Flavanone 3-hydroxylase genes in *Triticum aestivum* L.

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INTRODUCTION

An orthologue of the maize gene *c1* (which encodes a Myb-like transcriptional factor controlling tissue-specific anthocyanin biosynthesis[1]) was mapped earlier on each of the short arms of wheat homoeologous group 7 chromosomes[2] in positions highly comparable to those of *Rc-1* (red coleoptile) genes[3] and may represent the same loci. Recognizing elements (MRE) for *c1* have been identified in the promoter sequence of *Arabidopsis thaliana F3H* gene (flavanone 3-hydroxylase - one of the key enzymes involved in the biosynthesis of flavonoid compounds)[4], suggesting that *Rc-1* can exert a regulatory role for wheat *F3H*.

In this paper, we describe the cloning, sequence analysis, mapping and expression of *F3H* orthologues in bread wheat, and the interaction between *F3H* and the *Rc-1* homoeologues.

MATERIALS AND METHODS

The bread wheat cultivar 'Chinese Spring' ('CS') was used for PCR-based cloning. The complete set of 'CS' nulli-tetrasomic lines[5] and a subset of homoeologous group 2 chromosome deletion lines[6] were exploited to establish chromosome bin locations of *F3H* gene copies. Eight progeny from the cross 'CS' ('Hope' 7B) x 'TRI 2732'[3] and a set of six homozygous lines each containing a different chromosome 7D segment derived from *Ae. tauschii* in a 'CS' background[7] were used to investigate a correlation between the presence of dominant alleles at the *Rc-1* and expression of *F3H*. Quantitative examination of *F3H* expression was measured in 'CS' and 'Mironovskaya 808' ('M808') and the single chromosome substitution lines 'CS' ('Hope' 7A) and 'CS' ('Hope' 7B). RNA was extracted every 24h from two to six day old seedlings grown at 20ºC under a 12h day / 12h night regime using the QIAGEN (http://www1.qiagen.com/) Plant Rneasy Kit. Single-stranded cDNA was synthesized from 1mg total RNA using a $(dT)_{15}$ primer and the QIAGEN Omniscript Reverse Transcription kit in a 20µl reaction mixture. qRT-PCR was performed using a QIAGEN QuantiTect SYBR Green kit. UBC and GAPDH primers were used to standardize the cDNA template. The amplifications were performed in an Applied Biosystems 7900 HT fast real time PCR system. Pre-determined amounts of cloned cDNA were used to generate standard curves. Each sample was run in three replicates. Statistical significance of differences in expression level was assessed by a t-test. The specificity of the qRT-PCR products was confirmed by 2% agarose gel electrophoresis.

RESULTS AND DISCUSSION

Four *F3H* copies were isolated from bread wheat (Genbank accession numbers for copies *F3H1*, *2*, *3* and *4* are EF463100, DQ233636, EU402957 and EU402958, respectively). The length of the coding sequence, which was split into three exons, was 1137bp, and the first intron varied in length among the homoeologues by some hundreds of base pairs. The sequence of the segments of the first intron of the bread wheat copies *F3H1*, *F3H2* and *F3H3* not affected by deletions/insertions shared over 80% homology, but the first intron of *F3H4* was quite distinct. Gene copy-specific primer pairs were constructed for each of the four *F3H* copies and used for profiling the nulli-tetrasomic lines of 'CS'. This analysis showed that *F3H1* and *2* are on, respectively, chromosomes 2A and 2D, while *3* and *4* both map to chromosome 2B. A deletion line analysis was then used to define the intra-chromosomal location of *F3H1* to the sub-terminal bin (2AL3) of chromosome 2AL, both *F3H3* and *F3H4* to the terminal bin (2BL6) of chromosome 2BL, and *F3H2* to the terminal bin (2DL6) of chromosome 2DL. Additional analyses (data not shown) has revealed that *F3H3* along with *F3H1* and *F3H2*, belong to an *F3H* homoeoallelic series, whereas *F3H4* appears to be a non-homoeologous duplication. Accordingly, the genes were re-designated *F3H-A1* (*F3H1*), *F3H-B1* (*F3H3*), *F3H-D1* (*F3H2*), and *F3H-B2* (*F3H4*). To explore the role of the *Rc-1* (red coleoptile) genes as regulators for F3H expression, eight progeny from the cross 'CS' ('Hope' 7B) x 'TRI 2732', along with a set of six different chromosome 7D introgression lines 'CS'-*Ae. tauschii*, varying with respect to the dominant allele at either *Rc-B1* or *Rc-D1*, were subjected to RT-PCR analysis from cDNA derived from four day old seedlings. The parental genotypes with pigmented coleoptiles ('CS' ('Hope' 7B) and 'CS' (*Ae. tauschii* 7D) both showed a high level of F3H expression, whereas those with non-pigmented coleoptiles showed either little ('TRI 2732') or none ('CS'). When this result was compared with the microsatellite-based genotype of the lines[3,7], the regulator of F3H expression on chromosome 7B was mapped between *Xgwm0263* and *Xgwm0573*, co-segregating with *Rc-B1* (data not shown); similarly, the equivalent locus on chromosome 7D co-segregated with *Rc-D1* within the genetic interval *Xgwm0044* and *Xgwm0111* (Figure 1). RT-PCR was also used to study the contribution of single genes *F3H-A1*, *F3H-B1*, *F3H-B2* and *F3H-D1* to total F3H expression. It was shown

that *F3H-B2* is not expressed whether or not the coleoptiles are pigmented. In contrast, *F3H-A1*, *F3H-B1* and *F3H-D1* were actively expressed in lines with pigmented coleoptiles ('CS' ('Hope' 7B).

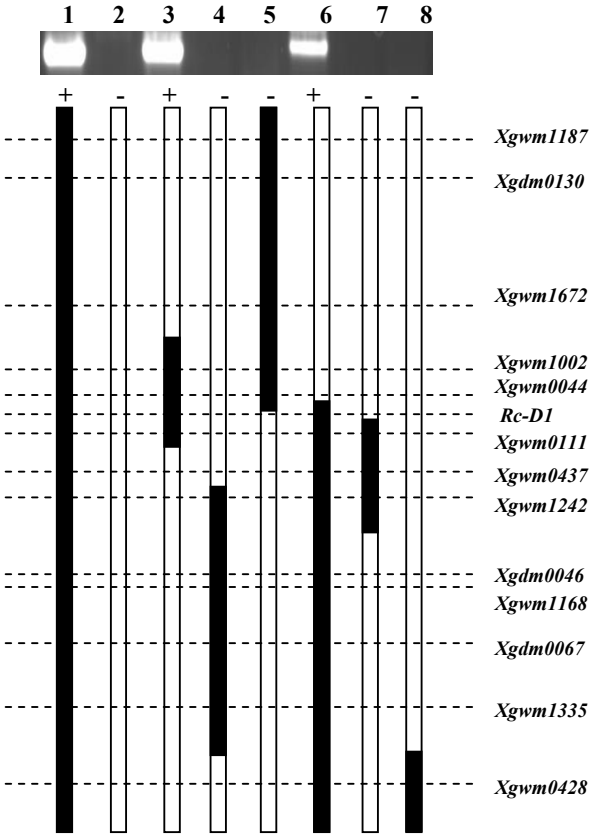

Figure 1. *F3H* RT-PCR analysis from four day old seedlings of the substitution 'CS' (*Ae. tauschii* 7D) (1), 'Chinese Spring' (2) and the 'CS'-*Ae. tauschii* 7D introgression lines (3-8). (+), (-) indicate presence/absence of anthocyanin pigmentation in the coleoptiles. The status of chromosome 7D of each line is indicated in the lower part of the panel**.**

To investigate the possibility of more subtle differences between expression levels of the *F3H* homoeologues in the presence of particular alleles of *Rc-1*, quantitative RT-PCR was applied to a set of cDNAs sampled from two to six day old seedlings (Figure 2). The test genotypes were 'CS' ('Hope' 7A) [*Rc-A1b*], 'CS' ('Hope' 7B) [*Rc-B1b*] and 'M808' [*Rc-D1b*], along with the control 'CS' which carries the non-pigmented alleles at all three *Rc-1* loci. In the latter, none of the *F3H* copies was expressed at any time during the sampling period. *F3H-B2* was not expressed in any of three test line seedlings, but *F3H-A1*, *F3H-B1* and *F3H-D1* were all expressed in these lines. No within genotype significant difference (p=0.05) in the expression level of the three homoeologues could be detected at any of the sampling times. Many sets of wheat homoeologous genes are known to be equally expressed in this way[8-10], but in others, the expression of one or more members may be either completely[8,11-12] or partially[10,13-14] suppressed.

The overall level of F3H expression differed very significantly between each pair of lines (Figure 2) - 'CS' ('Hope' 7A) *vs* 'CS' ('Hope' 7B) p>0.999, 'CS' ('Hope' 7A) *vs* 'M808' p>0.999, and 'CS' ('Hope' 7B) *vs* 'M808' p>0.95).

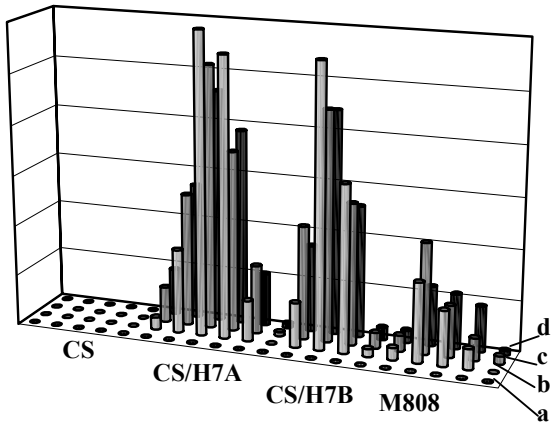

Figure 2. Quantitative RT-PCR analysis with respect to the various copies of *F3H* (a - *F3H-B2*, b - *F3H-A1*, c - *F3H-B1*, d - *F3H-D1*) in 'CS', 'CS' ('Hope' 7A), 'CS' ('Hope' 7B) and 'M808' (each genotyped is characterized with 5 rows, corresponding to 2, 3, 4, 5 and 6-day old seedlings, respectively).

Overall, therefore, each *Rc-1* gene appeared to regulate the expression of the three *F3H* homoeologues equally, but the level of expression was governed by which of the *Rc-1* genes was present as a dominant allele. The lack of any genome-specific relationship between *F3H-1* and *Rc-1* implies an integrative evolutionary process among the three diploid genomes, following the formation of hexaploid wheat.

Our general conclusion is that regulatory genes probably contribute more to the functional divergence between the wheat genomes than do the structural genes themselves. This is in line with the growing consensus which suggests that although heritable morphological traits are determined by the expression of structural genes, it is the regulatory genes which are the prime determinants of allelic identity.

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