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Screening for grain polyphenol variants from high-tannin sorghum somaclones

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Abstract Several hundred somaclones established from plants regenerated from embryogenic callus cultures of six high tannin sorghum lines were screened for variants with altered levels of polyphenols in the grain. Grain from over 6000 plants including the R_1 (primary), R_2, and R_3 generations were analyzed for total phenols, flavan-4-ols, and proanthocyanidins (condensed tannins). Although many variants which had lost the ability to synthesize chlorophyll were found, none of the somaclones tested had lost or greatly reduced the ability to synthesize any of the polyphenols assayed. However, we did observe statistically significant differences in polyphenol concentration between tissue culture-derived R_1 plants and the parental controls. In the R_2 generation the proportion of somaclones which differed significantly from the parents varied from 47% to 68% depending upon genotype. The average somaclonal variation rate and somaclonal variant frequency estimated in the tested population for the three polyphenol characteristics ranged from 37.3% to 40.7% and 5.3% to 7.8%, respectively. Variants with decreased levels of polyphenols were usually epigenetic and reverted back to normal levels in subsequent generations, but those with increased levels usually persisted after two meiotic cycles, indicating they are heritable. Variants with polyphenol levels increased up to 80% or decreased by 30% were selected for in the R_3 generation.

Key words Somaclonal variation · Plant tissue culture Polyphenols · *Sorghum bicolor*

Introduction

High-tannin sorghums have agronomic advantages, but tannin-rich grain has harmful nutritional effects (Butler 1989). Tissue culture procedures may complement conventional breeding programs for grain quality improvement (Gengenbach 1984). We are attempting to utilize in vitro tissue culture for overcoming problems associated with the utilization of high-tannin sorghums (Cai et al. 1990). Many sorghums contain no tannin, so tannin synthesis is clearly not essential for this crop. If it were possible to obtain, from a well-characterized, high-tannin sorghum, stable somaclonal variants unable to carry out one or more steps of polyphenol biosynthesis, these variants would be useful in establishing the tannin biosynthetic pathway.

Although the tissue culture technique has seldom been applied to sorghum grain quality improvement, variants in improved grain quality have been detected from tissue culture-derived plants of rye (Hoffman and Wenzel 1981), triticale (Jordan and Larter 1985), wheat (Ryan et al. 1987), and rice (Zheng et al. 1989). The purpose of the study presented here was to evaluate tissue culture-derived somaclones of high tannin sorghums for variations in biochemical (polyphenolic) and morphological traits. In a previous paper (Cai et al. 1990) we described a wide range of morphological and developmental variations observed in a field assessment of sorghum somaclones from high tannin cultivars. We expected that among these somaclones some polyphenol variation could be detected. We here report the results of chemical assays for screening variants with altered levels of polyphenols in grain.

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Materials and methods

Nomenclature

We use R_1, R_2, R_3..... to term the primary regenerants, the first selfed progeny, and successive generations, respectively. The term R_1 and R_2 families refer to the selfed progeny of R_1 or R_2 plants, respectively.

Establishment of Somaclones

Eight sorghum [*Sorghum bicolor* (L.) Moench] genotypes, 'IS0724', 'IS2830', 'IS3150', 'IS4225', 'IS6881', 'IS8260', 'SC0167-14E',

and 'IS8768' were selected for their high levels of tannin. 'IS8768' is a Group II sorghum, and all the others are Group III (conventional high-tannin) sorghums (Price et al. 1978). None of these genotypes are hybrid. Over 6000 plants (R_1) were regenerated from embryogenic calli derived from mature or immature embryos or from developing inflorescences (Cai et al. 1990). Nearly 2000 plants regenerated from calli which had been maintained in culture for 120–360 days were transferred to pots and subsequently grown in the greenhouse or the field with the corresponding parental controls. Their panicles were bagged for selfing and separately harvested to establish individual somaclone families. In the R_2 generation, self-pollinated seed from individual panicles of primary regenerants (R_1 plants) were planted separately in individual R_1 progeny field rows (henceforth called individual R_1 families) 5-m in length (20–35 R_2 plants). Control parents were planted every 12th to 40th row (Cai et al. 1990). In the R_3 generation, seeds from selected individual R_2 plants were planted separately in R_2 progeny rows (henceforth called individual R_2 families) with parental rows randomly planted every 8–12 rows. Except for some R_1 plants grown in a greenhouse in 1986, all of these regenerant and control plants were grown at the Purdue University Agronomy Research Center from 1987 (R_1 and R_2 generation) to 1989 (R_3 generation).

Sampling grain

Individual single panicle-derived grain samples used for the assay were taken from field-grown R_1, R_2, and R_3 plants and their corresponding parental controls planted alongside. Only 1 panicle was taken from each plant. These individual panicles were separately threshed and their grain samples were separately kept in individual seed bags. The regenerant plants from which the grain samples were taken were uniform and morphologically resembled their parents. All R_1 panicles were self-pollinated. Because of the labor cost and to avoid the influence of bags on polyphenol synthesis, most R_2 and successive generation samples were from open-pollinated panicles. Before the open-pollinated panicles were threshed, both ends of the panicles were removed, and grain was collected only from the middle part of the panicles to minimize the collection of cross-pollinated seeds. Grain from corresponding parental controls was also derived from single panicles and was sampled randomly from field-grown, self- or open-pollinated panicles (Table 1).

Assay procedures

Sorghum grain from individual threshed panicles was hand-cleaned to remove glumes and broken grain. About 10 g of grain from each regenerant and parental panicle was ground 40 s in an microanalytical mill. All of the ground grain was assayed within 2–3 days after grinding. Extracts were prepared from 500 mg of ground grain by continuous agitation with 10 ml of 1% concentrated HC1 in methanol in screw-top test tubes at room temperature for 20 min. The resulting extract was assayed for total phenols by the Prussian blue assay (Price and Butler 1977) and for flavan-4-ols and proanthocyanidins by the acid butanol assay (Watterson and Butler 1983). Primary assays showed that the blank absorbance for the butanol assay was zero for IS8768 and less than 0.05 for other genotypes, which is neglible in comparison with the content of flavan-4-ols and proanthocyanidins. Therefore the blanks for the acid butanol assay were not run unless variants for flavan-4-ols were to be determined. All data presented are optical densities not converted to percentage composition. Because these extractable polyphenols are located primarily in the pericarp and the testa, which are maternal tissue (Rooney et al. 1980), data from R_2, R_3, and R_4 grain harvested from the R_1, R_2, and R_3 plants were used to characterize the R_1, R_2, and R_3 generations, respectively. Parental samples were assayed along with the regenerant samples in every generation of each year (Table 1).

Table 1 Number of plants and families from which the analyzed plants were derived [a]

Genotypes	IS8260	IS8768	IS4225	Total	Others[b]
R_1 generation (1987)					
Parental plants	50 (s)	26 (s)	16 (s)	92 (s)	
R_1 plants	380 (s)	213 (s)	51 (s)	644 (s)	
R_2 generation (1987, 1988)					
Parental plant total	70	152	106	328	69
R_2 plant total	1291	1317	860	3468	393
Parental plants 1987		84 (s)	56 (o)		69 (o)
R_2 plants 1987		554 (s)	332 (o)		393 (o)
Parental plants 1988	70 (o)	68 (o)	50 (o)		
R_2 plants 1988	1291 (o)	763 (o)	528 (o)		
From R_1 families					
Total R_1 families	148	145	98	391	96
From un-analyzed R_1 plants	30	70	48	148	96
From analyzed R_1 plants	118	75	50	243	
Putative variants	47	38	16	101	
Selected R_1 plants	71	37	34	142	
R_3 generation (1988, 1989)					
Parental plants	60 (o)	50 (o)	110 (o)	220 (o)	
R_3 plants	312 (o)	269 (o)	632 (o)	1213 (o)	
From R_2 families					
Total R_2 families	35	30	68	133	
From variant R_2 plants	28	26	58	112	
From non-variants	7	4	10	21	
From R_1 families					
For variant R_2 plants	15	20	26	61	
For non-variant plants	5	2	7	14	

[a] (s) und (o) indicate grain samples derived from self- and open-pollinated panicles, respectively
[b] Including 'IS3150', 'IS6881', and 'IS0724' genotypes

Analysis of R_1 plants for grain polyphenols

Among 2000 R_1 regenerants grown to maturity, 644 plants of three cultivars ('IS8260', 'IS8768', and 'IS4225') grown in 1987 were assayed. The 644 R_1 plants were regenerated from the calli which had been maintained in culture longest (210–270 days) to see whether long-term culture affects the production of grain polyphenols. Assays were run without duplication. Data for the three polyphenol characteristics assayed from the 644 plants and corresponding parents were analyzed statistically. Analysis of variance and Duncan's multiple range tests were run for comparison among parental plants and R_1 plants derived from different explant sources (immature embryo and inflorescence) and different times in culture (210 days and 270 days before regeneration) (Tables 2, 3). The data array of R_1 plants and the corresponding parents were classified at equal intervals. Then the number and percentage of each class were calculated. Frequency distributions of grain polyphenol content were constructed with a frequency polygon for each pair of comparisons between R_1 plants and parents. The R_1 plants outside the range of the parents (transgressive phenotypes) were selected as putative R_1 variants for further screening in the R_2 generation.

Screening for polyphenol variation in the R_2 generation

Polyphenol variation in the R_2 generation was screened for in six genotypes in 1987 and 1988. For the three genotypes 'IS3150', 'IS6881', and 'IS0724', a total of 393 panicles were taken randomly from 96 R_1 family-rows grown in 1987 (Table 1). The 393 samples were assayed for three polyphenol characteristics along with corresponding parental control in duplicates of ground grain samples. Tests of significance for differences between the sampled R_2 population and their parents were accomplished using the standard *F*- and *t*-tests. The *F*-tests were run first for the equality of variance within each genotype, and then the *t*-test was run to determine the difference in the mean values. Frequency distributions (as mentioned in R_1 plants) were calculated for comparison of the range of grain polyphenols to detect the R_2 plants with values distributed beyond the range of the parent values. Those R_2 plants showing transgressive phenotypes were counted as grain polyphenolic variants.

For the remaining three genotypes ('IS8260', 'IS8768', and 'IS4225') from which the data described here were mainly obtained, screening in the R_2 generation was conducted by comparing individual R_1 families with their parents and selecting at the family level instead of with the total R_2 population and selecting at the R_2 plant level as described above for the first three genotypes. A total of 3468 R_2 panicles were tested from 391 R_1 families (Table 1). In order to test the effect of selection at the R_1 generation on the recovery of somaclonal variation in the R_2 generation, the families tested included some from R_1 plants not previously analyzed, as well as the putative R_1 variants mentioned above; other R_1 plants were selected from analyzed plants with polyphenol values at the lower or higher end of the parental range. From each of the 391 R_1 families, 9 R_2 panicles were harvested unless the row did not contain 9 plants. Grain samples from each of the panicles were assayed for these polyphenols along with parental samples. The data obtained from 9 samples of each R_1 family were compared statistically with those of the parents. Individual *F*-tests were run for equality of variance within each pair of R_1 family and control. Individual *t*-tests were processed for comparison with the mean value. The significance level was $P< 0.01$. R_1 families were considered to be variants if either of the tests showed significant differences in the characteristic(s) tested. Duplications were run only for the R_2 plants that needed to be confirmed for testing persistance in the R_3 generation.

Testing persistence of variation in subsequent generations

In order to test the persistence of polyphenol variation detected in the R_2 generation, seeds from 112 R_2 variants selected from 61 variant R_1 families (confirmed by repeat analysis) were grown separately in R_3 field rows (R_2 progeny, henceforth called individual R_2 families). In addition, 21 R_2 plants derived from non-variant R_1 families and similar to the control plants with respect to the three polyphenol characteristics were also grown for testing the recovery of polyphenol variations in the R_3 generation. A total of 133 R_2 families were tested in the R_3 generation (Table 1). Nine or more R_3 panicles from each R_2 family were analyzed, along with the parents in duplicates or triplicates, for the three polyphenol characteristics. Data from each family were compared with those for the parental control by the *F*- and *t*-tests at the $P< 0.01$ significance level, as mentioned above. An R_2 family was considered to be a persistent variant when the R_2 family showed the same deviation from the parent in the R_3 generation as did the R_2 variant (in the R_2 generation) from which the R_2 family was derived. An R_1 family was counted as a persistent variant if at least one of its R_2 families showed the same polyphenol variation as that detected from the R_1 family (in the R_2 generation) and as that detected from the R_1 plant (in the R_1 generation) from which the family derived.

Sample size of parental control

Original parental seeds of each genotype were threshed from 5 or 6 panicles harvested from 2 field rows. In each generation from 4 to 12 parental rows were planted, the exact number depending upon the number of rows of regenerant materials grown. Between 50 and 84 parental plants were randomly harvested from the 4–12 rows in each generation (Table 1), which was more than the original 5 or 6 plants from 2 rows. Seeds for planting the control rows in 1988 and 1989 were mixed seeds derived from 40 to 50 parental plants. It should be emphasized that because fewer embryogenic calli were available for maintenance after 120 days in culture, the regenerant plants assayed were derived from only a few explants for each parental genotype. Although fewer parental plants were assayed (Table 1) than regenerants, the number assayed was more than that of the original plants from which the explants were taken for initiating the cultures. Moreover, when running *F*- and *t*-tests for comparison between R_1 or R_2 families and their parental controls in genotypes 'IS8260', 'IS8768' and 'IS4225', the parental data were derived from all of the parental plants (50–70) harvested that year rather than from only the control plants grown in the row nearest the family row. So each pair of comparisons was analyzed between an R_1 or R_2 family consisting of 9 R_2 or R_3 plants and a parental control sample of 50–70 plants.

The levels of these grain polyphenols vary in a single genotype in different years. Samples of R_1, R_2, and R_3 generations were separately evaluated in successive years and were analyzed statistically along with the corresponding parental control harvested in the same year. The impact of the environment was considered to be same between regenerant and parental plants in each year.

All data were processed using MINITAB or SAS software packages in IBM/VM at the Purdue University Computer Center.

Calculation of somaclonal variation

As previously described by Cai et al. (1990), somaclonal variation rate (SVR) is based on 100 R_1 plants, and somaclonal variant frequency (SVF) is based on 100 R_2 plants. SVR describes the probability of variation of given somaclones through tissue culture. However, unlike mutagenesis, the rate includes variation resulting from independent mutation events as well as from proliferation of single, mutated cell clones. SVF refers to the proportion of detectable variants in a given somaclonal population. The formula:

$$\mathrm{SVR}(\%) = \frac{\text{Number of variations found in } R_1 \text{ families}}{\text{Number of total } R_1 \text{ families tested}} \times 100\%$$

$$\mathrm{SVF}(\%) = \frac{\text{Number of variants } R_2 \text{ detected}}{\text{Number of total } R_2 \text{ plants tested}} \times 100\%$$

In this paper the variation refers to altered grain polyphenolic phenotypes, and variant refers to individual plants which are different from the parental control in one, two, or three of the grain polyphenol contents tested.

Table 2 Analysis of variance of grain polyphenolic characteristics among R_1 plants derived from different cultures and parental plants. The samples assayed were harvested from self-pollinated panicles in 1987 field (SS: Sum of squares; F: Statistical F values)

	Df	Total phenols		Flavan-4-ols		Proanthocyanidins	
		SS	F	SS	F	SS	F
IS8260							
Treatments	4	745762	67.52**	0.24361	13.16**	4.50791	31.10**
Parent vs. R_1 plants	1	491976	178.17**	0.14001	30.24**	1.31067	36.21**
Explant sources[a]	1	111198	40.27**	0.00069	0.14^{ns}	0.02820	0.77^{ns}
Time in culture[b]	1	94607	34.26**	0.09369	20.24**	2.35051	64.93**
Explant × time	1	47981	17.38**	0.00921	1.99^{ns}	0.81853	22.61**
Error	435	1173520		1.96730		15.40219	
Total	429	1919281		2.21091		19.91011	
IS8768							
Treatments	4	18908	3.32*	0.05657	32.17**	0.67011	2.79*
Parent vs. R_1 plants	1	9864	6.92**	0.00038	0.86^{ns}	0.29773	4.95*
Explant sources	1	4811	3.38^{ns}	0.00042	0.95^{ns}	0.00352	0.06^{ns}
Time in culture	1	3869	2.72^{ns}	0.03477	79.02**	0.34591	5.76*
Explant × Time	1	364	0.26^{ns}	0.02100	47.72**	0.02295	0.38^{ns}
Error	234	333438		0.10285		14.05379	
Total	238	352345		0.15942		14.72391	
IS4225							
Treatments	2	14508	26.38**	0.24504	17.19**	0.18097	6.90**
Parent vs. R_1 plants	1	13248	48.17**	0.21389	29.99**	0.09028	6.89*
Explant sources	1	1260	4.85*	0.03115	4.37*	0.09069	6.92*
Error	64	17602		0.45603		0.83869	
Total	66	32110		0.70108		1.01966	

ns, not significant; * and **, significant at $P< 0.05$ and $P< 0.01$, respectively
[a] Explant source included immature embryos or inflorescences for initiating the cultures
[b] Time in culture to refer to 210 days or 270 days in culture before the plants regeneration

Results

Change in grain polyphenol characteristics observed in the R_1 generation

The analysis of variance (Table 2) and comparison of mean values (Table 3) indicated significant differences between the parents and R_1 plants derived from different culture sources. The differences between the parents and total R_1 plants were all significant except for flavan-4-ols of 'IS8768'. Between two explant sources, immature embryo and inflorescences, this difference was generally negligible, but was significant for the flavan-4-ols in 'IS4225' (P=0.05) and total phenols of 'IS8260'. Significant differences between 210–240 days and 270–300 days in culture were observed except for total phenols of IS8768. In some cases there were interactions between explant sources and time in culture (Table 2). Generally the mean values increased with increasing time in culture (Table 3). Genotype differences are apparent. The deviation of mean values of R_1 plants from their parents is toward increased levels for 'IS8260', but toward decreased levels for 'IS8768' and 'IS4225' (Table 3).

The frequency distribution of R_1 data (figures not shown) and the range of the polyphenol content shown in Table 3 indicated that the primary regenerants exhibited a wider range of grain polyphenol characteristics than their parental controls. Changes in both directions could be seen in each genotype. However, no variants in which polyphenol synthesis was greatly diminished were found. The R_1 plants with transgressive values were considered to be putative variants with respect to grain polyphenols. A total of 145 (22.5%) putative variants were obtained from the 644 R_1 plants of the three cultivars, and the 101 with the largest deviation were chosen for growth to the R_2 generation.

The results of testing the effect of R_1 selection on the appearance of polyphenol variants in the R_2 generation (data not shown) indicate that more than one-half of the polyphenol variations observed from the putative R_1 variants were epigenetic, especially the variants with decreased levels of polyphenols. The differences in the frequencies of R_1 families showing polyphenol variation(s) in the R_2 generation appear to be negligible between the putative R_1 variants and R_1 plants selected for the polyphenol values close to the highest or lowest values of the parental plants. Additional variations not detected in the R_1 generation could be recovered in the R_2 generation. Selection in the R_1 generation seems to be unnecessary for recovering polyphenol variation. However, R_1 plants selected from the analyzed R_1 plants had a higher frequency of R_1 families with polyphenol variation(s), 70% and 64% for the 101 putative variants and the 142 selected R_1 plants, respectively, relative to the 36.5% produced by the 148 randomly chosen unanalyzed R_1 plants (Table I).

Table 3 Comparison of R_1 plants with the parental controls in grain polyphenols. The samples assayed were harvested from self-pollinated panicles in 1987

Materials[a]	Number of plants tested	Total phenols A_{720}		Flavan-4-ols A_{550}		Proanthocyanidins A_{550}	
		Range	Mean ± SEM[b]	Range	Mean ± SEM	Range	Mean ± SEM
IS8260 CK	50	120 – 285	178.04 ± 44.74[d]	0.093 – 0.274	0.149 ± 0.050[d]	0.735 – 1.287	1.002 ± 0.165[c]
IS8620 R_1 IE210D	88	95 – 425	203.10 ± 57.38[c]	0.071 – 0.334	0.157 ± 0.043[cd]	0.606 – 1.412	1.023 ± 0.186[c]
IE270D	102	168 – 395	232.47 ± 39.51[b]	0.103 – 0.324	0.188 ± 0.045[b]	0.686 – 1.756	1.247 ± 0.193[a]
IF210D	96	131 – 330	239.75 ± 47.38[b]	0.099 – 0.295	0.172 ± 0.040[bc]	0.641 – 1.649	1.153 ± 0.210[b]
IF270D	94	176 – 485	307.61 ± 67.05[a]	0.085 – 0.426	0.218 ± 0.119[a]	0.850 – 1.805	1.255 ± 0.184[a]
IS8768 CK	26	52 – 154	97.92 ± 31.10[a]	0.010 – 0.095	0.045 ± 0.018[b]	0.445 – 1.013	0.663 ± 0.163[a]
IS8768 R_1 IE210D	68	24 – 139	68.46 ± 20.58[b]	0.000 – 0.108	0.044 ± 0.020[b]	0.135 – 0.974	0.512 ± 0.150[b]
IE270D	30	40 – 174	80.50 ± 26.15[b]	0.022 – 0.076	0.041 ± 0.010[b]	0.272 – 1.373	0.621 ± 0.206[ab]
IF210D	51	21 – 238	78.04 ± 63.06[b]	0.017 – 0.131	0.065 ± 0.026[a]	0.200 – 1.672	0.517 ± 0.404[b]
IF270D	64	30 – 176	84.58 ± 31.43[ab]	0.000 – 0.077	0.020 ± 0.023[b]	0.240 – 1.137	0.583 ± 0.200[ab]
IS4225 CK	16	96 – 126	107.7 ± 10.0[a]	0.332 – 0.525	0.429 ± 0.060[a]	0.546 – 0.870	0.674 ± 0.109[a]
IS4225 R_1 IE	14	55 – 98	82.8 ± 13.0[b]	0.213 – 0.691	0.336 ± 0.119[b]	0.435 – 0.817	0.656 ± 0.096[a]
IF	37	38 – 159	71.7 ± 19.7[c]	0.111 – 0.445	0.281 ± 0.078[c]	0.355 – 0.958	0.562 ± 0.123[b]

[a] IE und IF refer to immature embryo and inflorescence as explant source for initiating tissue cultures from which the plants regenerated. 210D and 270D refer to 210 days and 270 days in culture before plant regeneration from the culture
[b] Mean value and standard error of measurements (SEM). Means in a row of the same cultivar followed by the same letter do not differ at the 5% significant level by Duncan's Multiple Range Test

Screening grain polyphenolic variation in the R_2 generation

The results of screening for polyphenol variation in the R_2 generation were unexpected in that no variants with zero or very low contents of these polyphenols were found. However, variants with significantly increased or decreased levels of polyphenols were detected from R_2 plants that were phenotypically uniform and otherwise resembled their parents. From the 391 R_1 families of the three cultivars 'IS8260', 'IS8768', and 'IS4225' tested (Table 4), 216 (55.2%) were found to vary significantly from the parental control in one or more polyphenol characteristics. 'IS8260' had the highest frequency of R_1 families with polyphenolic variations. The number of variations in decreased or increased level of polyphenols carried by the 216 families included 146 in total phenols, 146 in flavan-4-ols, and 159 in proanthocyanidins (Table 4). Among the 451 variations, 382 were detected by the *t*-test, including 19 also with a significant *F*-ratio, and 69 were detected by the *F*-test, including 15 with a significant difference ($P<0.05$) in mean value. Table 4 also shows the comparison of mean values of polyphenol content between parents and variant R_1 families with the highest and the lowest mean value. The changes ranged from a 112% increase to a 41% decrease.

For the other three cultivars, 'IS3150', 'IS6881' and 'IS0724', analysis was carried out on the total R_2 population instead of individual R_1 families. Comparisons between the collective R_2 population and their parents (Table 5) indicated that the 'IS3150' R_2 population produced higher variability than their parental control. *F*-values were significant ($P<0.05$) in all of the three characteristics. R_2 plants of IS6881 had significantly different ($P<0.01$) mean values in total phenols and flavan-4-ols. For 'IS0724', the only significant ($P<0.01$) difference from the parental control was in flavan-4-ols. The 46 R_2 variants having transgressive characters were considered to be putative R_2 variants.

Table 6 shows the comparison of the range of grain polyphenol characteristics between R_2 plants and their parents. There was considerable variability in the grain polyphenols in the parental plants. Among the six parental genotypes tested (Table 6), 'IS4225' and 'IS8260' were more uniform in all three characteristics than the other four genotypes. The highest variability appeared in 'IS3150'. Among the three characteristics, flavan-4-ols were most uniform, while proanthocyanidins had the highest variability. Nevertheless, the frequency distribution curves for the R_2 plants (figures not shown) were shifted from their control parents in all three characteristics. As shown in Table 6, these R_2 plants had a wider range of the polyphenols than their parental plants, with increased levels more prevalent than decreased levels.

The average SVR estimated from the data of 391 R_1 families of the three genotypes 'IS8260', 'IS8768', and 'IS4225' was 37.3%, 37.3%, and 40.7% for total phenols, flavan-4-ols, and proanthocyanidins, respectively. The average SVF estimated from the data of 393 R_2 plants from 'IS3150', 'IS6881', and 'IS0724' three genotypes were 5.3%, 4.8%, and 7.8% for total phenols, flavan-4-ols, and proanthocyanidins, respectively. In comparison with the chlorophyll and other morphological and developmental variations described by Cai et al. (1990), the polyphenol variation reported here had six to ten-fold greater values for the SVR and SVF (Table 7). The chlorophyll and other morphological variations are macro-variations appearing as qualitative changes. The polyphenol variations de-

Table 4 R_1 families with polyphenolic variation detected in the R_2 generation

Genotypes	Number of R_1 families tested	R_1 families with variation		Number of variations detected from the R_1 family[a]						Range of mean value value of the R_1 families tested[b]								
				Total phenols		Flavan-4-ols		Proathantho-cyanidins		Total phenols A_{720}			Flavan-4-ols A_{550}			Proanthocyanidins A_{550}		
		n	%	+	−	+	−	+	−	H	CK	L	H	CK	L	H	CK	L
IS8260	148	101	68.2	60	7	39	30	69	5	309.5	168.5	138.8	0.415	0.195	0.115	1.748	1.073	0.797
IS8768	145	69	47.6	47	9	34	5	48	5	195.3	109.0	66.0	0.118	0.053	0.000	1.139	0.640	0.458
IS4225	98	46	46.7	4	19	12	26	4	25	170.7	96.9	64.1	0.602	0.479	0.320	0.973	0.784	0.586
Total	391	216	55.2	111	35	85	61	121	38									

[a] + and, not R refer to the direction of the variation toward an increase or decrease, respectively

[b] H and refer to the highest and lowest mean value of tested R_1 families, respectively. The column CK shows the mean value of the corresponding parent. The mean value data was from the 1988 R_2 generation

scribed here are micro-variations involving a quantitative change within a relatively minor range. The higher SVR and SVF values for polyphenols than for morphological variations (Table 7) found in our study is consistent with the estimation that micro-mutations occur 5–10 times more frequently than chlorophyll mutations and other vital macro-mutations (Gaul 1964).

Persistence of polyphenol variation in the R_3 generation

Among the 112 R_2 families (from 61 R_1 families) tested in the R_3 generation, 44 R_1 (72%) and 72 R_2 (64.3%) families retained their variant phenotypes (Table 8). Of the 222 variations carried by the 112 families, 136 persisted in the R_3 generation (61.3%) (Table 8). From 21 tested non-variant R_2 families, variations in all three of the polyphenol characteristics tested were detected in 6 families (28.6%). The failure in a few cases to recover polyphenol variation until the R_3 generation may result from chimeric regenerants with a small mutated sector, as previously reported for morphological and developmental variations (Cai et al. 1990). The results indicated that previously undetected variations could be found in the R_3 generation and that variation selected for in the R_2 generation will generally persist.

Genotypic differences in persistence were pronounced. The frequencies of persistent variant R_1 and R_2 families, and total variation from the R_2 to the R_3 generation were 100% and 96.4%, and 82.6% respectively for 'IS8260', which were higher than those produced by 'IS8768' and 'IS4225' (Table 8). Similarly, 'IS8260' had the most variant phenotypes in field assessment for morphological and developmental variation (Cai et al. 1990). There was no effect of polyphenol characteristics on the persistence. The average frequencies of persistent variants for total phenols, flavan-4-ols and proanthocyanidins were similar in each cultivar except for flavan-4-ols of 'IS8768', which contains very low levels of flavan-4-ols (Table 8). This frequency depends strongly upon whether the polyphenol levels were increased or decreased in the variation. The frequency of persistence for the increased level variation ranged from 65% to 90% (average 77%), but the frequency was only 22–44% (average 35%) for those with decreased levels of polyphenols (Table 8).

As expected because tannin is the major polyphenol component of high tannin sorghum grain, variants with altered levels of tannin almost always had similarly altered levels of total phenols. However, in some families, only one of these characteristics were significantly altered. For example, an 'IS4225' family produced 24% less tannin than the parental check, yet the level of total phenols was the same in both. In a case such as this, polyphenol metabolism may have been partially diverted from the production of tannin to other phenolic materials.

Table 9 illustrates somaclones that showed increased or decreased levels of grain polyphenols in the R_3 generation. Table 10 shows the persistence of changed polyphenol content in successive generations. Generally the percentage

Table 5 Comparison between R_2 populations (R_2) and their parental controls (CK) in grain polyphenols

Genotypes		Number of plants tested	Total phenols A_{720}			Flavan-4-ols A_{550}			Proanthocyanidins A_{550}		
			Mean ± SEM[a]	t^b	F^b	Mean ± SEM	t	F	Mean ± SEM	t	F
IS3150	R_2	310	240.7 ± 69.8	ns	*	0.867 ± 0.418	ns	*	1.111 ± 0.381	ns	**
	CK	42	249.4 ± 52.0			0.778 ± 0.311			1.132 ± 0.210		
IS6881	R_2	51	257.6 ± 26.5	**	ns	0.129 ± 0.019	**	ns	1.205 ± 0.219	ns	ns
	CK	16	221.8 ± 26.6			0.107 ± 0.024			1.266 ± 0.263		
IS0724	R_2	32	306.8 ± 63.7	ns	ns	1.229 ± 0.212	**	*	1.410 ± 0.255	ns	ns
	CK	11	280.7 ± 59.7			0.975 ± 0.116			1.264 ± 0.273		

ns, not significant; * and **, significant at $P< 0.05$ and $P< 0.01$, respectively
[a] Mean value and standard error of measurements (SEM)
[b] Difference between R_2 populations and their parents in variances and mean values by F- and t-test, respectively

Table 6 Comparison between R_2 plants (R_2) and their parents (CK) in the range of their grain polyphenol characteristics[a]

Genotypes		Number of plants tested	Total phenols A_{720}	Flavan-4-ols A_{550}	Proanthocyanidins A_{550}
IS8260	R_2	1291	100 – 363	0.084 – 0.539	0.667 – 1.939
	CK	70	125 – 212	0.131 – 0.286	0.848 – 1.300
IS8768	R_2	563	57 – 246	0.000 – 0.183	0.361 – 1.557
	CK	50	76 – 120	0.000 – 0.099	0.460 – 1.180
IS4225	R_2	528	58 – 226	0.175 – 0.802	0.424 – 1.328
	CK	50	75 – 134	0.335 – 0.584	0.622 – 1.005
IS3150	R_2	310	60 – 490	0.110 – 1.945	0.240 – 1.913
	CK	42	145 – 370	0.203 – 1.728	0.571 – 1.399
IS6881	R_2	51	210 – 320	0.082 – 0.173	0.831 – 1.771
	CK	16	165 – 259	0.071 – 0.155	0.942 – 1.760
IS0724	R_2	32	225 – 492	0.917 – 1.817	1.070 – 2.027
	CK	11	235 – 452	0.821 – 1.201	0.803 – 1.689

[a] The data listed here were obtained from 1988 R_2 plants for IS8260, IS8768, and IS4225; for the other three genotypes the data were from 1987. The grain samples were taken from open-pollinated panicles

Table 7 Somaclonal variation rate (SVR) and somaclonal variant frequency (SVF) for polyphenols and other characteristics

Characteristic	SVR (%)	SVF (%)
Total phenols	37.3[b]	5.3[c]
Flavon-4-ols	37.3[b]	4.8[c]
Proanthocyanidins	40.7[b]	7.8[c]
Chlorophyll[a]	4.6	0.72
Morphological and developmental characters[a]	6.7	0.87

[a] Data from field assessment of R_2 generation (Cai et al. 1990)
[b] Determined with IS8260, IS8768, and IS4225. The SVR value is the number of polyphenol variations detected from the assayed R_1 families divided by the total number of R_1 families assayed
[c] Determined with IS3150, IS6881, and IS0724. The SVF value is the number of variants, with the variation(s) detected from the assayed R_2 plants, divided by the total number of R_2 plants assayed

change for variants with increased levels of polyphenols was higher and more persistent than that for those with decreased levels (Table 10).

In the R_4 generation only 144 R_4 plants of 16 R_3 families from 2 somaclones were tested, both from ‘IS4225’ mature embryo culture. One variant maintained its increased levels of flavan-4-ols and the other retained high levels of total phenols and tannins.

Discussion

The results reported here demonstrate that although we obtained somaclonal variants with increased or decreased levels of tannins, flavan-4-ols, and total phenols in the

Table 8 Persistence of polyphenol variation in the R_3 generation

	R_1[b] families	R_2 families	Number of polyphenol variations recovered in R_3 generation[a]					
			Total	Total phenols	Flavan-4-ols	Proantho-cyanidins	+	–
	IS8260							
Number of tested	15	28	69	24	20	25	58	11
Number of recovered	15	27	57	19	17	21	52	5
%	100.0	96.4	82.6	79.2	85.0	84.0	89.7	45.5
	IS8768							
Number of tested	20	26	51	18	10	23	33	18
Number of recovered	11	12	28	10	8	10	24	4
%	55.0	46.2	54.9	55.6	80.0	43.5	72.7	22.2
	IS4225							
Number of tested	26	58	102	29	47	26	48	54
Number of recovered	18	33	51	14	22	15	31	20
%	69.2	56.9	50.0	48.3	46.8	57.7	64.6	37.0
	Total							
Number of tested	61	112	222	71	77	74	139	83
Number of recovered	44	72	136	43	47	46	197	29
%	72.1	64.3	61.3	60.6	61.0	62.2	77.0	44.9

[a] + and – refer to the direction of the variations toward an increase or decrease, respectively
[b] The R_1 families from which the tested R_2 families were selected

Table 9 Selected somaclones (SC) showing polyphenolic variation(s) in the R_3 generation

Materials	Total phenols A_{720} Mean ± SE	Flavan-4-ols A_{550} Mean ± SE	Proanthocyanidins A_{550} Mean ± SE[a]
	IS8260		
Parental control	264 ± 19.1	0.310 ± 0.033	1.534 ± 0.166
SC IF911	338 ± 38.1**	0.436 ± 0.057**	1.844 ± 0.154**
SC IF707	312 ± 16.7**	0.563 ± 0.114**	1.966 ± 0.231**
SC IF909	222 ± 35.7**	0.259 ± 0.099*	1.129 ± 0.137**
	IS8768		
Parental control	114 ± 31.7	0.036 ± 0.025	0.731 ± 0.201
SC ME429	166 ± 45.8**	0.023 ± 0.033[ns]	1.058 ± 0.229**
SC ME418	104 ± 17.4[ns]	0.025 ± 0.012[ns]	0.510 ± 0.092**
	IS4225		
Parental control	138 ± 8.45	0.417 ± 0.068	0.867 ± 0.101
SC IF406	221 ± 14.0**	0.460 ± 0.061*	1.514 ± 0.012**
SC IF511	159 ± 13.4**	0.618 ± 0.036**	1.051 ± 0.138**
SC IF607	130 ± 8.77**	0.342 ± 0.025**	0.749 ± 0.082**

ns not significant; * and ** significant at $P < 0.05$ and $P < 0.001$, respectively
[a] Mean value and standard error (SE) difference between R_3 and control in mean values

grain, we did not identify a single somaclone in which the capacity to produce these polyphenols was lost or greatly reduced. No variant having a qualitative change from a high tannin to low tannin phenotype was observed. Most of the variants produced more, rather than less, polyphenols than their parental control. Several aspects of this finding warrant comment.

In addition to the possibility of recovering new genetic variants improved in grain quality or agronomic characters, we also hoped to obtain variants blocked at different steps in flavonoid and/or tannin biosynthesis. Condensed tannins are oligomers of flavan-3-ols (Gupta and Haslam 1978). Anthocyanidins, the major pigments of sorghum grain, are alternative end products, with tannin, of flavonoid metabolism in the seed coat. The occurrence of tannin in sorghum grain is controlled by two complementary dominant genes *B1* and *B2* (Rooney et al. 1980). The homozygous recessive condition of either loci results in the absence of a pigmented testa and the absence of tannin in the grain. Woodruff et al. (1982) found that tannin quantity in sorghum grain is a highly heritable trait controlled by relatively few genes and that the high-tannin trait is dominant to the low-tannin trait, although simple one-or two-gene models did not adequately describe the segregation. Haskins and Gorz (1986) found that flavan-4-ol (leucoanthocyanidin in their terminology) content of sorghum

Table 10 Maintenance of changed grain polyphenol content in successive generations of tissue culture-derived sorghum somaclones

Characteristics	Somaclones	Increase %[a]		
		R_1	R_2	R_3
Total phenols	82IF911	54.5	30.6	28.2
	87ME429	–	8.3	46.5
	42IF406	42.0	66.2	59.4
Flavan-4-ols	82IF707	–	79.5	81.6
	82IE682	85.9	59.5	69.7
	42IE802	71.0	65.2	67.7
Proanthocyanidins	82IF911	42.5	25.8	20.2
	87ME429	–	45.2	44.7
	42IF406	44.5	37.2	74.6
Total phenols	82IF909	–	24.6	16.0
	87ME418	–	34.0	9.7
	42IF607	43.4	16.4	6.1
Flavan-4-ols	82IF909	–	43.1	16.5
	42IF607	30.4	32.5	18.0
	42IF604	32.7	33.4	20.1
Proanthocyanidins	82IF909	–	16.1	26.4
	87ME418	–	28.4	30.2
	42IF607	23.2	28.7	13.6

[a] Comparison with the parental control

forage is primarily controlled by a single allelic pair. We did not find variants in which the capability for polyphenol production was lost due to a mutation altering a biosynthetic enzyme. A possible explanation for our unexpected result is that sorghum has multiple metabolic pathways, each consisting of several steps, leading to the production of polyphenolic materials not separately distinguished by our assays for total phenols, flavan-4-ols, and proanthocyanidins. The loss of one of these pathways or only one step of a pathway may have little effect on overall polyphenol content.

There was variability in the level of grain polyphenols produced within the parental plants even without in vitro culture. It is unlikely that these or other crop cultivars were selected for uniformity in polyphenol production during their development. The origins of somaclonal variation are genetic differences pre-existing in the somatic cells of the explants as well as genetic events induced by culture conditions (Bajaj 1990). Heterozygosity pre-existing in the parents might have contributed to the polyphenol variations with lower percentage change from the parental control identified here.

Our observation that variants with increased levels of polyphenols appeared more frequently and persisted over more generations than variants with decreased levels of polyphenols may indicate that the culture conditions we used somehow favored variations with increased levels rather than decreased levels of polyphenols. By way of contrast, we found many chlorophyll-deficient variants (Cai et al. 1990), although chlorophyll is an essential metabolite for normal growth and development and proanthocyanidins and flavan-4-ols are not (because many sorghums do not produce them). The biochemical basis for this curious difference is unknown.

Many genetically controlled factors including pericarp color and thickness, the presence, color and thickness of the testa, endosperm, glume and plant color, and others affect the polyphenol content of sorghum grain (Rooney et al. 1980). Environmental factors (Hahn and Rooney 1986) as well as grain maturity (Butler 1982) also influence the level of sorghum grain polyphenols. Finding genuine polyphenol variants is more difficult than finding morphological variants. Variations that did not persist to subsequent generations possibly resulted not only from culture-induced epigenetic variation but also from other factors. The loss of variation in subsequent generations has previously been reported in tissue culture derived sorghum somaclones (Bhaskaran et al. 1987).

Our demonstration that useful agronomic variation occurs only rarely in tissue cultures is consistent with reports by others (Zehr et al. 1987; Lee et al. 1988; Dunwell et al. 1986; Maddock and Semple 1986; Ryan et al. 1987). Despite favorable reports (Hanning et al. 1989; Waskom et al. 1990), somaclonal variation is not likely to replace other strategies for crop improvemement. Among the polyphenol variations detected here the variant families with a high content of high flavan-4-ols (Tables 9, 10) may be useful. Studies by Jambunathan et al. (1990) have demonstrated that high levels of flavan-4-ols are indicative of mold resistance. These culture-derived somaclones, especially single family-derived sister lines, have a near-isogenic background. They may provide valuable material for elucidating genetic factors controlling polyphenol production.

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