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Isoflavones in Seeds of Field-Grown Soybean: Variation Among Genetic Lines and Environmental Effects

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Abstract This study was undertaken to determine how seed isoflavones from soybean [*Glycine max* (L.) Merr.] lines differing in their maturity group, change between locations and years with different weather conditions. Seeds from 15 lines representing four maturity groups grown at three locations in Maryland (full season at all three and double crop at one location) were analyzed from 2001 to 2002, representing one relatively normal and one warm and dry year, respectively. Comparing lines, total isoflavones averaged for both years and all locations/planting dates ranged between 4.7 μmol [g seed dry matter (SDM)]$^{-1}$ in MD95-5358 and 8.7 μmol (g SDM)$^{-1}$ in Stressland. Isoflavones were reduced by about 50% in early maturing soybean lines in 2002 compared to 2001 under warmer conditions on the Eastern Shore of Maryland but not in the cooler central Maryland location. Isoflavones were not affected consistently or to a large extent in later maturity lines at any location or planting date combination. Relative changes in genistein, daidzein, and total isoflavones were similar to each other, whereas glycitein was much more variable. The results suggest that early maturing soybean lines are more likely to be affected by changes in temperature and precipitation.

Keywords Isoflavone · Genistein · Daidzein · Glycitein · *Glycine max* · Soybean · Maryland · Weather · Climate · Global change

Introduction

Soybean [*Glycine max* (L.) Merr.] seeds are the major dietary source of isoflavones, a class of flavonoids with significant albeit weak estrogenic activity [1] and other possible biological effects such as antioxidant activity [2]. In spite of continued positive reports on health benefits of isoflavones [3, 4], as well as advances in assessing isoflavone effects in mammals at a molecular level [5], questions continued to be raised about the efficacy and/or safety of isoflavones, either in supplements or foods [6, 7].

Reported large differences in isoflavone concentration, and to some extent composition, in soybean lines indicate it should be possible to breed soybeans with either high or low isoflavone levels as desired. However, genetic differences are complicated by correspondingly large differences in isoflavones within lines planted in different locations or years [8]. Numerous studies manipulating controlled environments, plant location, or planting date have shown that cooler temperatures, as well as, adequate soil moisture consistently increase isoflavone concentration [9–17]. Soybean lines with later maturity dates, and hence subject to cooler conditions during seed development, also tend to have higher levels of isoflavones [18].

It is still unclear how isoflavones in seeds from soybean lines differing in their maturity group will be affected by location-to-location or year-to-year fluctuations in weather

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and potential adverse changes in climate (e.g., increasing average and extreme temperatures, more variable precipitation) such as may accompany global change [19]. The Maryland uniform soybean trials provide the opportunity to examine phytochemical concentration and composition in a number of soybean lines spanning several maturity groups grown under agronomically relevant conditions at three distinct locations in the state of Maryland with small but predictable differences in climate. Moreover, both full season and double crop are available at one location, so soybeans grown in nearby fields but planted several weeks apart can be compared.

In an earlier study using these seeds [20], significant differences in the proportion of various tocopherols (vitamin E) were observed dependent on location and planting date, with higher levels of α-tocopherol relative to total tocopherol found in seeds that matured under warmer conditions. These results were consistent with independent controlled environment studies. The differences were very large when comparing early maturity groups from 1999 to 2001, relatively "normal" years, with the same lines grown in 2002, a year distinguished by above average temperatures and severe drought. The differences in tocopherols were less pronounced in soybean lines that matured later in the season.

Experimental Procedures

Seeds from 15 soybean lines representing four maturity groups (MG-III, MG-IV, MG-IVS [i.e., late maturing MG-IV lines having a relative maturity of 4.6–4.9], and MG-V) were obtained in 2001 and 2002 through the University of Maryland uniform state cultivar field testing program from Clarksville, MD [Central Maryland Research and Education Center (39.25N, 76.93W, 113 m above sea level); Queenstown, MD [Wye Research and Education Center (38.92N, 76.13W, 6 m above sea level); and Quantico, MD [Lower Eastern Shore Research and Education Center (38.37N, 75.78W, 6 m above sea level)]. Tillage, cultivation and development of maturity groups are described in more detail elsewhere [20] and at the Maryland Cropping Systems website (http://psla.umd.edu/extension/crops/soybeans).

Temperatures during months corresponding to seed development were compared for Clarksville, Queenstown and Quantico. Average values for August, in order, were 23.8, 24.8, and 24.7 °C in 2001 and 24.4, 25.7, and 25.1 °C in 2002. Average September temperatures were 17.7, 18.7, and 19.7 °C in 2001 and 20.5, 20.9, and 21.6 °C in 2002. In general, 2001 was cooler than 2002 at all locations. Consistent with 30-year average temperature data (not shown), Clarksville was the coolest location, while Quantico was the warmest location in October. Although the temperature differences were small, they were sufficient to affect cropping. Quantico was mild enough to support economic yields for both full season (FS) and double crop (DC) soybeans. In contrast, only one later maturity MG-V line was grown with economic yields at Clarksville (cv. Holladay).

All three locations have a similar rainfall based on 30-year averages (ca. 1,000–1,173 mm per year). Precipitation for the 12-month period ending in August 2001 was 1,011, 1,235, and 984 mm for Clarksville, Queenstown, and Quantico, in that order. Rainfall was drastically lower in 2002 at all locations (648, 637 and 734 mm, in order), reflecting deficits for 10–11 out of 12 months.

Soybeans were harvested, freeze-dried, ground and stored at −20 °C as described earlier [20]. The extraction procedure was slightly modified from Griffith and Collison [21] and Charron et al. [22]. Approximately 100 mg of soy flour was weighed into 2-ml polypropylene screw-cap tubes and extracted with acetonitrile (1 ml), deionized water (0.6 ml) and dimethylsulfoxide (0.1 ml) for 15 min while sonicating. Tubes were vortexed once before, once during, and once after sonication. After adding 0.3 ml deionized water to bring the final volume to 2.0 ml, samples were vortexed once more, centrifuged for 10 min at approximately 3,700×*g*, and filtered through 0.45-μm nylon syringe filters into HPLC vials. Extracts were stored at −20 °C if not used the same day.

HPLC analyses were done with a Model 2695 and Model 996 photodiode array detector (Waters Corp., Milford, MA) injecting 10 μl of extract onto a C-18 column (ODS–Hypersil, 250 × 4.6 mm, 5 μm particle size; Thermo Fisher Scientific Inc., Waltham, MA). The column temperature was 29 °C [12]. Isoflavones were separated using a linear gradient of 10–17% solvent B (acetonitrile) in solvent A (deionized water with 1% v:v acetic acid) for 30 min followed by 17–30% solvent B for 20 min, held at 30% solvent B for 1 min, ramped from 30 to 80% solvent B over 4 min and then held at 80% solvent B for 5 min. Elution rate was 0.9 ml min^{-1}. Solvent was returned to initial conditions over 4 min and held for 8 min for equilibration before the next injection. Spectral data were collected at 240–350 nm at 0.5 s intervals for 72 min. Chromatograms were integrated at 262 nm.

A typical chromatogram is included with Supplemental Material. Major peaks (A, daidzin; B, glycitin; D, genistin; F, malonyl daidzin; G, malonyl glycitin; I, malonyl genistin; J, daidzein; K, acetyl genistin; and L, genistein) were identified on the basis of co-chromatography with authentic standards (LC Laboratories, Woburn, MA, USA) as well as absorption spectra. In addition, malonyl glucoside peaks were confirmed by heating extracts for 2 h at 83 °C and observing decreases in presumptive malonyl glucoside

peak areas and corresponding increases in presumptive glucoside peak areas [23, 24].

Aglycones were quantified using daidzein and genistein standards as appropriate, while the remaining glucoside species were quantified using daidzin, glycitin and genistin, assuming the presence of malonyl or acetyl moieties did not affect absorption properties [15]. All values were expressed as μmol (g SDM)$^{-1}$ to facilitate calculations of absolute and relative isoflavone composition. Three unknown peaks (C, E, and H) with spectral properties corresponding to daidzin or genistin derivatives were noted and appeared similar to compounds described by other investigators [21, 22]. They were not included in values reported here, but were determined to constitute a relatively constant proportion (6–8%) of total isoflavones. Thus, their absence from calculated values does not qualitatively influence conclusions concerning differences in isoflavone distribution between lines, years or locations/planting dates.

The stability of isoflavones in samples before and after extraction is indicated by the relatively high proportion of native malonyl species [82.2 ± 0.2% (standard error of the mean) of total isoflavones] and the corresponding low proportion of acetyl species and aglycones. Acetyl genistin was always less than 0.5% of the total, while acetyl glycitin, acetyl daidzin, and glycitein were not detectable.

The significance of treatment effects and differences between means was determined using ANOVA (fixed effects model) and Tukey Pairwise Multiple Comparison tests (SigmaStat ver. 2.03, SPSS, Chicago, IL). Data were checked for adherence to normality and equal variance.

Results and Discussion

Total isoflavones are listed in Table 1 for all 15 lines grown at Queenstown and Quantico (both FS and DC) in 2001 and 2002. ANOVA indicated highly significant effects of genetic line ($p < 0.001$), year ($p < 0.001$), and location/planting date ($p < 0.001$) with a highly significant interaction between year and line ($p = 0.001$) and a nearly significant interaction between year and location/planting date ($p = 0.068$). Note that data from Clarksville were not included in Table 1 because 3 of the 4 MG V lines, Clifford, Essex, and Hutcheson, were not grown at that location. A second analysis was prepared to compare isoflavones from Clarksville, Queenstown and Quantico (FS and DC) based on the 12 lines grown at all three locations. Essentially identical results (data not shown) were obtained for genetic lines and years. In addition, total isoflavones at Clarksville and Quantico DC were equivalent and in each case were significantly greater than total isoflavones at Queenstown and Quantico FS.

The highest concentration of total isoflavones was found (Table 1) in Stressland [8.7 μmol (g SDM)$^{-1}$] and the lowest in MD95-5358 [4.7 μmol (g SDM)$^{-1}$]. However, most of the soybean lines in this study (12 of 15) assembled into a large intermediate group with average values for total isoflavones ranging between ca. 5.6 and 7.6 μmol (g SDM)$^{-1}$, none of which were not significantly different. Correcting for the mol proportion of the three isoflavones and converting to aglycone mass, these values correspond to a range of 1.27–2.33 mg isoflavone aglycone (g SDM)$^{-1}$. These values are underestimates, since three unidentified peaks (C, E, and H) constituted 6–8% of total A_{262} and this amount was not included in the total.

Although a higher isoflavone level tended to be associated with later seed maturation (Table 1), Stressland and MD95-5358 are both assigned to MG IV, indicating that the maturity group was not the most important criterion in isoflavone accumulation. It is also interesting that Stressland had been previously shown to have relatively high levels of total tocopherols while MD95-5358 had relatively low levels [20]. Nonetheless, isoflavones and tocopherols do not necessarily segregate, since MD92-5769 was high in isoflavones but low in tocopherols, while General was low in isoflavones but high in tocopherols.

Isoflavone composition was compared based on the proportions of total daidzein (daidzin, malonyldaidzin, and daidzein), total genistein (genistin, malonylgenistin, acetylgenistin, and genistein), and total glycitein (glycitin and malonylglycitin) relative to total isoflavones (Table 1). The proportions were all significantly different with respect to the soybean line ($p < 0.001$ for daidzein and genistein; $p = 0.002$ for glycitein), but variation occurred within relatively narrow ranges. IA3010 had the smallest proportion of genistein and the largest proportion of daidzein (both approximately 46% of total isoflavones). General had the most genistein (56% of total isoflavone) and nearly the least daidzein (34% of total isoflavones, ranking 14 out of 15). Macon had the highest proportion of glycitein (13% of total isoflavones) and Manokin the lowest (7%). Most of the difference in glycitein between Macon and Manokin can be ascribed to changes in the proportion of daidzein, since the two lines were not significantly different with respect to total isoflavones or the proportion of genistein. In fact, the proportions of genistein and glycitein are negatively correlated with the proportion of daidzein ($r^2 = 0.87$ and 0.58, respectively).

The environment is also important for total isoflavone concentration, since levels were significantly greater in 2001 compared to 2002 comparing either all 15 lines at two locations (Table 1) or 12 lines at all three locations (data not shown). Thus, total isoflavones were significantly greater at Quantico (DC) than Queenstown or Quantico (FS). Average isoflavone composition, in contrast, was scarcely influenced

Table 1 Total isoflavones and the mol fractions of total daidzein, genistein and glycitein from Queenstown, Quantico full season (FS) and Quantico double crop (DC) plantings in 2001 and 2002

Line	MG	Total isoflavones μmol (g SDM)$^{-1}$	Daidzein Fraction of total (rank)	Genistein	Glycitein
Stressland	IV	8.7a	0.42a–c (6)	0.48ef (14)	0.11a–c (4)
MD92-5769	IVS	7.6ab	0.41bc (8)	0.51a–f (9)	0.09a–c (8)
Holladay	V	7.0a–c	0.41bc (7)	0.51a–e (8)	0.08bc (14)
LS93-0375	IV	7.0a–c	0.38cd (11)	0.52a–e (6)	0.10a–c (5)
Hutcheson	V	6.8a–c	0.35de (13)	0.54ab (3)	0.12ab (2)
HS93-4118	IV	6.8a–d	0.44ab (3)	0.48b–f (11)	0.09a–c (7)
Essex	V	6.7a–d	0.43ab (4)	0.48d–f (13)	0.09a–c (10)
Clifford	V	6.7a–d	0.38cd (10)	0.53a–d (5)	0.09a–c (9)
Manokin	IVS	6.6b–d	0.46a (2)	0.48c–f (12)	0.07c (15)
Macon	III	6.5b–d	0.33e (15)	0.53a–c (4)	0.13a (1)
KS4694	IVS	6.0b–d	0.35de (12)	0.54ab (2)	0.11ab (3)
IA3010	III	5.7b–d	0.46a (1)	0.46f (15)	0.09bc (12)
General	III	5.6b–d	0.34 de (14)	0.56a (1)	0.10a–c (6)
Corsica	IV	5.2cd	0.43ab (5)	0.49b–f (10)	0.08b–c (13)
MD95-5358	IV	4.7d	0.41bc (9)	0.51a–e (7)	0.09a–c (11)
Year					
2001		7.3a	0.39b	0.51a	0.10a
2002		5.8b	0.40a	0.50a	0.10a
Location/date					
Quantico DC		7.2a	0.41a	0.50a	0.09b
Quantico FS		6.3b	0.39b	0.51a	0.10ab
Queenstown		6.1b	0.39b	0.51a	0.10a

Significance determined by ANOVA for year, line and location/date. Within a column, values not followed by the same letters are significantly different ($p < 0.05$)

by planting year, location or date, even though statistically significant differences were obtained in some cases.

As indicated above for total isoflavones, the interaction between year and line was significant ($p < 0.001$), while the interaction between year and location/planting date was nearly significant ($p = 0.068$). ANOVA on genetic line and year revealed that total isoflavones were significantly higher in 2001 compared to 2002 for Macon, IA3010, General, Corsica, KS4694 and HS93-4118 but not for the other lines. These effects are illustrated graphically by plotting the concentration ratios between 2001 and 2002 values calculated for daidzein, glycitein and genistein for each line and location/planting date (Fig. 1). Changes in genistein and daidzein were generally similar as were changes in total isoflavones (data not shown). This is not surprising, since genistein and daidzein constitute the bulk of total isoflavones, whereas glycitein is only 6–13% of the total isoflavones and even large relative changes have a small effect on the total.

The 15 soybean lines segregate into three "response" groups based on maturity group and similarity of responses: "early" (General, Mg III; IA3010, MG III; Macon, MG III; and Corsica; MG IV); "late" (Manokin, MG IVS; MD92-5769, MG IVS; Clifford, MG V; Essex, MG V; Holladay, MG V; and Hutcheson, MG V); and "intermediate" (HS93-4118, MG IV; LS93-0375, MG IV; MD95-5358, MG IV; Stressland, MG IV; and KS4694, MG IV). ANOVA comparing 2001/2002 ratios for early, intermediate and late response groups at all four location/planting date combinations indicated a significant effect for response group ($p = 0.002$) and a significant interaction ($p = 0.014$) with location/planting date associated with the early response group.

Early group: total isoflavones, total daidzein and total genistein were significantly higher in 2001 than in 2002 for samples from Queenstown (1.7 to 2.5-fold more), Quantico FS (1.5 to 2.5-fold more) and Quantico DC (1.4 to 2.0-fold more). At Clarksville, in contrast, total isoflavones in three of the four lines are about 40% greater in 2002 compared to 2001 and the fourth line showed no change.

Late group: small relative increases ($\leq$20%) or decreases ($\leq$30%) in total isoflavones, total daidzein and total genistein, were observed in 2002 compared to 2001. These changes are much smaller and more variable that those for the early group and indicate environmental differences between 2002 and 2001 had no significant effect on isoflavone concentration and composition with later maturing soybean lines.

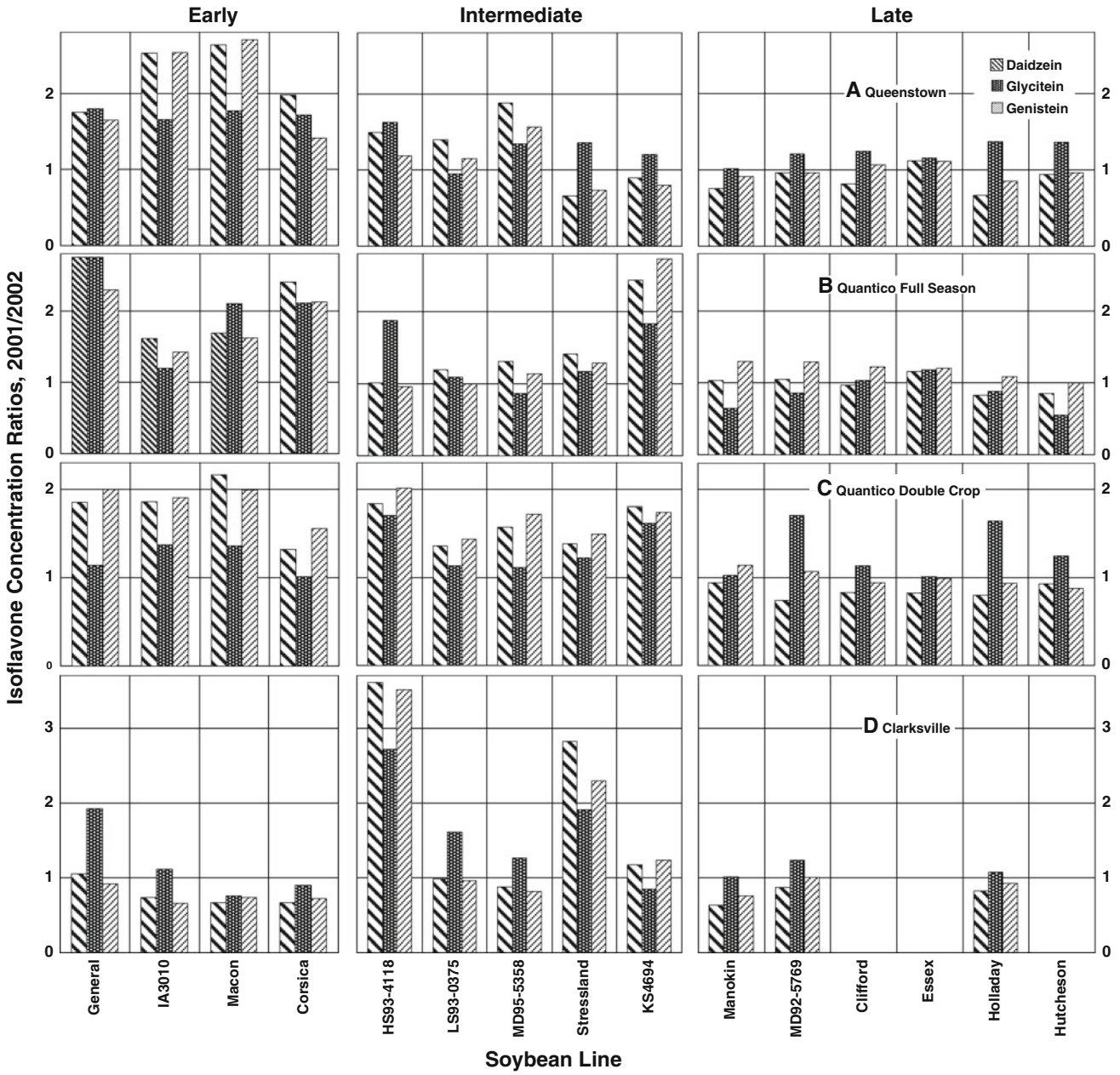

Fig. 1 Relative changes in total daidzein, total glycitein and total genistein families, 2001/2002, at four location/planting dates, Queenstown (**a**), Quantico full season (**b**), Quantico double crop (**c**) and Clarksville (**d**). Ratios were calculated based on μmol (g SDM)$^{-1}$. Data were obtained from 15 soybean lines representing four maturity groups (MG). MG-III: General; IA3010; and Macon. MG-IV: Corsica; HS93-4118; LS93-0375; MD95-5358; and Stressland. MG-IVS: KS4694; Manokin; and MD92-5769. MG-V: Clifford; Essex; Holladay; and Hutcheson. Early, intermediate and late response groups are described in more detail under "Results and Discussion"

Intermediate group: differences between 2001 and 2002 depended strongly on line, location and planting date and may reflect interactions between seed development and temperature. At Queenstown and Quantico FS, the concentration ratios were intermediate between early and late groups, but cool temperatures during seed maturation for Quantico DC in 2001 may have resulted in especially high isoflavone concentrations and hence elevated ratios for 2001/2002. Several exceptions stand out that have no clear explanation (i.e., KS4694 at Quantico FS as well as HS93-4118 and Stressland at Clarksville).

Glycitein was more variable between 2001 and 2002, consistent with indications that glycitein synthesis, localized in the germ, may be regulated independently of genistein and daidzein [25]. Thus, about one-third of all the samples were characterized by extreme values for the glycitein

concentration ratio, either greater than 1.5 times the average ratio for daidzein and genistein or one-quarter to one-half that of the daidzein-genistein ratio. This knowledge may have important functional ramifications. Although glycitein is a weak estrogen comparable to daidzein and genistein [1], glycitein may have specific neuroprotective effects not shared with genistein or daidzein [26]. In addition, glycitein was more effective than genistein, daidzein, or equol in activating extracellular signal-regulated kinase and in decreasing proliferation of RWPE-1 cells [27].

In conclusion, it appears that elevated temperatures and drought are most likely to affect isoflavone concentrations in seeds of soybean lines representing earlier maturity groups, and then mostly when grown under warmer conditions. The results suggest that variability in weather and possibly future climate change may impact soybean seed phytochemical composition. Increased variability in isoflavones may result, especially in soybeans from northern regions. However, given the limited 2-year duration of this study, as well as anomalous results for some lines, it is clear that additional studies are required to confirm the observations and to understand how varying environmental conditions interact with specific stages of seed development.

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