting that SoSGV was the main cause of SGS in this study (Fig. 6A). Then, new cloning primers were designed (P11–P22, Supplementary Table S7), and a SoSGV isolate, here named the LCU-1 isolate, was obtained (GenBank: OR759770.1) (Fig. 6B). The full

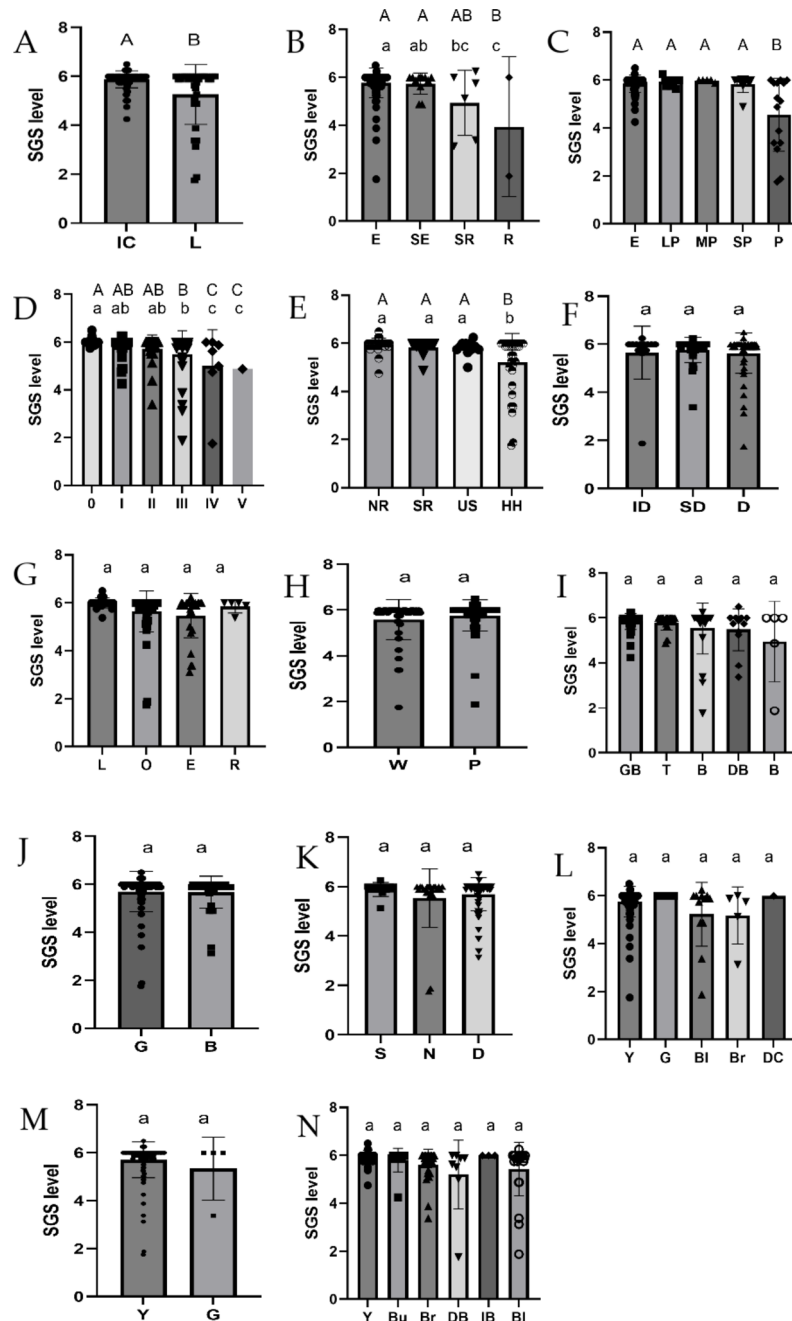


Fig. 4. The relationship between the SGS levels and other morphological traits in soybean. **(A)** Biological status of accession: IC (improved-cultivar), L (landrace); **(B)** Growth habit: E (erect), SE (semi-erect), SR (semi-rampant), R (rampant); **(C)** Lodging: E (erect), LP (lightly prostrate), MP (much prostrate), SP (severe prostrate), P (prostrate); **(D)** Maturity group: 0 (MG 0), I (MG I), II (MG II), III (MG III), IV (MG IV), V (MG V); **(E)** Eco-region: US (United States of America), NR (northern eco-region of China), HH (Huang-Huai eco-region of China), SR (southern eco-region of China); **(F)** Stem termination: ID (indeterminate), SD (semi-determinate), D (determinate); **(G)** Leaf shape: L (lanceolate), O (ovate), E (ellipse), R (round); **(H)** Flower color: W (white), P (purple); **(I)** Pod color: GB (grayish brown), T (tan), Br (brown), DB (dark brown), B (black); **(J)** Pubescence color: G (gray), B (brown); **(K)** Pubescence density: S (sparse), N (normal), D (dense); **(L)** Seed coat color: Y (yellow), G (green), Bl (black), Br (brown), DC (di-color); **(M)** Cotyledon color: Y (yellow), G (green); **(N)** Hilum color: Y (yellow), Bu (buffer), Br (brown), DB (dark brown), IB (imperfect black), Bl (black). The dots in the columns indicated the genotype number. Sample groups with the same lowercase letter were indistinguishable at $p < 0.05$, sample groups with the same uppercase letter were indistinguishable at $p < 0.01$. The standard deviation bars were shown in the columns.

Eigenvectors								
Characteristics	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Biological status (BS)	0.19	−0.01	0.21	−0.32	0.08	0.08	0.00	−0.08
Maturity group (MG)	0.15	0.33	−0.24	−0.02	0.03	−0.04	0.00	−0.16
Eco-region (ER)	0.17	0.13	−0.2	0.18	−0.03	−0.07	−0.38	−0.03
Leaf shape (LS)	0.08	0.22	0.07	−0.06	−0.32	−0.12	−0.07	−0.05
Growth habit (GH)	0.21	−0.12	0.21	−0.09	−0.25	0.10	0.10	−0.15
Seed coat color (SCC)	0.21	−0.07	0.39	0.15	0.01	0.04	−0.07	0.03
Stem termination (ST)	−0.05	0.08	0.09	−0.14	0.61	−0.04	−0.02	0.18
Pod color (PodC)	0.11	−0.06	0.01	0.31	−0.09	0.31	0.16	−0.25
Cotyledon color (CC)	0.01	0.05	0.21	0.17	0.16	−0.24	0.54	−0.17
Hilum color (HC)	0.22	0.09	0.01	0.33	0.04	0.00	−0.15	0.11
Pubescence color (PubC)	0.16	0.01	0.09	0.48	0.14	0.07	−0.29	0.09
Flower color (FC)	0.00	−0.10	−0.03	−0.11	−0.04	0.64	0.07	0.04
Pubescence density (PubD)	−0.07	−0.06	−0.17	0.21	0.26	−0.06	0.39	−0.23
Lodging (Lodg)	0.23	0.02	0.15	−0.10	0.08	0.11	0.10	−0.13
Flowering days (FD)	0.26	0.13	0.00	−0.16	0.25	−0.03	−0.01	0.01
Growth period (GP)	0.19	0.30	−0.22	0.03	0.02	−0.07	0.02	−0.25
Plant height (PH)	0.27	0.10	−0.16	0.00	−0.15	0.15	0.18	−0.02
Height of bottom pod (HBP)	0.03	0.20	0.00	0.00	0.39	0.40	−0.15	−0.29
Nod number on main stem (NNMS)	0.25	0.04	−0.26	0.02	−0.10	0.08	0.22	0.06
Effective branch number (EBN)	0.26	0.12	0.04	−0.11	0.05	−0.10	−0.04	0.28
Pod number per plant (PNPP)	0.29	−0.04	−0.04	−0.03	−0.08	−0.05	0.18	0.19
Pod length (PodL)	−0.22	0.24	0.14	0.05	−0.05	0.20	0.03	0.24
Pod width (PodW)	−0.19	0.26	0.22	0.01	0.04	−0.02	0.12	0.12
Seed weight per plant (SWPP)	0.16	0.30	−0.09	0.00	−0.02	−0.09	0.22	0.36
Seed size (100 seeds weight) (SS)	−0.20	0.35	0.06	0.05	−0.07	0.04	0.01	−0.13
Seed length (SL)	−0.10	0.33	0.29	0.00	−0.23	0.12	0.05	0.04
Seed width (SW)	−0.19	0.36	0.10	0.08	−0.05	0.04	−0.01	−0.08
Protein content (ProC)	−0.11	0.10	−0.35	−0.33	0.02	0.20	0.02	0.12
Oil content (OC)	−0.25	0.00	−0.27	0.08	−0.08	−0.12	−0.07	−0.18
Seed number per pod (SNPP)	−0.13	−0.07	−0.19	0.34	0.04	0.22	0.21	0.43
Eigenvalue (explained variance)	9.32	4.04	2.39	1.90	1.52	1.39	1.10	1.01
Proportion of total variance (%)	31.07	13.47	7.97	6.34	5.07	4.64	3.67	3.36
Cumulative variance (%)	31.07	44.54	52.51	58.86	63.93	68.57	72.23	75.59

Table 2. Principal component analysis for 30 characteristics.

genome length of the SoSGV LCU-1 isolate is 2762 nt. Seven open reading frames were annotated on this isolate genome, based on its sequence homology information. These frames included V2 (312 nt) and CP (786 nt) on the viral sense strand, and pre-CP (303 nt), C1 (1086 nt), C2 (417 nt), C3 (456 nt), and C4 (291 nt) on the viral complementary strand.

The phylogenetic tree of 59 homogeneous SoSGV sequences showed that these isolates could be divided into three groups. Group I included 35 isolates from the Huang–Huai region and northeastern China, and Group III included 21 isolates from the Huang–Huai region and South Korea. However, group II contained only two isolates. One was an isolate from Anhui Province, and the other was the SoSGV LCU-1 isolate (Fig. 6C).

The qPCR results indicated a linear positive correlation ($r^2=0.96$, $p<0.0001$) between the copy number of SoSGV and the flat pod rate (Fig. 6D), indicating that the flat pod rate is a reliable indicator of SGS severity.

Discussion

During domestication and modern breeding of crops, a decline in genetic diversity has been observed²⁴. This trend is also evident in soybean breeding practices²⁵. Modern soybean cultivars have been developed by pyramiding desired alleles in a specific eco-region, resulting in enhanced yields and improved quality. However, this selective process has led to the loss of certain so-called unfavorable alleles and their linked segments on chromosomes. For example, variations in allele frequencies key genes such as *E2* and *GmSPA3c*, which regulate flowering time, as well as the seed dormancy gene *G* have been documented among the improved cultivars, landraces, and wild soybeans²⁶. The soybean cyst nematode resistant alleles *rhg1-b*, *rhg1-a*, and *Rhg4*, which are widely used in the United States, are present in two landraces, PI88788 and Peking, but are absent in improved cultivars^{27–29}. Similarly, the high-protein haplotype of the *cqProt-003* locus is prevalent in wild accessions (94.7%), uncommon in landraces (10.6%), and nearly absent in cultivated breeding pools (4.1%)³⁰. In this study,

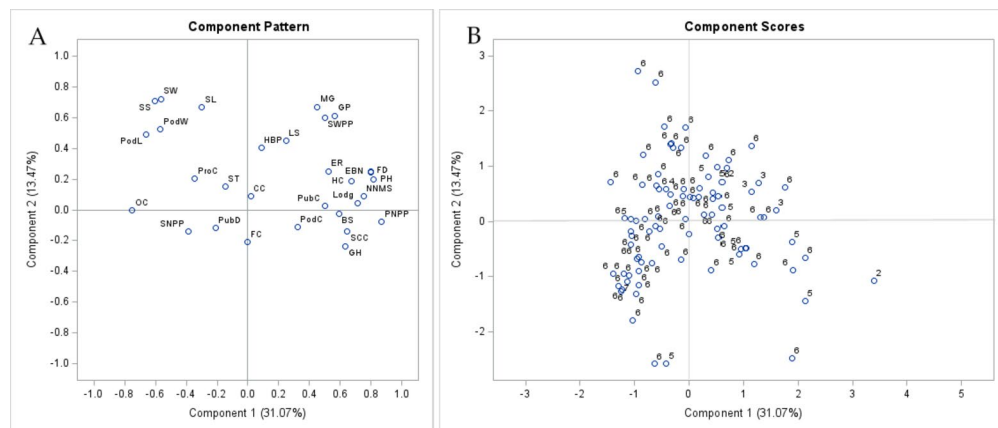


Fig. 5. Principal component analysis loading plot for 30 characteristics and score plot for different SGS levels based on the PC1 (Component 1) and PC2 (Component 2). **(A)** Loading plot for 30 characteristics. The location of a characteristics indicated its influence strength and direction on the PC1 and PC2. **(B)** Score plot for different SGS levels. The circle dots show the position of accessions in the quadrant with SGS levels marker near to them. *BS* biological status of accession, *MG* maturity group, *ER* eco-region, *LS* leaf shape, *GH* growth habit, *SCC* seed coat color, *ST* stem termination, *PodC* pod color, *CC* cotyledon color, *HC* hilum color, *PubC* pubescence color, *FC* flower color, *PubD* pubescence density, *Lodg* lodging, *FD* flowering days, *GP* growth period, *PH* plant height, *HBP* height of bottom pod, *NNMS* nod number on main stem, *EBN* effective branch number, *PNPP* pod number per plant, *PodL* pod length, *PodW* pod width, *SWPP* seed weight per plant, *SS* seed size (100 seeds weight), *SL* seed length, *SW* seed width, *ProC* protein content, *OC* oil content, *SNPP* seed number per pod.

landraces exhibited significantly lower level of SGS than improved cultivars. Accessions with unfavorable traits generally outperformed those with desirable traits. This was consistent with the results of PCA analysis that the PC1, primarily attributed to domestication-related traits, accounted for 31.07% of total variance. These results collectively suggest that SGS-resistant alleles may have been lost during the cultivar improvement process. Future efforts to rediscover these resistant alleles could potentially benefit from exploring landraces or wild soybeans.

In this study, there was consistent and conclusive variations observed among germplasms in both 2021 and 2022, aligning with the results of Guo et al.²¹. The orthogonal experiment conducted in 2022, revealed that the application of insecticide significantly reduced the severity of SGS, which is in agreement with previous studies^{1,22}, suggesting that insects were either directly or indirectly responsible for the occurrence of SGS in this study. However, the utilization of organic fertilizer and soil sterilization had no discernible impact on SGS severity, implying that soil nutrition and soil-borne diseases were not contributing factors in this particular field. Furthermore, the severity of SGS in 2021 was significantly higher in early-planted accessions compared to late-planted ones, which was consistent with the findings of Liu et al.³¹. Considering the fact that the insect pests may be the cause of SGS, the effect of planting date on SGS might be caused indirectly by altering the chance of insect feeding. Accessions from the Huang-Huai region of China had significantly lower incidences of SGS compared to those from other regions, possibly due to a higher proportion of SGS resistant accessions in this area than those in other areas because of the natural selection under SGS stress. Additionally, a significant negative correlation was found between SGS level and growth period. Specifically, accessions that matured later usually had a lower SGS level than those that matured earlier. This phenomenon may be attributed, at least in part, to the eco-regions of the accessions. In the early maturing maturity groups (0 and I), the majority of accessions were from the northern eco-region. In the later maturing maturity groups (II to IV), more than 40% of accessions were from the Huang-Huai eco-region.

The PCR results showed that all 83 SGS DNA samples could be successfully amplified by at least one pair of geminivirus-specific primers, indicating that, in accordance with previous studies^{14–16}, geminivirus was the primary cause of SGS in this study. Based on the available data, it can be preliminarily inferred that the SGS on the East Campus of Liaocheng University was primarily attributed to geminivirus, which was likely transmitted by insects.

The co-evolutionary battle between plants and geminiviruses persist. Plants have developed intricate defense mechanisms to combat viral infections, including RNA-silencing machinery, protein-kinase-mediated antiviral immunity, effector-triggered immunity, autophagy-mediated antiviral defense, an ubiquitin-proteasomal protein degradation system^{32,33}, phytohormone-mediated machinery^{32–34}, and the instability of the genome³⁵, among others. In this study, it was found that several SGS-resistant accessions harbored significantly lower levels of SoSGV compared to SGS-sensitive accessions, indicating that impeding the proliferation of SoSGV or repelling the insect feeding may be the ways exploited by SGS-resistant accessions. However, the strategies employed by these resistant accessions remain elusive and merit further exploration.

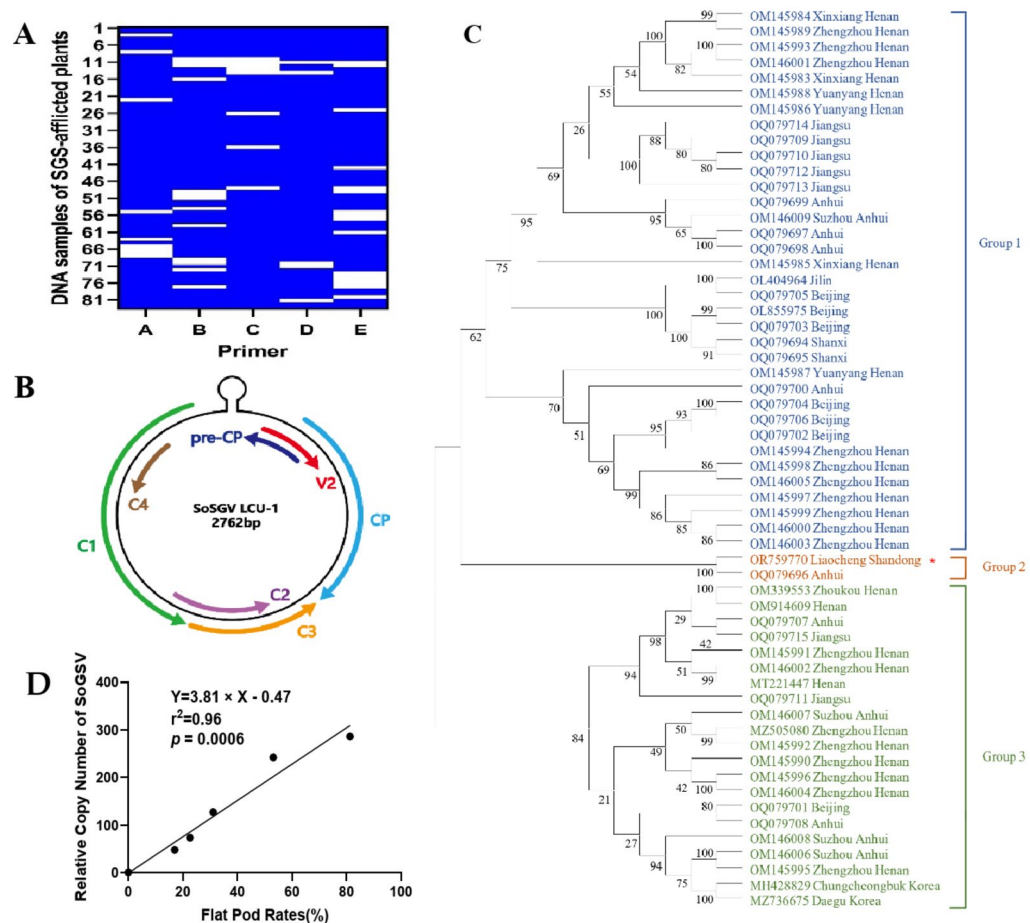


Fig. 6. The detection of SoSGV in DNA samples of SGS-afflicted plants and the SoSGV LCU-1 isolate cloning. **(A)** Amplification efficiency of different primers on 83 soybean DNA samples of SGS-afflicted plants. **(A–E)** are pairs of primers GMGAC1–5 (P1–P10, Supplementary Table S7), respectively. The blue color indicates that SoSGV could be amplified successfully, the white color indicates that no SoSGV was found. **(B)** Genome schematic of SoSGV LCU-1 isolate. The genome is a circular DNA with a full length of 2762 nt and consists of seven open reading frames. **(C)** The phylogenetic tree of homologous SoSGV isolates from different regions. The tree was constructed using the Neighbor-joining method with 1000 bootstrap replicates in the MEGA 11 software based on the nucleotide sequence of the full-length genome of different SoSGV isolates. The isolates were divided into three groups: Group 1 (blue), Group 2 (red), and Group 3 (green). The SoSGV LCU-1 isolate belonged to Group 2. For each isolate, the GenBank accession number and sample origin were provided. **(D)** The relationship between the flat pod rate and the relative copy number of SoSGV in the plants with SGS. The Y was the relative copy number of SoSGV, X was the flat pod rates, and r^2 was determination coefficient.

Conclusions

After three consecutive years of natural inoculation, a landrace called ZaoShuHeiDou out of 1553 accessions was identified as a SGS resistant germplasm. On the whole, landraces exhibited conclusively lower SGS incidence than improved cultivars. In the field, insecticide utilization other than organic fertilizer utilization and soil sterilization could significantly alleviate SGS severity of accessions. Soybean stay-green-associated virus (SoSGV) was found in all 83 soybean SGS DNA samples, indicating that the insect-borne geminivirus is the primary cause of SGS in this study.

Materials and methods

Experimental material

A total of 1553 soybean accessions were supplied by the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences (Supplementary Table S1). These germplasms were extensively evaluated in Liaocheng, a prefecture-level city in Shandong Province, China, between 2017 and 2020.

Methods

Preliminary assessment of Stay-Green Syndrome resistance via natural inoculation in 2020

In 2020, a preliminary assessment was conducted to determine the SGS resistance levels of 1553 soybean accessions. The experimental field on the East Campus of Liaocheng University (36.43481 N, 116.01311 E) was

Treat	Insecticide	Organic fertilizer	Soil sterilization
1	Yes	Yes	Yes
2	Yes	Yes	No
3	Yes	No	Yes
4	Yes	No	No
5	No	Yes	Yes
6	No	Yes	No
7	No	No	Yes
8	No	No	No

Table 3. Orthogonal experimental design for assessing the effects of factors on the SGS severity.

used for this purpose. Thirty seeds per accession were planted uniformly in a single row, which had a length of two meters and was spaced fifty centimeters from other rows. The soil in this field is sandy loam. Due to the primary goal and the evaluation workload, a quick and easy ocular estimate approach was used to determine the SGS levels of these accessions. At the R8 development stage²³, plants with more than 30% flat pods were treated as having SGS. The SGS level of a soybean accession was assessed in the field, based on the rate of SGS-afflicted plants in a row and the severity of SGS in a single plant. The severity levels were rated on a scale of one to seven. One (1) indicated that every plant in a row was in a normal state. Two (2) indicated that the rate of SGS-afflicted plants in a row was between 0% and 25%. Three (3) indicated that the rate of SGS-afflicted plants in a row was between 25% and 50%. Four (4) indicated that the rate of SGS-afflicted plants in a row was between 50% and 75%. Five (5) indicated that the rate of SGS-afflicted plants in a row was between 75% and 100%. Six (6) indicated that the rate of SGS-afflicted plants in a row was 100% and the normal pod rate on plants was between 5% and 20%. Finally, seven (7) indicated that the rate of SGS-afflicted plants in a row was 100% and the normal pod rate on plants was 5% or less.

The Stay-Green Syndrome evaluation of 121 accessions via natural inoculation in 2021

A panel of 121 soybean accessions was planted in 2021, using a randomized complete block design with two replicates. Of these, 113 were SGS-resistant at SGS level 1, while 8 were SGS-sensitive at SGS level 7 in 2020. Supplementary Table S2 provides details on these accessions. They were all planted on June 15 and July 1 in single rows with the same consistent row lengths, plant spacings, and row spacings as in the previous year (see Supplementary Table S8 for the climatic data of Liaocheng City from June to October in 2021). The SGS evaluation for these germplasms was also implemented using an ocular estimation approach as mentioned in 5.2.1.

Relationships between Stay-Green Syndrome levels and other characteristics in 2021

In this part of the study, the correlations between the SGS levels in 2021 and another 30 measured characteristics were determined. The original data for 11 quality traits and 16 quantitative traits were collected between 2017 and 2020. The quality traits included cotyledon color, flower color, growth habit, hilum color, leaf shape, lodging, pod color, pubescence color, pubescence density, seed coat color, and stem termination. The quantitative traits included seed number per pod, seed width, seed length, oil content, protein content, pod width, pod length, seed size, seed weight per plant, pod number per plant, effective branch number, node number on main stem, growth period, plant height, pod height at bottom, and flowering date. The geographical origin and the biological status of the accessions were provided by²². These accessions were split into eco-regions of the United States of America (US) and three other Chinese eco-regions: the northern eco-region, the Huang-Huai eco-region, and the southern eco-region³⁶. The maturity group classification of maturity group reference varieties was obtained from³⁷ (Supplementary Table S9). The maturity groups of the accessions in this study were determined by comparing their growth periods with those of maturity group reference varieties. The evaluation standards were based on³⁸.

Factors influencing Stay-Green Syndrome in the field in 2022

The effects of three factors—namely, insecticide use, soil nutrition (organic fertilizer), and soil sterilization—on the severity of SGS were investigated using a L8(2⁷) orthogonal experimental design with three replicates, without considering the interactions between different factors (Table 3). Each factor had two levels, resulting in eight treatments for each replicate. Fourteen accessions, including the seven most SGS-resistant accessions and seven SGS-susceptible accessions from 2021, were planted in each treatment, with a row length of 2 m, a row spacing of 50 cm, and a plant spacing of 7 cm. The order of the treatments in each replicate and the soybean accessions in each treatment were both randomized.

Clothianidin (Hebei Li Shi Jie Biotech. Co. Ltd., 20% suspension concentrate) was applied once every 10 days starting on July 24, 2022 (the R1 stage), for the insecticide-sprayed treatments. The remaining treatments were sprayed with water only, which served as the control.

Thirty kilograms of organic fertilizer, specifically fermented chicken manure from a local manufacturer, was evenly spread on each treated plot, and all plots were then tilled to a depth of at least 30 cm.

Soil sterilization was achieved through fumigation with dazomet (Zhejiang Dapeng Pharmaceutical Co. Ltd, 98% active ingredient particulate). To ensure the complete sterilization of the treated plots, the field was first

irrigated on May 7, 2022, and 667 g of dazomet per plot was evenly spread seven days later. The field was then plowed to a minimum depth of 30 cm and covered with film for 25 days. On June 10, 2022, the film was removed for aeration for 7 days, and the treated plots were plowed again on June 17.

To ensure a more precise evaluation of SGS severity, the average flat pod rates of at least ten plants at the R8 development stage were measured, instead of relying on ocular estimates.

Detection of Soybean Stay-Green-Associated Virus in Stay-Green Syndrome-afflicted plants

At the R8 development stage, 83 leaf samples of the 14 soybean germplasms with varying SGS severity were obtained and their genomic DNAs were subsequently isolated with a modified CTAB method³⁶. Based on the sequence OM145986.1, five pairs of SoSGV geminivirus-specific primers were designed with Primer 3 on the NCBI website (see Supplementary Table S7 for P1–P10 primers).

The fragments of interest were amplified by polymerase chain reaction (PCR) using an Authorized Thermal Cycler 5331 (Eppendorf). The PCR mixture, consisting of approximately 100–200 ng of genomic DNA, 0.2 μmol/L primers, and 1 × PCR mix (Beijing LabLead Trading Co. Ltd.), was prepared as a volume of 20 μL. The PCR process consisted of predenaturation at 94 °C for 5 min, followed by 35 cycles of denaturation for 30 s, annealing for 30 s at different annealing temperatures for different primer pairs, and extension for 1 min at 72 °C. Lastly, an additional extension for 7 min at 72 °C was performed. The PCR products were stored at 4 °C until they were analyzed by 1% agarose gel electrophoresis.

The PCR products were Sanger sequenced by Sangon Biotech (Shanghai) Co., Ltd. Subsequently, the specificity in the amplification of these PCR products was evaluated through nucleotide BLAST analysis on the NCBI website.

Isolation of the Soybean Stay-Green-associated Virus LCU-1 isolate

To clone the full-length SoSGV isolate, six pairs of new cloning primers (P11–P22, Supplementary Table S7) which were used to amplify six target fragments with successive overlaps of 44–197 bp, were designed based on the PCR product sequences mentioned above. The amplification conditions were similar to those in 2.2.5 except for the primers and the DNA polymerase. In this section, a high-fidelity and high-efficiency DNA polymerase, KOD (Toyobo Biotech), was used to amplify target fragments. The PCR products were sequenced using the Sanger method and the resulting sequences were manually assembled into the full-length sequence of SoSGV. The isolate of SoSGV cloned in this study was subsequently named SoSGV LCU-1.

Phylogenetic analysis

A total of 59 sequences of SoSGV isolates, which included the SoSGV LCU-1 isolate and 58 other homologous sequences obtained from NCBI, were utilized to construct neighbor-joining trees (see Supplementary Table S10 for 59 sequences). The phylogenetic analysis was conducted using MEGA (version 11), with 1000 bootstraps³⁹.

Soybean Stay-Green-associated Virus content in DNA samples with varying flat pod rates

The contents of SoSGV were evaluated using DNA samples from SGS-afflicted plants with varying rates of flat pods: 16.95%, 22.66%, 31.08%, 53.20%, and 81.31%. The genomic DNA of the normal seedling (cultivar L85-1467) germinated in the laboratory was used as the negative control for SoSGV content, representing a 0.00% flat pod rate. For quantitative polymerase chain reaction (qPCR) analysis, three DNA samples with similar flat pod rates were used as biological replicates, with water serving as the no-template control and the soybean EF-1α gene as the reference gene (P25–P26, Supplementary Table S7)⁴⁰. A pair of qPCR-specific primers (P23–P24, Supplementary Table S7) was designed based on the SoSGV LCU-1 isolate sequence (GenBank: OR759770.1). The qPCR mix contained 100 ng of genomic DNA, 0.3 μmol/L primers, and 1 × BeyoFast SYBR Green qPCR MIX (Beyotime Biotech. Inc.) as a total volume of 20 μL. The qPCR was conducted using a CFX96 temperature cycler (Bio-Rad). The procedure began with a predenaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 30 s per cycle. After the final PCR cycle, a melting curve analysis was conducted to assess the reaction's specificity. The reaction was incubated at 95 °C for 15 s, with annealing at 60 °C for 15 s, and then the temperature was raised to 95 °C for 15 s. The qPCR results were analyzed using the Bio-Rad CFX Maestro, and the relative content of SoSGV in the DNA samples was determined using the $2^{-\Delta\Delta CT}$ method⁴¹.

Data analysis

The data were processed using Microsoft Excel 2010 and statistical analysis was performed using SAS 9.4 (SAS Institute Inc., Cary, North Carolina). The effects of various factors on SGS were analyzed using ANOVA with a general linear model (GLM) procedure. The relationship coefficients between SGS and other quantitative traits were calculated using the CORR procedure. The relationships between SGS and other quality traits were determined using a one-way ANOVA procedure. A principal component analysis was carried out with a SAS PRINCOMP procedure to demonstrate the association between SGS severity of accessions and the observed traits. For the convenience of display and calculation, the SGS severity of accessions were rounded to the nearest integer and the 14 quality characteristics were digitized (Supplementary Table S11).

The broad-sense heritability (H^2) was calculated as.

$$H^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_{gp}^2 / n + \sigma_e^2 / nr),$$

where σ_g^2 is the genetic variation, σ_{gp}^2 is the genotype × planting date interaction variation, σ_e^2 is the error, n is the number of the planting date, and r is the number of replication. A SAS GLM procedure was used to estimate the phenotypic variation, including genetic variation, planting date variation, genotype × planting date interaction variation, and error. At the same time, the effects of genotype and plant date were tested using the mean square of the genotype × planting date interaction as the denominator.

Data availability

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Received: 7 August 2024; Accepted: 12 December 2024

Published online: 10 March 2025

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Acknowledgements

Professor Lijuan Qiu of the Chinese Academy of Agricultural Sciences supplied the germplasms. Professor Zenglu Li of University of Georgia provided critical advices for manuscript writing. Professor Jun Wang of Yangtze University offered help for PCA analysis. Jingying Zhang, Xiaotong Luan, Minyang Yu, Xin Gao, Xin Zhao, Jialiang Ma, Xuanyu Ji, Lan Yang and many other students from the School of Life Sciences of Liaocheng University participated in the field investigation and data collection. In addition, three anonymous reviewers provide professional and helpful advice.

Author contributions

X.Z., L.L., H.G., W.W., Y.Z., Y.G. and G.L. mainly carried out the field experiments and data collection; X.Z. and L.L. did the data analysis and figures and table preparation; X.Z., W.W., and L.L. Wrote the original draft; Q.Y., S.W., C.L. and R.L. reviewed the manuscript and helped to analyse data. All authors participated in contributing to the text and the content of the manuscript, including revisions and edits. All authors approve of the content of the manuscript and agree to be held accountable for the work.

Funding

This research was funded by the National 13th Five-Year Plan Key Research and Development Plan (2016YFD0100201-09).

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-83227-4>.

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