SUPPLEMENTARY MATERIALS

Enrichment of grain anthocyanin content through marker-assisted breeding for *Ant1*, *Ant2* or *HvMyc2* genes in barley (*Hordeum vulgare* L.)

Tatjana V. Kukoeva[1,2], Camilla A. Molobekova[1,3], Igor V. Totsky[1], Gennady V. Vasiliev[1,2], Artem Yu. Pronozin[1,2], Dmitry A. Afonnikov[1,2,3], Elena K. Khlestkina[1,4], Olesya Yu. Shoeva[1,2,3]*

[1] Institute of Cytology and Genetics (ICG), Siberian Branch of Russian Academy of Sciences (SB RAS), Novosibirsk 630090, Russia

[2] Kurchatov Genomic Center of ICG SB RAS, Novosibirsk 630090, Russia

[3] Novosibirsk State University, Novosibirsk 630090, Russia

[4] N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), St. Petersburg 190000, Russia

Email: olesya_ter@bionet.nsc.ru

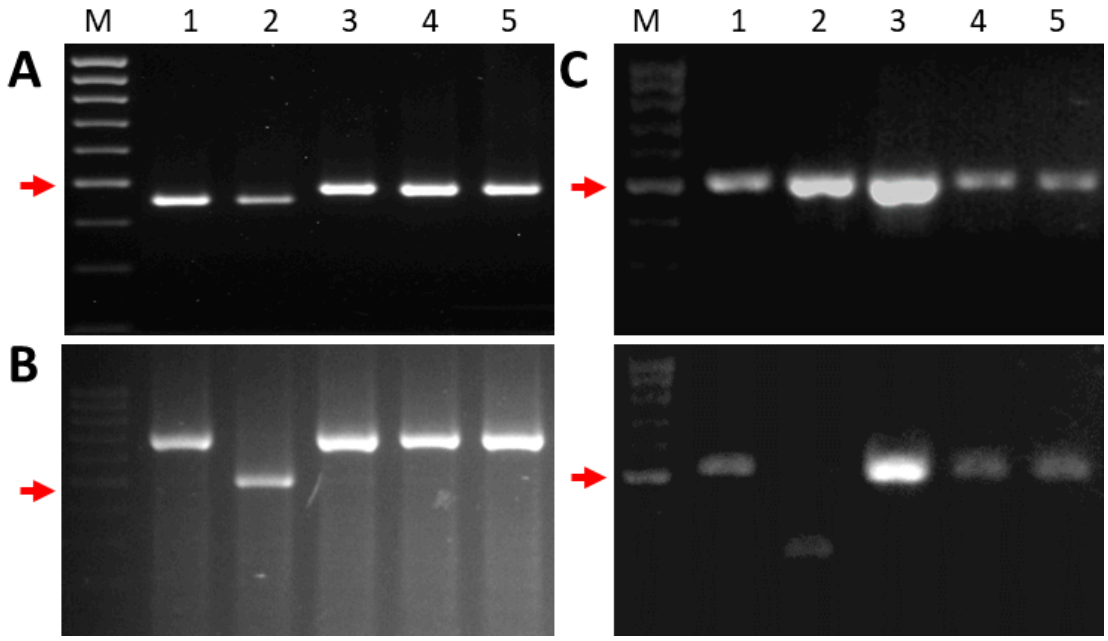


Figure S1. Electropherograms of PCR products obtained using molecular markers for genes *Ant1* (A), *Ant2* (B), and *HvMyc2* (C); Bowman (lane 1), BW648 (in A and B) or BW418 (in C) (2), *cv.* Aley (3), *cv.* Tanay (4), or *cv.* Vorsinsky-2 (5); M: 100-bp DNA marker ladder; the red arrow shows the DNA marker band corresponding to 500 bp.

In C, the upper and lower panels show amplicons before and after digestion by restriction enzyme *Bse1 I*, respectively.

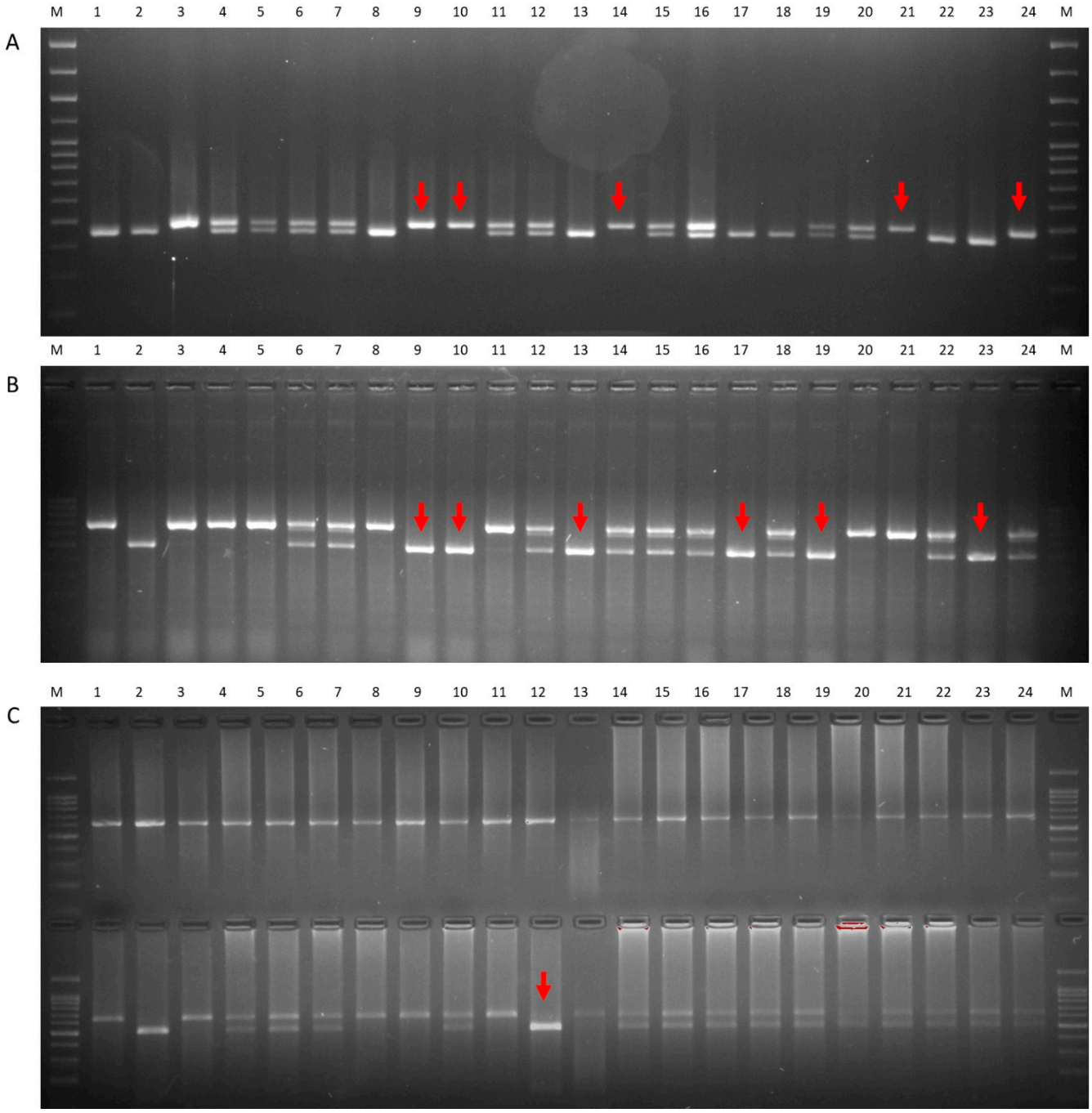


Figure S2. Electropherograms of PCR products obtained using molecular markers for genes *Ant1* (A), *Ant2* (B), and *HvMyc2* (C) from the DNA of parental genotypes and their resulting F_2 hybrids. In first and last lanes, a 100-bp DNA marker ladder (M) was run. The red arrows show the homozygous hybrids carrying donors' alleles of the target genes.

In A: Bowman (line 1), BW648 (2), *cv.* Vorsinsky 2 (3), and hybrids of F_2 population Vorsinsky 2 x BW648 (4-24),

In B: Bowman (1), BW648 (2), *cv.* Vorsinsky 2 (3), and hybrids of F_2 population Vorsinsky 2 x BW648 (4-24),

In C: Bowman (line 1), BW418 (2), *cv.* Tanay (3), and hybrids of F_2 population Tanay x BW418 (4-24), the upper and lower pictures show PCR-products before and after digestion by restriction enzyme *Bse1 I*, respectively.

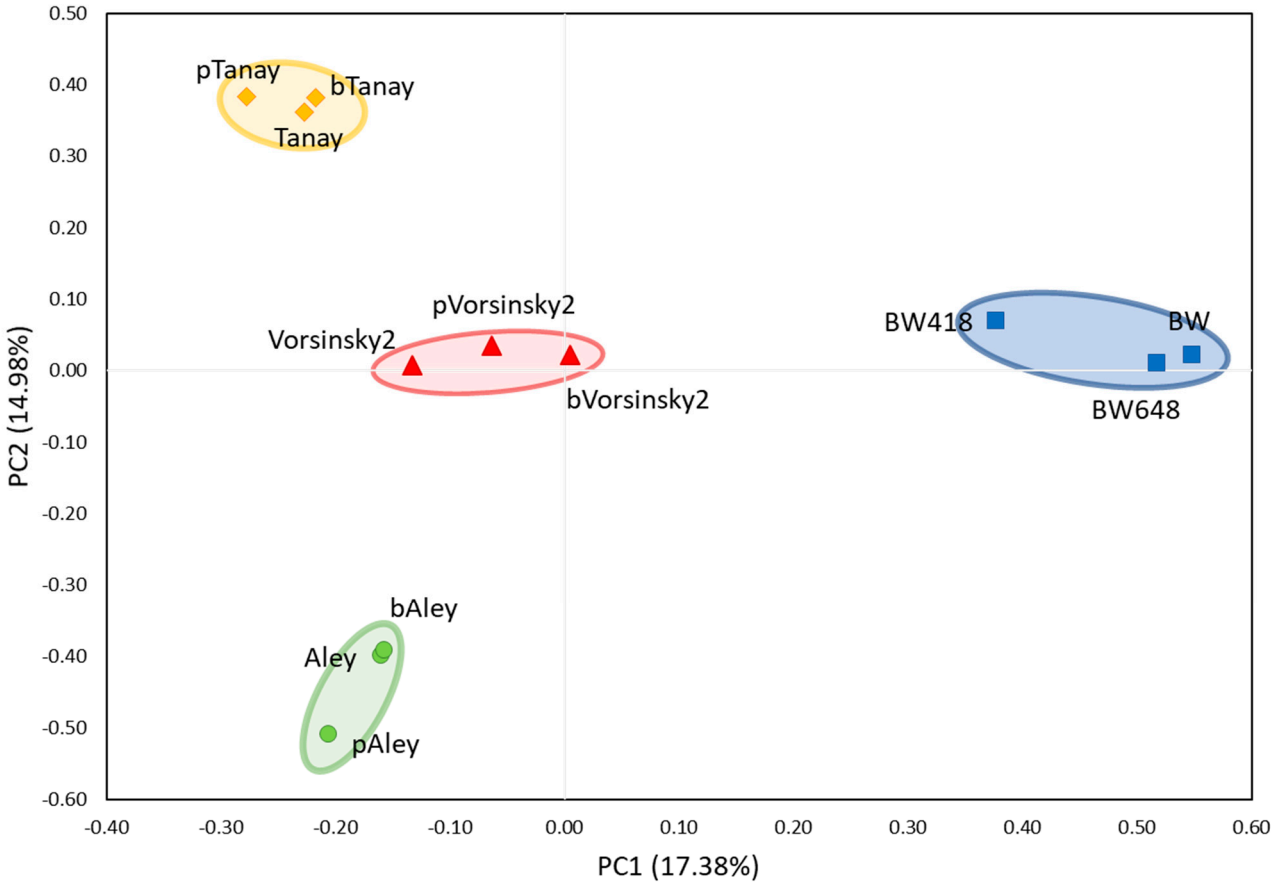


Figure S3. Scatter plots of the genotypes of newly developed barley NILs and their parental cultivars (recurrent parents and donors) in the space of the first two components derived from SNP data of the genomes retrieved by GBS analysis. The ovals highlight a set of lines based on Aley (green), Tanay (yellow), Vorsinsky-2 (orange), and Bowman donor NILs (blue).

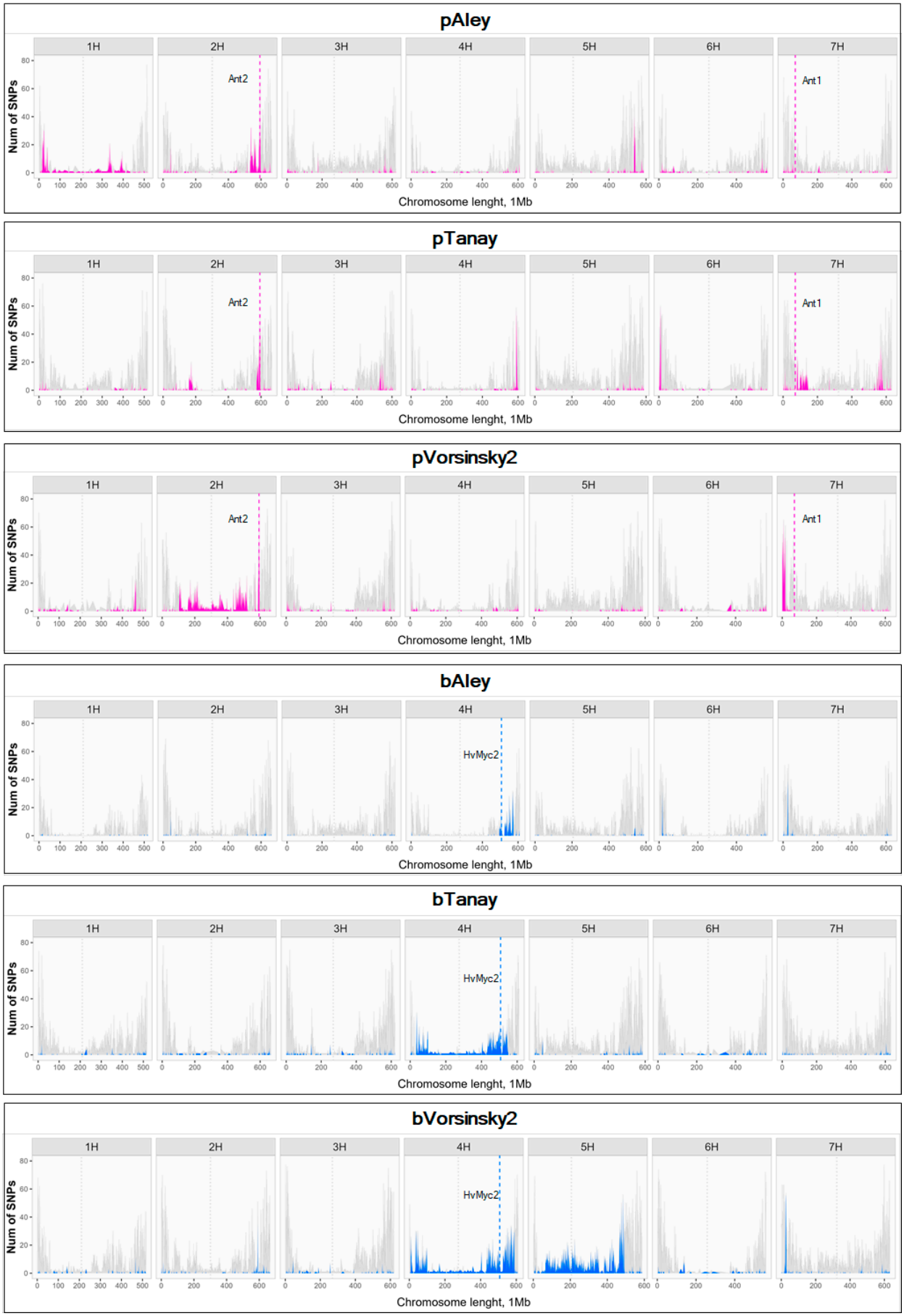


Figure S4. The number of donor SNPs (are shown in a purple and blue color) and total number of SNPs (in a gray color) per megabase pair.

Table S1. Segregation of the *Ant1*, *Ant2* and *HvMyc2* genes in F_2 populations developed by crossing recurrent cultivars with the donor lines

Crossing combination	Trait/Gene	Genotypic Class	Number of plants			Segregation ratio	X^2	*p*
			Observed	Expected	Total			
Breeding of purple-grained lines								
cv. Aley x BW648	*Ant1*	*Ant1/Ant1*	6	10	40	1:2:1	2.15	0.3413
		Ant1/ant1	23	20				
		ant1/ant1	11	10				
	Ant2	*Ant2/Ant2*	18	10	40	1:2:1	10.20	0.0061
		Ant2/ant2	18	20				
		ant2/ant2	4	10				
cv. Tanay x BW648	*Ant1*	*Ant1/Ant1*	17	14.75	59	1:2:1	2.08	0.3526
		Ant1/ant1	24	29.50				
		ant1/ant1	18	14.75				
	Ant2	*Ant2/Ant2*	14	14.75	59	1:2:1	4.45	0.1082
		Ant2/ant2	35	29.50				
		ant2/ant2	10	14.75				
cv. Vorsinsky-2 x BW648	*Ant1*	*Ant1/Ant1*	7	10.25	41	1:2:1	2.17	0.3378
		Ant1/ant1	25	20.50				
		ant1/ant1	9	10.25				
	Ant2	*Ant2/Ant2*	15	10.25	41	1:2:1	5.68	0.0583
		Ant2/ant2	13	20.50				
		ant2/ant2	13	10.25				
Breeding of blue-grained lines								
cv. Aley x BW418*	*HvMyc2*	*HvMyc2/hvmyc2*	52	55.3	83	2:1	0.59	0.4424
		HvMyc2/ HvMyc2	31	27.7				
cv. Tanay x BW418*	*HvMyc2*	*HvMyc2/hvmyc2*	47	48	72	2:1	0.06	0.8026
		HvMyc2/ HvMyc2	25	24				
cv. Vorsinsky-2 x BW418*	*HvMyc2*	*HvMyc2/hvmyc2*	35	42.7	64	2:1	4.17	0.0411
		HvMyc2/ HvMyc2	29	21.3				

* the grains of generation F_1 with blue color were selected and propagated to get F_2 populations, in which the segregation of the *HvMyc2* gene is analyzed. The recessive homozygotes for the *HvMyc2* gene are not present in this population.

Table S2. SNPs identified by GBS in chromosomes of the newly developed NILs

NIL	Chr	Number of SNPs	SNP density (number of SNPs per Mbp)	Number of donors' SNPs	Frequency of donors' SNPs, %
pAley	1H	1 691	3.27	461	27.26
	2H	4 208	6.32	820	19.49
	3H	3 971	6.39	147	3.70
	4H	1 786	2.93	71	3.98
	5H	4 344	7.39	255	5.87
	6H	2 078	3.70	120	5.77
	7H	4 233	6.69	151	3.57
	Total	22 311	5.32	2025	9.08
bAley	1H	2 146	4.15	49	2.28
	2H	4 056	6.09	107	2.64
	3H	4 265	6.86	66	1.55
	4H	2 267	3.71	492	21.70
	5H	4 115	7.00	87	2.11
	6H	2 517	4.48	105	4.17
	7H	4 492	7.10	185	4.12
	Total	23 858	5.69	1091	4.57
pVorsinsky-2	1H	2 007	3.89	178	8.87
	2H	5 044	7.58	1845	36.58
	3H	3 921	6.31	152	3.88
	4H	1 889	3.10	93	4.92
	5H	4 998	8.50	111	2.22
	6H	2 426	4.32	77	3.17
	7H	5 484	8.67	692	12.62

	Total	25 769	6.14	3148	12.22
bVorsinsky-2	1H	2 977	5.76	92	3.09
	2H	5 069	7.62	185	3.65
	3H	4 596	7.39	112	2.44
	4H	3 020	4.95	2092	69.27
	5H	5 361	9.11	2489	46.43
	6H	2 904	5.17	82	2.82
	7H	6 362	10.06	271	4.26
	Total	30 289	7.22	5323	17.57
pTanay	1H	2 955	5.72	85	2.88
	2H	4 557	6.85	572	12.55
	3H	4 019	6.47	240	5.97
	4H	1 938	3.18	248	12.80
	5H	5 071	8.62	115	2.27
	6H	2 834	5.04	254	8.96
	7H	5 107	8.07	704	13.79
	Total	26 481	6.31	2218	8.38
bTanay	1H	3 273	6.34	76	2.32
	2H	4 268	6.41	104	2.44
	3H	4 897	7.88	137	2.80
	4H	2 839	4.65	1310	46.14
	5H	5 201	8.84	129	2.48
	6H	3 213	5.72	67	2.09
	7H	5 958	9.42	107	1.80
	Total	29 649	7.07	1930	6.51
Average		26 393	6.29	2 623	9.72

Table S3. Effects of different factors on the TAC, TPC, and AOA according to the Kruskal–Wallis *H* test

Factors	Group	Group Size	Sum of Ranks	df	*H*	*p*-value	Significant
Total anthocyanin content							
Growth conditions	greenhouse	33	1157.0	1	0.5773E-3	0.9808	no
	field	36	1258.0				
Growth conditions. yellow	greenhouse	9	46.0	1	14.2053	0.0002	yes
	field	12	185.0				
Growth conditions. purple	greenhouse	12	187.5	1	4.6895	0.0303	yes
	field	12	112.5				
Growth conditions. blue	greenhouse	12	123.0	1	2.4300	0.1190	no
	field	12	177.0				
Grain color	yellow	21	231.0	2	45.1529	0.0000	yes
	purple	24	995.0				
	blue	24	1189.0				
Grain color. greenhouse	yellow	9	45.0	2	19.3601	0.0001	yes
	purple	12	271.0				
	blue	12	245.0				
Grain color. field	yellow	12	78.0	2	27.8219	0.0000	yes
	purple	12	239.5				
	blue	12	348.5				
Genetic background	Aley	18	640.5	3	2.9940	0.3925	no
	Tanay	18	554.0				
	Vorsinsky 2	18	587.5				
	Bowman	15	633.0				
Total phenolic content							
Growth conditions	greenhouse	33	613.0	1	42.3917	0.0000	yes
	field	36	1802.0				
Growth conditions. yellow	greenhouse	9	52.0	1	11.1566	0.0008	yes
	field	12	179.0				
Growth conditions. purple	greenhouse	12	78.0	1	17.2800	0.0000	yes
	field	12	222.0				
Growth conditions. blue	greenhouse	12	78.0	1	17.2800	0.0000	yes
	field	12	222.0				
Grain color	yellow	21	598.0	2	3.3431	0.1880	no
	purple	24	881.0				
	blue	24	935.5				
Grain color. greenhouse	yellow	9	113.5	2	2.5749	0.2760	no
	purple	12	220.0				
	blue	12	227.5				
Grain color. field	yellow	12	133.5	2	9.3492	0.0093	yes
	purple	12	247.5				
	blue	12	285.0				

Genetic background	Aley	18	496.0	3	7.5289	0.0568	no
	Tanay	18	812.0				
	Vorsinsky 2	18	635.5				
	Bowman	15	471.5				
Antioxidant activity							
Growth conditions	greenhouse	33	1198.0	1	0.2668	0.6055	no
	field	36	1217.0				
Growth conditions. yellow	greenhouse	9	106.0	1	0.2475	0.6189	no
	field	12	125.0				
Growth conditions. purple	greenhouse	12	149.0	1	0.0033	0.9540	no
	field	12	151.0				
Growth conditions. blue	greenhouse	12	157.0	1	0.1633	0.6861	no
	field	12	143.0				
Grain color	yellow	21	554.0	2	11.1741	0.0037	yes
	purple	24	766.0				
	blue	24	1095.0				
Grain color. greenhouse	yellow	9	118.0	2	3.7704	0.1518	no
	purple	12	190.0				
	blue	12	253.0				
Grain color. field	yellow	12	150.0	2	10.3799	0.0056	yes
	purple	12	203.0				
	blue	12	313.0				
Genetic background	Aley	18	291.0	3	25.4695	0.0000	yes
	Tanay	18	871.0				
	Vorsinsky 2	18	630.0				
	Bowman	15	623.0				

Table S4. Spearman's coefficients of correlation between the TAC, TPC, and AOA in the grains assessed in the combined dataset (without suffixes) or datasets collected from grains harvested in the greenhouse (with the "-GH" suffix) or in the field (with the "-F" suffix) processed individually. Correlations marked in red are significant at $p < 0.05$

	TAC	TPC	AOA	TAC-GH	TPC-GH	AOA-GH	TAC-F	TPC-F	AOA-F
TAC	1.00	0.27	0.42						
TPC	0.27	1.00	0.37						
AOA	0.42	0.37	1.00						
TAC-GH				1.00	0.28	0.51	0.43		
TPC-GH				0.28	1.00	0.76		0.16	
AOA-GH				0.51	0.76	1.00			0.05
TAC-F				0.43			1.00	0.60	0.75
TPC-F					0.16		0.60	1.00	0.82
AOA-F						0.05	0.75	0.82	1.00

Table S5. Characteristics of the anthocyanin-rich NILs and their parental recurrent cultivars. Statistically significant differences between the lines and recurrent parental lines are marked by an asterisk (*U* test, * $p < 0.05$, ** $p < 0.001$).

Growth conditions	Trait	Cultivars/Lines		
		Aley	**pAley**	**bAley**
Field, 2022	Plant height, cm	87.73 ± 6.06	79.77 ± 7.24**	86.95 ± 6.89
	Spike number per plant	5.03 ± 2.19	3.52 ± 1.44**	4.62 ± 1.74
	Main spike length, cm	7.15 ± 1.22	6.80 ± 1.07	7.92 ± 0.98**
	Spike density	12.00 ± 0.00	12.03 ± 0.26	12.17 ± 0.85
	Number of seeds per plant	93.88 ± 46.25	63.55 ± 29.47**	88.55 ± 33.12
	Grain weight per plant, g	6.45 ± 2.85	4.49 ± 1.75**	6.19 ± 1.88
	Thousand-grain weight, g	70.20 ± 6.89	73.02 ± 9.51*	72.54 ± 9.05
		Tanay	**pTanay**	**bTanay**
	Plant height, cm	90.63 ± 4.00	93.32 ± 4.17*	92.77 ± 4.33*
	Spike number per plant	5.58 ± 1.93	4.03 ± 1.71**	4.68 ± 1.20*
	Main spike length, cm	6.43 ± 1.01	6.77 ± 0.72*	6.87 ± 0.93*
	Spike density	13.95 ± 0.71	13.63 ± 0.78	13.93 ± 0.63
	Number of seeds per plant	114.30 ± 43.59	82.78 ± 35.48**	97.53 ± 27.37
	Grain weight per plant, g	7.54 ± 2.55	5.72 ± 2.06**	6.44 ± 1.46
	Thousand-grain weight, g	68.46 ± 7.22	71.34 ± 8.67*	67.91 ± 10.90
		Vorsinsky 2	**pVorsinsky 2**	**bVorsinsky 2**
	Plant height, cm	84.80 ± 4.33	86.52 ± 3.54*	84.87 ± 4.17
	Spike number per plant	5.27 ± 1.73	5.83 ± 1.49*	3.72 ± 1.11**
	Main spike length, cm	6.68 ± 1.02	6.07 ± 0.63**	6.20 ± 1.04*
	Spike density	12.27 ± 0.69	12.83 ± 0.99**	12.25 ± 0.65
	Number of seeds per plant	88.90 ± 28.13	87.87 ± 22.32	62.10 ± 20.37**
	Grain weight per plant, g	5.92 ± 1.71	6.10 ± 1.20	4.47 ± 1.29**
	Thousand-grain weight, g	67.66 ± 7.61	70.54 ± 5.81**	73.89 ± 15.22*
Field, 2023		**Aley**	**pAley**	**bAley**
	Plant height, cm	91.05 ± 5.15	87.55 ± 4.60*	90.68 ± 5.48
	Spike number per plant	5.00 ± 1.58	5.73 ± 2.12*	6.83 ± 2.97**
	Main spike length, cm	6.90 ± 0.84	7.87 ± 0.89**	7.95 ± 0.89**
	Spike density	12.07 ± 0.36	12.43 ± 0.93*	12.15 ± 0.52
	Number of seeds per plant	96.15 ± 32.62	112.90 ± 40.49*	135.20 ± 62.16**
	Grain weight per plant, g	4.38 ± 1.52	5.02 ± 1.96	6.01 ± 2.93*
	Thousand-grain weight, g	45.62 ± 7.10	45.64 ± 3.09	45.36 ± 13.35
		Tanay	**pTanay**	**bTanay**
	Plant height, cm	98.75 ± 6.00	98.03 ± 7.29	94.20 ± 5.06**
	Spike number per plant	5.20 ± 1.82	5.82 ± 2.34	4.97 ± 1.99
	Main spike length, cm	7.17 ± 0.69	6.92 ± 0.87*	6.67 ± 0.77**
	Spike density	13.05 ± 1.01	13.53 ± 0.85*	13.87 ± 0.50**
	Number of seeds per plant	117.20 ± 39.28	121.60 ± 46.20	110.30 ±47.01
	Grain weight per plant, g	5.18 ± 1.76	5.53 ± 2.12	4.90 ± 2.16
	Thousand-grain weight, g	44.29 ± 3.37	45.55 ± 4.08	44.34 ± 3.67
		Vorsinsky 2	**pVorsinsky 2**	**bVorsinsky 2**
	Plant height, cm	87.12 ± 4.14	84.73 ± 5.28*	89.45 ± 5.45*
	Spike number per plant	5.77 ± 2.40	7.08 ± 2.75*	5.87 ± 2.13
	Main spike length, cm	6.68 ± 0.86	6.07 ± 0.78**	7.23 ± 0.81*
	Spike density	12.72 ± 0.94	12.67 ± 0.95	11.95 ± 0.32**

	Number of seeds per plant	110.00 ± 44.36	116.70 ± 48.35	116.00 ± 44.37
	Grain weight per plant, g	4.70 ± 1.98	5.32 ± 2.33	5.40 ± 2.07
	Thousand-grain weight, g	42.61 ± 4.33	45.39 ± 4.08**	46.70 ± 3.25**
		Aley	**pAley**	**bAley**
Greenhouse, 2022	Plant height, cm	104.40 ± 5.45	106.10 ± 5.05	103.20 ± 5.63
	Spike number per plant	7.75 ± 3.45	7.25 ± 1.94	6.40 ± 2.01
	Main spike length, cm	6.40 ± 0.60	7.35 ± 0.88**	7.30 ± 0.92**
	Spike density	12.10 ± 0.31	11.80 ± 0.89*	11.75 ± 0.44*
	Number of seeds per plant	108.40 ± 45.84	120.30 ± 31.82	105.10 ± 30.55
	Grain weight per plant, g	5.10 ± 2.08	5.13 ± 1.47	4.78 ± 1.41
	Thousand-grain weight, g	47.41 ± 2.45	42.46 ± 2.33**	45.57 ± 2.66*
		Tanay	**pTanay**	**bTanay**
	Plant height, cm	113.00 ± 11.11	114.30 ± 8.75	112.60 ± 9.75
	Spike number per plant	9.40 ± 2.11	6.80 ± 2.07**	5.63 ± 1.42**
	Main spike length, cm	5.40 ± 0.82	6.15 ± 0.59*	5.79 ± 0.71
	Spike density	12.15 ± 0.67	12.70 ± 0.98	12.74 ± 0.99
	Number of seeds per plant	121.40 ± 28.38	104.80 ± 27.03*	80.00 ± 22.42**
	Grain weight per plant, g	4.74 ± 1.26	4.04 ± 0.99	2.94 ± 0.99**
	Thousand-grain weight, g	38.97 ± 3.88	38.90 ± 4.81	36.18 ± 4.17*
		Vorsinsky 2	**pVorsinsky 2**	**bVorsinsky 2**
	Plant height, cm	100.70 ± 6.64	97.40 ± 3.62	103.30 ± 5.27
	Spike number per plant	10.05 ± 2.80	10.65 ± 2.93	14.85 ± 5.05**
	Main spike length, cm	6.90 ± 0.79	6.30 ± 0.86*	8.20 ± 0.89**
	Spike density	11.80 ± 0.62	11.60 ± 0.50	11.40 ± 0.50*
	Number of seeds per plant	142.90 ± 49.04	142.50 ± 35.80	237.10 ± 38.84**
	Grain weight per plant, g	5.10 ± 2.24	5.76 ± 1.65	10.89 ± 1.81**
	Thousand-grain weight, g	34.83 ± 4.81	40.27 ± 6.52*	45.46 ± 2.85**
		Aley	**pAley**	**bAley**
Greenhouse, 2023	Plant height, cm	104.00 ± 4.48	108.90 ± 5.26*	105.40 ± 6.15
	Spike number per plant	7.05 ± 2.70	6.85 ± 3.00	4.40 ± 1.31**
	Main spike length, cm	7.60 ± 0.75	7.80 ± 1.11	7.40 ± 0.82
	Spike density	11.90 ± 0.45	12.00 ± 0.00	12.00 ± 0.00
	Number of seeds per plant	121.30 ± 48.30	131.10 ± 57.69	78.30 ± 25.52*
	Grain weight per plant, g	6.50 ± 2.68	6.64 ± 2.94	3.80 ± 1.60**
	Thousand-grain weight, g	53.24 ± 2.27	50.55 ± 1.66**	50.54 ± 4.27*
		Tanay	**pTanay**	**bTanay**
	Plant height, cm	119.00 ± 6.29	117.80 ± 8.48	117.60 ± 8.20
	Spike number per plant	5.75 ± 1.07	7.15 ± 1.95*	6.60 ± 1.85
	Main spike length, cm	6.45 ± 0.94	6.00 ± 0.79	5.55 ± 0.69**
	Spike density	13.20 ± 1.01	12.00 ± 0.00**	12.00 ± 0.00**
	Number of seeds per plant	100.10 ± 19.10	113.80 ± 34.31	109.80 ± 31.11
	Grain weight per plant, g	4.51 ± 1.09	5.64 ± 1.68*	5.19 ± 1.73
	Thousand-grain weight, g	44.61 ± 4.86	49.64 ± 2.87**	46.92 ± 3.28
		Vorsinsky 2	**pVorsinsky 2**	**bVorsinsky 2**
	Plant height, cm	118.70 ± 6.18	106.70 ± 4.43**	121.40 ± 5.63
	Spike number per plant	8.55 ± 2.93	8.05 ± 3.73	6.30 ± 2.45*
	Main spike length, cm	8.35 ± 0.81	7.05 ± 0.60**	8.85 ± 0.75
	Spike density	11.85 ± 0.67	12.00 ± 0.00	12.00 ± 0.00
	Number of seeds per plant	159.20 ± 59.41	139.40 ± 61.71	125.00 ± 41.65
	Grain weight per plant, g	8.25 ± 3.04	7.34 ± 3.58	7.13 ± 2.87
	Thousand-grain weight, g	51.93 ± 2.92	52.06 ± 4.72	56.68 ± 10.31*

Table S6. Eigenvalues, eigenvectors, and percentages of variance explained by the principal components assessed for five traits in 11 barley genotypes evaluated in the greenhouse and in the field (Novosibirsk).

	Principal Component Axis				
	1	2	3	4	5
Eigenvalues	2.71	1.42	0.70	0.17	0.01
PC variation (%)	54.14	28.32	13.96	3.36	0.23
Trait	Eigenvectors				
Spike number per plant	0.53	-0.18	-0.38	0.73	-0.02
Plant height	0.38	-0.19	0.89	0.14	0.02
Number of seeds per plant	0.58	0.10	-0.16	-0.46	0.64
Grain weight per plant	0.45	0.56	-0.02	-0.22	-0.66
Thousand-grain weight	-0.18	0.78	0.17	0.43	0.39