Identification and validation of QTLs for green plant percentage in barley (*Hordeum vulgare* L.) anther culture

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Abstract In cereals, albinism is a major obstacle to produce doubled haploids (DH) for breeding programs. In order to identify QTLs for green plant percentage in barley anther culture, a specific population was developed. This population, consisting of 100 DH lines, was generated by crossing the model cultivar for anther culture "Igri" with an albino-producing DH line (DH46) selected from Igri x Dobla, in search of a maximum segregation for the trait and minimum for the other anther culture variables. A combination of bulked segregant analysis and AFLP methodology was used to identify markers linked to the trait. A linkage map was constructed using these AFLPs, together with RAPD, STS and SSR markers. This study identified a new QTL for green plant percentage on chromosome 3H and confirmed the previously reported one on chromosome 5H. Up to 65.2% of the phenotypic variance for this trait was explained by the additive effects of these two QTLs. Thirty elite cultivars of barley from different origin, row type, growth habit and end use, were selected to validate these QTLs. Since two of the markers linked to the QTLs were AFLPs, we successfully converted them into simple PCR-based SCAR markers. Only the SSR HVM60, on chromosome 3H, was significantly associated with the trait, explaining near 20% of the phenotypic variance. Among the allelic variants identified for this marker, HVM60-120bp was associated with the highest values of green plant percentage.

Keywords Albinism – Anther culture – Barley – Doubled haploids – Green plant percentage – QTLs

INTRODUCTION

Doubled haploid (DH) production is an important tool to rapidly generate homozygous lines for plant breeding and genetic studies (Forster and Thomas 2005). In barley, doubled haploidy has been widely used in breeding programs and has contributed to the release of many cultivars. DH populations have also enabled the mapping of many molecular markers as well as qualitative and quantitative trait loci (QTL) (Devaux and Pickering 2005).

Microspore embryogenesis, via anther or microspore culture, is one of the most commonly used methods for DH production in barley due to its potential to generate a large number of plants from a single anther. However, the disadvantage of this method is its dependence on genotype (Foroughi-Wehr et al. 1982; Larsen et al. 1991). Although the improvements achieved during the last few years have led to the production of DH from most genotypes (Cistué et al. 1994, 1999 and 2004; Castillo et al. 2000), there are genotypes of agronomic importance that still render none or a low number of green plants (Cistué et al. 2004).

In barley anther and microspore culture, regeneration of chlorophyll-deficient (albino) plants is a serious problem affected by genotype. This genotypic effect has been reported to account for over 70% of the total variation in percentage of green plants in studies of barley anther culture (Knudsen et al. 1989; Larsen et al. 1991). It has also been demonstrated, by means of reciprocal crosses, that genes controlling percentages of green plants are inherited chromosomally (Larsen et al. 1991). When plastid differentiation during anther culture has been studied, abnormal features have been found, mainly affecting the plastid size, thylakoid and granum organization, as well as starch accumulation (Caredda et al. 1999 and 2000). From molecular studies in barley, wheat and rice, deletions and rearrangements in the plastid genome of many microspore-derived albino plants have been observed (Day and Ellis 1984 and 1985; Dunford and Walden 1991; Harada et al. 1991). However, plastid DNA deletions cannot be the primary reason for albinism, since albino plants with an intact wild-type plastid genome have been described (Day and Ellis 1984; Dunford and Walden 1991; Harada et al. 1991; Hofinger et al. 2000). Hofinger et al. (2000) showed that, in wheat, all albino plants had an altered transcription and translation pattern when compared to green

plants. These results were in agreement with the study of Dunford and Walden (1991), who found different transcript levels of some plastid-related genes in barley albino plants. The deficiencies in plastids of albino plants could indicate that nuclear genes exert their effect via interactions with events in plastid development (Torp et al. 2004).

Identification of QTLs affecting green/albino plant formation may provide new tools for the study of albinism. QTLs for green plant percentage were mapped in wheat (Torp et al. 2001 and 2004), rye (Grosse et al. 1996) and triticale (González et al. 2005) anther culture. In barley, Manninen (2000) identified QTLs for anther culture response, although none of them was associated with green plant percentage. Recently, in a study conducted by our group, new QTLs affecting barley anther culture response were mapped in an Igri x Dobla DH population (Chen et al. 2007). Only a QTL for green plant percentage, located on chromosome 5H, was identified, explaining 21.07% of the phenotypic variance. Thus, a substantial portion of the phenotypic variance for this trait remained unexplained.

In the present study, a population of 100 DH lines derived from the cross between Igri and DH46 (DH line derived and selected from the F1 (Igri x Dobla)) was developed for further identification of chromosomal regions associated with green plant percentage. In order to validate the QTLs identified in this new population, a total of 30 elite cultivars of barley were also characterized.

MATERIALS AND METHODS

Plant material and anther culture

Plant material consisted of a DH population of 100 lines obtained via anther culture from a cross between Igri and DH46 (51 lines from Igri x DH46, and 49 lines from DH46 x Igri). The winter cultivar Igri is a model variety for microspore embryogenesis due to its good response to anther culture and it is known to have a high green plant percentage. DH46 is a doubled haploid line obtained from the cross between cultivars Igri and Dobla. Dobla is a facultative spring/winter cultivar with an intermediate response to anther culture, with 49.6% of green plants (Chen et al. 2007).

DH46 was selected due to its low green plant percentage, although had numbers of dividing microspores and embryos similar to Igri. Moreover, this DH line had 81.2% of the Igri genome (81.2% of the markers analyzed were not polymorphic between them). Polymorphic regions between Igri and DH46 were restricted to chromosomes 3H, 5H and 6H and their location are shown in Figure 1. A total of 30 elite cultivars of barley with different origin, row type, growth habit and end use were selected from the recommended lists of France, Spain, United Kingdom and United States of America for validation.

Donor plants were grown in growth chambers with controlled temperature, relative humidity, quality and intensity of light, as described by Cistué et al. (2003). Parents, DH lines and barley cultivars were evaluated for anther culture response, following the well-established standard protocol described by Cistué et al. (2003). Ten replications (each replication from one spike) of fifteen anthers were used for characterization. For parents and DH lines, variables recorded were the numbers of dividing microspores (nDM), embryos (nEMB), green plants (nGP), albino plants (nAP) and total plants (nTP), all referred to 100 cultured anthers, as well as the percentages of embryogenesis (number of embryos per 100 dividing microspores; pEMB), regeneration (number of total plants per 100 embryos; pREG) and green plants (number of green plants per 100 total plants; pGP). For elite cultivars, percentages of green plants (pGP) were evaluated using two independent batches of plants. Ten to twelve replications of fifteen anthers from the same spike were used for each batch.

Standard SAS/STAT procedures were used for statistical analysis (SAS Institute Inc. Cary, NC, USA). Analysis of variance of anther culture variables was performed separately for parents and for the two subpopulations with the Generalized Linear Model (GLM) procedure using the individual data of each replication. For the DH population, correlation among variables and transgressive segregation analysis were performed using the regression (REG) procedure and the LSD test, respectively.

Molecular marker and bulked segregant analysis

Genomic DNA was extracted from young leaf tissues by a modification of the method described by Saghai-Maroof et al. (1984). Amplification reaction for the RAPD

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was performed as in Chen et al. (2007) and amplified products were separated on 1.5% agarose gels. Both microsatellite (SSR) and STS primers, and amplification reaction conditions, were based on Ramsay et al. (2000), Blake et al. (1996) and Künzel et al. (2000). SSR amplification products were resolved in 5% denaturing polyacrylamide gels (7M Urea) and subsequently silver-stained as described by Briard et al. (2000). STS polymorphisms were detected without enzyme digestion for all of them except for MWG2029, which was digested with HaeIII. Fragments were resolved in 2.5% agarose gels and stained with ethidium bromide.

Bulked segregant analysis (Michelmore et al. 1991) with AFLPs was used to saturate with markers those regions associated with green plant percentage. Aliquots of DNA from ten doubled haploid lines from each of the two extremes of the DH-distribution for green plant percentage were combined to produce the bulks, which were subsequently used with the parents to screen for polymorphisms. AFLP analysis was performed according to the procedure described by Vos et al. (1995), with a commercially available kit (AFLP® Analysis System I, Invitrogen Life Technologies) and following the manufacturer's instructions. Amplification reactions were resolved in 5% denaturing polyacrylamide gels (7M Urea) and visualized with silver staining according to Briard et al. (2000). Each polymorphic AFLP marker was named by the primer pair combination together with the position of the fragment on the sequence gel.

Map construction and QTL analysis

Linkage groups were constructed using the software package MAPMAKER/EXP version 3.0 (Lander et al. 1987; Lincoln et al. 1993). The recombination values were converted to map distances (cM) using the Kosambi mapping function (Kosambi, 1944). SSRs with known chromosomal locations, based on previously published maps (Ramsay et al. 2000), were used as anchor markers to assign linkage groups to chromosomes. Chi-square was used to test for deviations of alleles from the expected 1:1 segregation ratio.

QTL analysis was performed with MQTL software (Tinker and Mather 1995) using simple interval mapping (SIM) and simplified composite interval mapping (sCIM). Threshold values were calculated using 3,000 permutations and QTLs were

considered real when both SIM and sCIM showed the presence of a significant peak. For a single environment, the test statistic can be converted to a LOD score by multiplying by 0.22 (Tinker and Mather 1995). Estimates of the positions of QTL corresponded to the peaks of the sCIM scans. The percentage of phenotypic variation explained by each QTL was calculated with a single factor regression (R^2). The total phenotypic variation explained by all QTLs detected for a given trait was estimated by fitting a multivariate regression and was confirmed by stepwise multiple regression analysis (SAS/STAT software).

For QTL validation, stepwise multiple regression analysis was performed to establish associations between the anther culture response and molecular markers. Maximum R-square analysis was also performed to find out the allele explaining the highest percentage of variance for pGP. Differences among the alleles were established using the GLM procedure and the Duncan's mean separation test.

Development of SCARs from AFLP markers

Two AFLP markers associated with green plant percentage were excised from polyacrylamide gels and transferred to 25 μl of sterile dH$_2$O for one day at 4ºC. Samples were stirred frequently and finally centrifuged for 10 min at 13000 rpm. Supernatant was used as template for PCR amplification using primers and reaction conditions similar to those used for the AFLP reaction. The amplified products were resolved on a 2% agarose gel, gel-purified using the Gel Band Purification Kit (Amersham Biosciences), cloned into the pGEM®-T Easy Vector I (Promega) and transformed into *Escherichia coli* DH5α competent cells. The plasmid DNA from positive clones was purified using the NucleoSpin Plasmid Purification Kit (Macherey-Nagel). The inserts were sequenced using the universal primers T7 and SP6 and an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Sequence homologies were analysed with the BLASTN program at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

The SCAR primers were designed based on the DNA sequence of the cloned fragments. Amplification reactions were carried out in a 20 μl reaction volume containing 50 ng of genomic DNA template, 1 unit of T*th* DNA polymerase (Biotools),

1x PCR buffer, 0.2 mM of each dNTP, 0.3 μM of each primer and the optimal $MgCl_2$ concentration (Table 1). PCR conditions were optimized to 94ºC for 3 min, 35 amplification cycles (45 s denaturation at 94ºC, 45 s at the appropriate annealing temperature (Table 1), 1 min of extension at 72ºC), and a final extension of 10 min at 72ºC. Amplified products were resolved on 2% agarose gels and visualized by ethidium bromide staining.

RESULTS

Anther culture response characterization of the DH population

To characterize the anther culture response of the parents and the DH population, eight variables were recorded (Table 2). As expected, due to the particular characteristics of the DH46 line selected for the cross, the parents only differed significantly for nGP, nAP and pGP (Table 2). Igri had values of 276.2 and 11.4 for nGP and nAP, whereas DH46 values were 8.3 and 240.4 for the same variables. The green plant percentages from Igri and DH46 were 93.9 and 2.3%, respectively.

Analysis of variance of the two subpopulations obtained by reciprocal crosses showed no significant differences between them for any trait. Thus, the subpopulations were considered as one population for further analysis.

The mean values of the population, for all traits, were close to those between the parents, being 32.2 for pGP, 100.6 for nGP and 137.7 for nAP. These three variables were significantly correlated: pGP was correlated with nGP (0.87) and nAP (-0.62), and nGP was correlated with nAP (-0.44). Significant transgressive segregation (LSD α=0.05) was observed for all traits except for pGP (Table 2).

Linkage map construction and QTL mapping

For map construction, we initially used 10 RAPD, STS and SSR markers known to be polymorphic between Igri and DH46 from the previous study based on the Igri x Dobla population (Chen et al. 2007). The Igri x Dobla linkage map had a total length of

1,228 cM and included 92 genetic markers grouped in 11 linkage groups assigned to the seven barley chromosomes. The low number of polymorphic markers between Igri and DH46 found in the Igri x Dobla map were located on three chromosomal regions (3H, 5H and 6H). Subsequently, we looked for SSRs and STSs on those chromosomal regions, according to the Ramsay et al. (2000) and Künzel et al. (2000) barley maps. From the new 34 SSRs and 19 STSs analysed, only 5 SSRs and 3 STS generated polymorphism between the parents and were mapped in the DH population. Bulked segregant analysis (BSA) was used with AFLPs, as a 'short cut' to saturate with markers the regions associated with pGP. From the 64 AFLP primer combinations used, 15 showed one or more polymorphisms between the parents and between the green and albino bulks, and were used to screen the entire DH population. Most of these AFLP primer combinations also identified additional polymorphisms not related to the trait, which were scored and included in the data set. Finally, the Igri x DH46 linkage map comprised a total of 47 AFLP, SSR, RAPD and STS markers, and covered approximately 160 cM (Fig. 1). Mapped regions were located on the long arm of chromosomes 3H and 5H, and along chromosome 6H. Markers were tightly-linked and, although the average interval was 3.4 cM, areas with less than 1 cM between markers were obtained. None of the markers analysed in the DH progeny showed distorted segregation.

Simple interval mapping and simplified composite interval mapping were used to identify putative QTLs in the DH population. Two chromosomal regions associated with green plant percentage were found on chromosomes 3H and 5H (Fig. 1). QTLs for nGP and nAP, variables correlated with pGP, were located in the same regions. Furthermore, one QTL was identified for nDM on the centromeric region of chromosome 6H (Fig. 1).

One of the QTLs for pGP was located on the long arm of chromosome 3H (Bin8 and 9), linked to the marker interval Bmag225-MWG2132 (LOD=10.0), and the other was mapped on the long arm of chromosome 5H (Bin9) and was linked to ABC717-E40M62A marker interval (LOD=10.1) (Table 3). These QTLs explained 28.14% and 37.05% of the phenotypic variance, respectively, with a significant additive effect of 65.20%. QTLs identified for nGP and nAP had LOD scores around 7.0 and explained respectively a 46.93 and 49.32% of the total variance for each trait (Table 3). The QTL

mapped on chromosome 6H for nDM explained the lowest percentage of phenotypic variance (25.03%), and had also the lowest LOD score (5.7). This QTL was linked to the marker interval HVM11a-Bmag173 (Table 3).

Developing of SCAR markers

The AFLP markers E40M48A and E40M62A, linked to the QTL for pGP on chromosome 5H, were cloned and sequenced to convert them into simple PCR-based markers. Homology searches were performed and no significant homology was found in nucleotide databases for any sequence.

Based on the sequence of the cloned AFLP fragments, candidate SCAR primers were designed. PCR reaction conditions for each primer pair were optimized and, then, these SCAR primers were tested on all DH lines of the population. As expected, the PCR banding pattern for each of the two candidate SCAR primer pairs was exactly the same as that of its corresponding AFLP analysis. Each developed SCAR marker was designated as ACS (AFLP converted SCAR, as the terminology of Xu et al. 2001) followed by its corresponding length. Information of SCAR markers (ACS-637 and ACS-174), including primer sequences, is shown in Table 1.

Validation of QTLs for green plant percentage

Thirty elite cultivars with different row type, growth habit and end use were selected from the recommended lists of four countries (France, Spain, UK and USA), and were evaluated for green plant percentage in anther culture. Cultivars Cebu, Legacy and Scarlett were eliminated from the analysis due to their very low number of dividing microspores. Cultivars were grouped according to their growth habit and row type, and their pGP values are shown in Figure 2. The highest values were obtained from Volley (100%), Orelie (95.36%) and Hispanic (90.0%), and the lowest from Colter, Kamiak Nikel, Prudentia and Vanessa (0%). Analysis of variance showed significant differences between row-types, with means of 41.38% and 15.86% for two- and six-rowed types, respectively. Although no significantly, winter cultivars had higher percentages of green

plants (mean=42.5%) than spring ones (mean=26.4%). In general, the two-rowed winter cultivars had the highest values for pGP (mean=60.52%).

In order to validate the markers linked to QTLs for pGP identified in this study, a total of eight markers located on the QTL regions were selected. These markers included: Bmag225, MWG2132, HVM60 and GMS116, on chromosome 3H, and Bmag223, Bmag812, ACS-637 and ACS-174, on chromosome 5H. Although microsatellites HVM60 and GMS116, on chromosome 3H, were non-polymorphic between Igri and DH46, they were selected due to the shortage of markers on this chromosomal region. Stepwise multiple regression analysis was used to identify those markers associated with pGP in the 27 cultivars. Only the SSR HVM60 was significantly associated with this trait, explaining 19.39% of phenotypic variance. This marker was non-polymorphic between Igri and DH46 and was located on the 3H chromosomal region where the new QTL for pGP was identified. Four allelic variants (113, 115, 117 and 120 bp) were identified for HVM60 among the elite cultivars (Fig. 2). Maximum R-square analysis showed that the allele HVM60-120bp explained the highest percentage of variance for pGP (22.48%). This allele was associated with the highest values for the trait.

DISCUSSION

In cereal anther and microspore culture, regeneration of albino plants is a limitation to the efficient use of doubled haploids in breeding programs. To our knowledge, only one previous report identified a QTL for green plant percentage in barley anther culture, using a small Igri x Dobla DH population (Chen et al. 2007). This QTL, located on chromosome 5H, explained 21.07% of the phenotypic variance. In the present work, a new population was developed to identify new QTLs for pGP and to confirm the previous one. This population was generated by crossing Igri with DH46 (DH Igri x Dobla), in search of a maximum segregation for green plant percentage and minimum for the other variables related to anther culture. The specificity of the population was confirmed as the variability of the parents was limited to pGP, nGP and nAP.

QTL analysis identified two chromosomal regions for pGP with high LOD-scores (10.0-10.1), explaining up to 65.2% of the phenotypic variance. One of these QTLs was located on the long arm of chromosome 3H, in a region where it was difficult to find polymorphic markers. Only one SSR and one STS could be mapped in this area, and not even AFLPs could identify any polymorphism. In this chromosome, no QTL associated with green plant percentage has been previously described in barley, wheat, or rye anther culture (Manninen 2000; Chen et al. 2007; Torp et al. 2001; Grosse et al. 1996). However, a region of chromosome 3R has been recently associated with this trait in triticale anther culture (González et al. 2005). In barley somatic embryogenesis, a QTL has been identified on chromosome 3H for green plant regeneration from callus (*Qsr2*, Mano et al. 1996; Bregitzer and Campbell, 2001), although this QTL was located on the short arm of the chromosome. The other QTL, located on chromosome 5H, had a major effect on green plant percentage. In this study, we have increased the marker density on this region, partly due to the combination between AFLP markers and bulk segregant analysis (BSA). We have also confirmed the QTL previously described by Chen et al. (2007) and have increased the phenotypic variance explained by it, from 21.07 to 37.05%. The presence of QTLs associated with pGP has been previously reported in anther culture of wheat (5B) (Torp et al. 2001; Agache et al. 1989) and rye (5R) (Grosse et al. 1996). Moreover, two QTLs for green plant regeneration from embryogenic barley callus were also described on this chromosome, one of them (*Qsr4*) in the same region as ours (Mano et al. 1996; Bregitzer and Campbell 2001). This could indicate a possible role of cereal group 5 chromosomes in the control of green plant regeneration in tissue culture.

QTLs for nGP and nAP were identified on chromosomes 3H and 5H, exactly at the same position as those for pGP. The specific type of population developed in this study marks the relations among variables nGP, nAP and pGP. In the previous Igri x Dobla study, QTLs for nEMB and nAP were identified on chromosome 2H. This chromosome 2H was not polymorphic in the Igri x DH46 population, eliminating the major influence of QTL for nEMB. Thus, correlations among variables nGP, nAP, pGP were increased, probably leading to the identification of QTLs for these variables in the same regions. Surprisingly, a new QTL for number of dividing microspores (nDM) was identified on the centromeric region of chromosome 6H, explaining the 25.03% of the

phenotypic variance. Although there were not significant differences between the parents for this variable, the high degree of transgressive segregation found among the DH population could explain the identification of this QTL.

In this study, none of the markers analyzed showed distorted segregation. Studies in barley have indicated that some markers showing distorted segregation were linked to genes involved in anther culture response, with a prevalence of the allele of the better-responding parent (Graner et al. 1991; Zivy et al. 1992; Devaux and Zivy 1994). However, results of other studies do not support this hypothesis (Manninen 2000; Sayed et al. 2002). In our previous Igri x Dobla population, 15% of the markers showed skewed segregation and these were distributed on all chromosomes with the exception of 3H (Chen et al. 2007). However, the only relation of a QTL for anther culture response to segregation distortion was found on chromosome 2H. None of the markers that showed segregation distortion in Igri x Dobla were polymorphic in the present Igri x DH46 population.

QTL mapping studies not only should be confirmed but also validated in different genetic backgrounds, by testing the reliability of markers associated with them to predict phenotype (Collard et al. 2005). To our knowledge, the only validation study in anther culture response was performed in rice (Kwon et al. 2002), where a RFLP marker was associated with green plant regeneration in 43 cultivars.

In this study, a group of 27 elite barley cultivars was characterized for green plant percentage, showing a great variability among them. The two-row cultivars had significantly higher mean values than the six-row ones. These results were coincident with the study of Cistué et al. (1999), performed with ten cultivars grown in Spain. In general, the winter cultivars presented higher percentages of green plants than the spring ones. However, we found exception as some winter cultivars had lower percentages than the spring ones. In the same way, Knudsen et al. (1989) found several winter varieties with a low capacity for green plant formation, similar to the spring types. Therefore, it was suggested by Larsen et al (1991) that capacity for green plant formation was unrelated to growth habit. Other studies in barley indicated a different origin of albinism in the winter and the spring cultivars (Caredda et al. 2000 and 2004).

Further investigations would be necessary to clarify the possible relationship between green plant percentage and the growth habit.

To validate the QTLs for pGP in the selected cultivars, a set of eight markers linked to these QTLs was used. Since two of them were AFLPs, we successfully converted these into simple PCR-based SCAR markers. When multiple regression analysis was performed with the pGP data, only the SSR HVM60 was significantly associated with pGP, explaining 19.39% of phenotypic variance. This marker was linked to the QTL on chromosome 3H identified for the first time in this study. This indicates that not necessarily the most tightly linked marker to a QTL in a population is the most effective for validation. The QTL for pGP on chromosome 5H, confirmed in this new DH population, could not be validated in the elite cultivars. This might be due to the fact that the distance between the locus and the QTL could not be short enough to show linkage disequilibrium. Another possibility is that the validation of this QTL could be masked by other anther culture related traits not evaluated in these cultivars. Moreover, some studies have indicated the problems in validating the marker-QTL linkages since, in segregating populations, only a small fraction of the allelic variation at QTL is sampled and these alleles are frequently not representative for elite germoplasm due to the choice of parents with extreme phenotypes (Melchinger et al. 2004).

For the first time, a study maps, confirms and validates QTLs associated with green plant percentage in barley anther culture. Using a specific DH population, a new QTL has been mapped on chromosome 3H and the QTL on chromosome 5H has been confirmed. The marker HVM60, linked to the QTL on 3H, was validated using a group of barley elite cultivars. Further studies should be carried out to validate all QTLs associated with the anther culture response in barley. This could help breeders in the identification of genotypes prior to their entry into the breeding programmes, allowing the selection of the best-responding ones and optimizing the inputs needed for doubled-haploid production.

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Table 1 Length, nucleotide sequence, MgCl$_2$ concentration and annealing temperatures (Ta) of SCAR markers derived from AFLPs

SCAR marker	Corresponding AFLP marker	Length (base pairs)	Primer sequence (5'→3')	[MgCl$_2$] (mM)	Ta (ºC)
ACS-637	E40M48A	637	For: TAACACTAATGGTTTCATTTTACTG	2.5	62
			Rev: CCTTGTATGAGAAATACGACTTAA		
ACS-174	E40M62A	174	For: TAACTTTGCATCCCCGACCAGAAC	1.0	68
			Rev: TGAGTGAACGTCTCCTCGACTTAA		

Table 2 Anther culture response of Igri, DH46 and the doubled haploid population. Means for the studied variables are given. nDM, nEMB, nGP, nAP and nTP are referred to 100 anthers. The type of transgressive segregation in the DH population is also presented

Trait	Parents		Doubled-haploid population					
	Mean value ± SD		Subpopulations		All lines			
	Igri	DH46	Igri x DH46	DH46 x Igri	Mean	Minimum	Maximum	Transgressive segregation[‡]
nDM	2846.2 ± 942.7	3143.4 ± 1794 (ns)[a]	2891.3	3120.9 (ns)[b]	3006.1	667.6	6092.8	+/–
nEMB	377.6 ± 160.6	522.4 ± 277.4 (ns)	444.5	400.0 (ns)	421.3	35.2	822.3	+/–
nGP	276.2 ± 140.6	8.3 ± 17.1 (**)	120.8	80.0 (ns)	100.6	0.0	609.2	+
nAP	11.4 ± 13.42	240.4 ± 148.3 (**)	138.5	136.8 (ns)	137.7	3.8	380.2	+
nTP	287.6 ± 146.2	248.7 ± 161.4 (ns)	259.2	219.1 (ns)	239.1	20.0	625.1	+/–
pEMB	13.5 ± 5.8	18.8 ± 8.6 (ns)	16.9	14.5 (ns)	15.7	1.5	41.0	+/–
pREG	62.3 ± 15.4	50.8 ± 15.3 (ns)	52.1	47.9 (ns)	50.0	23.3	76.7	+/–
pGP	93.9 ± 6.1	2.3 ± 3.5 (**)	36.0	28.5 (ns)	32.2	0.0	98.0	/

[a] Analysis of variance for the parents (**:significant at $P<0.01$, ns: non-significant)
[b] Analysis of variance for the two subpopulations (ns: non-significant)
[‡] Type of transgressive segregation (+: positive transgresive segregation, –: negative transgressive segregation, +/–: transgressive segregation in both directions, /: no transgressive segregation

Table 3 Characteristics of QTLs associated with anther culture response in the Igri x DH46 doubled haploid population

Trait	Chromosome	Marker interval	LOD score	Estimated additive effect[a]	R^2_p (%)[b]	mR^2_p (%)[c]
nDM	6H	HVM11a – Bmag173	5.7	547.1	25.03	25.03
nGP	3H	Bmag225-MWG2132	7.3	81.6	26.02	46.93
	5H	ABC717-E40M62A	6.4	64.2	20.92	
nAP	3H	Bmag225-MWG2132	7.4	-54.0	17.00	49.32
	5H	Bmag223-E32M47B	7.6	-49.2	32.31	
pGP	3H	Bmag225-MWG2132	10.0	21.8	28.14	65.20
	5H	ABC717– E40M62A	10.1	19.4	37.05	

[a] Additive effects of QTLs expressed in the trait unit. A positive value means that the allele from Igri increases the value of the trait
[b] Percentage of phenotypic variation explained by each QTL
[c] Total phenotypic variation explained by all QTLs detected for a given trait

Fig. 1 Linkage map of Igri x DH46 and location of QTLs for green plant percentage (pGP) and numbers of dividing microspores (nDM), green plants (nGP) and albino plants (nAP). Boxes indicate significant QTLs identified with both SIM and sCIM, and adjacent lines indicate significant QTLs only with sCIM. The Igri x DH46 map is compared with chromosomes 3H, 5H and 6H of the Igri x Dobla map (Chen et al. 2007), and common markers are underlined. The black areas on Igri x Dobla chromosomes represent the polymorphic regions between Igri and DH46. The QTLs for these variables identified previously in the Igri x Dobla population are also shown. Distances between markers are in centiMorgans (cM)
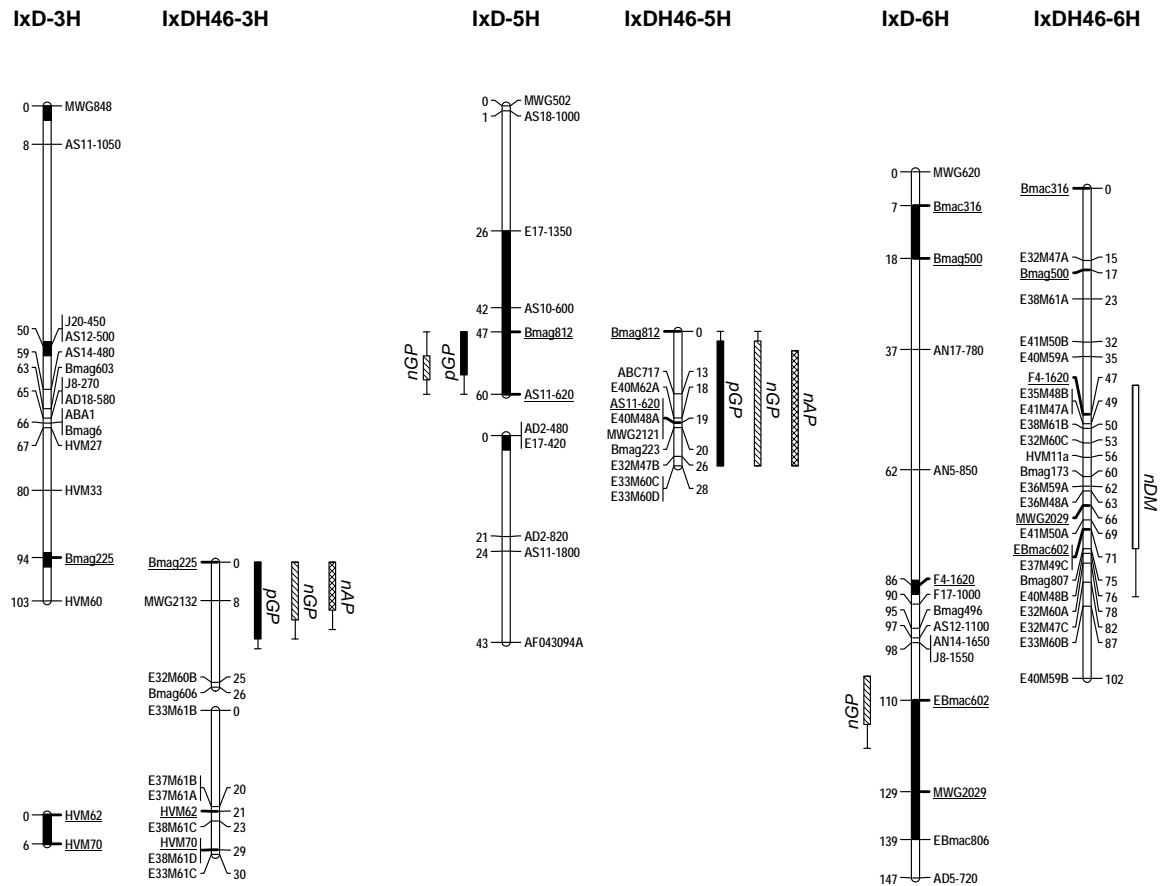
Fig. 2 Green plant percentages of the 27 barley elite cultivars used, grouped according to the growth habit and row type. Fragment size of the HVM60 allele in each cultivar is shown above the bars. (*): no amplification