Characterization of Mutations in Barley fch2 Encoding Chlorophyllide a Oxygenase

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(Received February 22, 2012; Accepted April 18, 2012)

The barley (Hordeum vulgare L.) mutants fch2 and clo-f2 comprise an allelic group of 14 Chl b-deficient lines. The genetic map position of fch2 corresponds to the physical map position of the gene encoding chlorophyllide a oxygenase. This enzyme converts chlorophyllide a to chlorophyllide b and it is essential for Chl b biosynthesis. The fch2 and clo-f2 barley lines were shown to be mutated in the gene for chlorophyllide a oxygenase. A five-base insertion was found in fch2 and base deletions in clo-f2.101, clo-f2.105, clo-f2.2800 and clo-f2.3613. In clo-f2.105 and clo-f2.108, nonsense base exchanges were discovered. All of these mutations led to a premature stop of translation and none of the mutants formed Chl b. The mutant clo-f2.2807 was transcript deficient and formed no Chl b. Missense mutations in clo-f2.102 (leading to the amino acid exchange D495N) and clo-f2.103 (G280D) resulted in a total lack of Chl b, whereas in the missense mutants clo-f2.107 (P419L), clo-f2.109 (A94T), clo-f2.122 (C320Y), clo-f2.123 (A94T), clo-f2.133 (A376V) and clo-f2.181 (L373F) intermediate contents of Chl b were determined. The missense mutations affect conserved residues, and their effect on chlorophyllide a oxygenase is discussed. The mutations in clo-f2.102, clo-f2.103, clo-f2.133 and clo-f2.181 may influence electron transfer as illustrated in the active site of a structural model protein. The changes in clo-f2.107, clo-f2.109, clo-f2.122 and clo-f2.123 may lead to Chlb deficiency by interfering with the regulation of chlorophyllide a oxygenase. The correlation of mutations and phenotypes strongly supports that the barley locus fch2 encodes chlorophyllide a oxygenase.

Keywords: Barley (Hordeum vulgare L.) • chlorina-f2 • Chlorophyllide a oxygenase • fch2.

Abbreviations: Dicamba-MO, 3,6-dichloro-2-methoxybenzoic acid monoxygenase; EST, expressed sequence tag; SNP, single nucleotide polymorphism.

The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers JQ627619 (chlorophyllide a oxygenase in Sejets Tron), JQ627620 (chlorophyllide a oxygenase in Svalofs Bonus) and JQ627621 (chlorophyllide a oxygenase in Ackermanns Donaria).

Introduction

Leaves with aberrant Chl pigmentation are easily spotted in large groups of plants even at an early stage of growth. Thus, Chl mutation frequencies have been used to evaluate the success rates of various mutagenic treatments (Lundqvist 1992). Many of those Chl mutants have been deposited in mutant stock collections of various species (e.g. Arabidopsis Biological Resource Center; National Small Grains Collection; Maize Genetics Cooperation Stock Center; Nordic Genetic Resource Center—NordGen). In barley (Hordeum vulgare L.), the Chl-related mutants kept at NordGen and the Carlsberg Laboratory have been assigned to 105 different loci (Simpson and von Wettstein 1992). The genetic identity of the loci has been clarified at the DNA level in five cases only: Xantha-f, -g and -h encode the three subunits of magnesium chelatase (Hansson et al. 1999, Olsson et al. 2004, Axelsson et al. 2006); Xantha-l encodes a component of the magnesio protoporphyrin IX monomethyl ester cyclase (Rzeznicka et al. 2005); and Tigrina-d encodes a tetrapyrrrole biosynthesis regulatory component (Lee et al. 2003).

The barley mutant 28-3398/fch2.c (Franckowiak 1997) has a light-green chlorina phenotype. It is Chl b deficient and survives as a homozygous mutant (Highkin 1950, Highkin and Frenkel 1952). The mutant occurred spontaneously in a cross of the cultivars Moister, CI 2799 (Lion × Manchuria), and California Coast, CI 6115 (Tsuchiya 1972). The locus of the monofactorial, recessive trait was designated chlorina seedling 2 and initially...
given the symbol f2 (Tsuchiya and Robertson 1971). Later, the symbol was changed to fch2 and the locus was mapped to the long arm of barley chromosome 3H (3HL), about 7.7 cM distal to the locus curly 2 (cur2; Franckowiak, 1997).

Additional alleles of fch2 are light green 5 (lg5), which was designated fch2·y (Takahashi et al. 1975, Franckowiak 1997), and the three mutants 2800, 2807 and 3613 that were induced by X-ray treatment of seeds of the cultivar Ackermanns Donaria (Mahold et al. 1977). Ten more allelic mutants were induced by sodium azide treatment and referred to as clo-f2 (Simpson et al. 1985). The mutants clo-f2·101, clo-f2·102, clo-f2·103, clo-f2·105, clo-f2·107, clo-f2·108 and clo-f2·109 were induced in the cultivar Sejets Tron, whereas clo-f2·122, clo-f2·123 and clo-f2·133 were induced in the cultivar Svalöfs Bonus. The three mutants derived from Donaria were included in this nomenclature: clo-f2·2800, clo-f2·2807 and clo-f2·3613 (Simpson et al. 1985). Another mutant induced in Sejets Tron was clo·106, which was reported to have a reduced Chl b content, yet it was not an allele of fch2 (Simpson et al. 1985).

The fch2 and clo-f2 lines are characterized by a reduced amount or total lack of Chl b (Highkin 1950, Highkin and Frenkel 1952, Apel 1967, Sagromsky 1974, Simpson et al. 1985). Absence of Chl b results in a different composition of the light-harvesting apparatus, which is the reason why many studies have been conducted with fch2 (see, for example, Goodchild et al. 1966, Thornber and Highkin 1974, Henriques and Park 1975, Hiller and Raison 1980, Mullet et al. 1980, Bhalla and Bennett 1987, Harrison et al. 1993, Preiss and Thornber 1995, Bossmann et al. 1997, Havaux and Tardy 1997, Rudoi and Shcherbakov 1998, Saito et al. 2010, Tytereva and Voitsekhovskaja 2011a, Tytereva and Voitsekhovskaja 2011b). Despite the detailed characterization of the fch2 and clo-f2 mutants, the identity of the fch2 locus has not yet been described.

Chl b is formed by the enzyme chlorophyllide a oxygenase, which converts the 7-methyl group of chlorophyllide a to the 7-formyl group of chlorophyllide b (Tanaka et al. 1998, Espineda et al. 1999, Oster et al. 2000). Alignment of the chlorophyllide a oxygenase sequences of Arabidopsis thaliana, Oryza sativa and the prochlorophyte Prochlorothrix hollandica led to the assignment of three domains in the polypeptide. Domain A is regulatory, B is a linker, and C is the catalytic domain (Nagata et al. 2004, Sakuraba et al. 2007, Yamasato et al. 2008). Domain A contains a degradation signal sequence (degron) which has been suggested to be involved in the regulation of chlorophyllide a oxygenase activity by interaction with a protease (Sakuraba et al. 2009). The C domain contains three conserved sequence motifs: a Rieske FeS cluster-binding site, a mononuclear non-heme iron-binding site and a conserved sequence unique to chlorophyllide a oxygenase polypeptides (Espineda et al. 1999). The oxygen atom incorporated in the 7-formyl group of chlorophyllide b originates from O2 (Schneegurt and Beale 1992) and, to activate O2 for the oxygenation reaction, electron transfer from a reduced donor through the FeS cluster and the mononuclear iron has been discussed (Tanaka et al. 1998). This electron transfer sequence suggests an essential functional and structural involvement of the conserved metal-binding sites of chlorophyllide a oxygenase in the synthesis of Chl b.

The Chl b-deficient phenotype of the fch2 and clo-f2 mutants and genetic mapping data suggested that the locus fch2 and the barley gene encoding chlorophyllide a oxygenase (HvCAO) are identical. To test this and to identify the barley fch2 locus, HvCAO cDNA in fch2 and the 13 allelic clo-f2 mutants was amplified and sequenced. This revealed severe mutations in HvCAO in the fch2 and clo-f2 barley genetic stocks. We suggest structural and functional consequences of the missense mutations on chlorophyllide a oxygenase by comparison with a model structure. The findings provide strong evidence that fch2 does encode barley chlorophyllide a oxygenase.

Results

The fch2 and clo-f2 phenotype

Barley mutated in the locus fch2 has a light green chlorina phenotype due to the reduction in Chl b. The color varies between alleles (Fig. 1). Homozygous plants are viable, but they are delayed in their development (Highkin 1950, Franckowiak 1997). The pigment contents of the fch2 and clo-f2 plants are given in Table 1.

The analysis of the pigment content of the individual fch2 and clo-f2 lines (Table 1) detected no Chl b in the mutants fch2, clo-f2·101, clo-f2·102, clo-f2·103, clo-f2·105, clo-f2·107, clo-f2·108, clo-f2·2800, clo-f2·2807 and clo-f2·3613.

The wild type had a Chl a to Chl b (Chl a/b) ratio of 3.7–3.8. The weakest Chl b-deficient phenotype was that of mutant clo-f2·107, which had a Chl a/b ratio of 5.7. In the lines clo-f2·109, clo-f2·122, clo-f2·123, clo-f2·133 and clo-f2·181 the ratio was 9.5, 21, 9.3, 8.5 and 15, respectively.

The Chl a content of the Chl b-deficient lines compared with wild-type barley varied between 30 and 133% (clo-f2·123 and clo-f2·107, respectively), while the majority of lines had between 56 and 93% Chl a.

The pigment analyses showed that the mutations in the fch2/clo-f2·lines specifically affect the biosynthesis of Chl b, while the ability per se to synthesize Chl was not affected in any of the lines. As chlorophyllide a oxygenase is the enzyme that catalyzes the formation of Chl b, it was hypothesized that locus fch2 encodes barley chlorophyllide a oxygenase.

Mapping identifies HvCAO as a candidate gene for fch2

In the GrainGenes Hordeum-Bins-3H map, fch2 is listed at position 166.8 cM on chromosome arm 3HL. The distance to curly2 (cur2 at 155.5 cM) is somewhat greater than the 7.7 cM reported by Franckowiak (1997). Recently, Druka et al. (2011) reported on a collection of 881 near-isogenic barley lines representing mutant alleles in various loci, which were genotyped.

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**Table 1**

<table>
<thead>
<tr>
<th>Line</th>
<th>Chl a (mg g⁻¹ Fw)</th>
<th>Chl b (mg g⁻¹ Fw)</th>
<th>Chl a/b ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>24.3</td>
<td>0.35</td>
<td>7</td>
</tr>
<tr>
<td>fch2</td>
<td>0.1</td>
<td>0</td>
<td>5.7</td>
</tr>
<tr>
<td>clo-f2·101</td>
<td>0.1</td>
<td>0</td>
<td>5.7</td>
</tr>
<tr>
<td>clo-f2·102</td>
<td>0.1</td>
<td>0</td>
<td>5.7</td>
</tr>
<tr>
<td>clo-f2·103</td>
<td>0.1</td>
<td>0</td>
<td>5.7</td>
</tr>
<tr>
<td>clo-f2·105</td>
<td>0.1</td>
<td>0</td>
<td>5.7</td>
</tr>
<tr>
<td>clo-f2·107</td>
<td>0.1</td>
<td>0</td>
<td>5.7</td>
</tr>
<tr>
<td>clo-f2·2800</td>
<td>0.1</td>
<td>0</td>
<td>5.7</td>
</tr>
<tr>
<td>clo-f2·2807</td>
<td>0.1</td>
<td>0</td>
<td>5.7</td>
</tr>
<tr>
<td>clo-f2·3613</td>
<td>0.1</td>
<td>0</td>
<td>5.7</td>
</tr>
</tbody>
</table>
using 3,072 single nucleotide polymorphisms (SNPs). Line BW358 contains the original fch2 mutation (22-3398/fch2.c) and resulted from six backcrosses to the barley cultivar Bowman. In BW358, four SNP markers co-segregated with the fch2 phenotype. Two markers are located at position 167.8 cM (GrainGenes map Hordeum-OPA-2009-3H, Pilot OPA markers 1_1410 and 1_0681), and one is located at 168.4 cM (1_0694). The fourth marker, 1_1411, was not mapped. Interestingly, the marker 1_0629, which is also at 167.8 cM in this map, is the HarvEST barley unigene HAR35_2495 that has been annotated as chlorophyllide a oxygenase (HvCAO). This finding suggested that fch2 and HvCAO might be identical.

To corroborate the location of HvCAO at the position of the barley genome to which fch2 was mapped, the synteny of barley 3HL to the chromosomes of rice and Brachypodium distachyon was analyzed (Fig. 2). In rice, the genes for two isoforms of chlorophyllide a oxygenase are located on chromosome 10 (LOC_Os10g41760.1 and LOC_Os10g41780.1), while the orthologs to the barley markers flanking unigene HAR35_2495 and the position of fch2 are found on rice chromosome 1 (Fig. 2). On B. distachyon chromosome 2, however, the order of open reading frames including that annotated chlorophyllide a oxygenase is the same as the orthologous markers in barley flanking unigene HAR35_2495 and the position of fch2. This conserved

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### Table 1 Pigment content in wild-type, fch2 and clo-f2 barley

<table>
<thead>
<tr>
<th>Line</th>
<th>Chl a (nmol g⁻¹ fresh leaf)</th>
<th>Chl b (nmol g⁻¹ fresh leaf)</th>
<th>Chl a/b ratio</th>
<th>Chl a as percentage of Chl a in Tron</th>
<th>Mother cultivar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tron</td>
<td>613 (±11)</td>
<td>161 (±6)</td>
<td>3.8</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>Tron*</td>
<td>567</td>
<td>152</td>
<td>3.7</td>
<td>92</td>
<td>–</td>
</tr>
<tr>
<td>Bonus</td>
<td>640 (±16)</td>
<td>171 (±6)</td>
<td>3.7</td>
<td>104</td>
<td>–</td>
</tr>
<tr>
<td>Donaria</td>
<td>602</td>
<td>162</td>
<td>3.7</td>
<td>98</td>
<td>–</td>
</tr>
<tr>
<td>fch2</td>
<td>386</td>
<td>ND</td>
<td>–</td>
<td>63</td>
<td>(Lion × Manchuria) × California Coast</td>
</tr>
<tr>
<td>clo-f2.101</td>
<td>529</td>
<td>ND</td>
<td>–</td>
<td>86</td>
<td>Tron</td>
</tr>
<tr>
<td>clo-f2.102</td>
<td>343</td>
<td>ND</td>
<td>–</td>
<td>56</td>
<td>Tron</td>
</tr>
<tr>
<td>clo-f2.103</td>
<td>233</td>
<td>ND</td>
<td>–</td>
<td>38</td>
<td>Tron</td>
</tr>
<tr>
<td>clo-f2.105</td>
<td>385</td>
<td>ND</td>
<td>–</td>
<td>63</td>
<td>Tron</td>
</tr>
<tr>
<td>clo-f2.107</td>
<td>816</td>
<td>142</td>
<td>5.7</td>
<td>133</td>
<td>Tron</td>
</tr>
<tr>
<td>clo-f2.108</td>
<td>616</td>
<td>ND</td>
<td>–</td>
<td>100</td>
<td>Tron</td>
</tr>
<tr>
<td>clo-f2.109</td>
<td>563</td>
<td>59</td>
<td>9.5</td>
<td>92</td>
<td>Tron</td>
</tr>
<tr>
<td>clo-f2.122*</td>
<td>342</td>
<td>16</td>
<td>21</td>
<td>60</td>
<td>Bonus</td>
</tr>
<tr>
<td>clo-f2.123</td>
<td>185</td>
<td>20</td>
<td>9.3</td>
<td>30</td>
<td>Bonus</td>
</tr>
<tr>
<td>clo-f2.133</td>
<td>571</td>
<td>67</td>
<td>8.5</td>
<td>93</td>
<td>Bonus</td>
</tr>
<tr>
<td>clo-f2.181</td>
<td>398</td>
<td>26</td>
<td>15</td>
<td>65</td>
<td>Tron</td>
</tr>
<tr>
<td>clo-f2.2800*</td>
<td>459</td>
<td>ND</td>
<td>–</td>
<td>81</td>
<td>Donaria</td>
</tr>
<tr>
<td>clo-f2.2807</td>
<td>464</td>
<td>ND</td>
<td>–</td>
<td>76</td>
<td>Donaria</td>
</tr>
<tr>
<td>clo-f2.3613</td>
<td>420</td>
<td>ND</td>
<td>–</td>
<td>69</td>
<td>Donaria</td>
</tr>
<tr>
<td>clo-106</td>
<td>452</td>
<td>131</td>
<td>3.4</td>
<td>74</td>
<td>Tron</td>
</tr>
<tr>
<td>* The measurements of Tron and Bonus were done in triplicate; the standard deviations are given. The other values are based on single or duplicate measurements. An asterisk denotes leaves grown in ambient light in a greenhouse. ND, not detected.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
synteny between the genetic map of barley chromosome 3HL and the physical map of B. distachyon chromosome 2 supported the strong connection between fch2 and HvCAO.

**Cloning and characterization of HvCAO**

In Arabidopsis, AtCAO is a gene of 3,273 bp that encodes a protein of 536 amino acid residues (GenBank AF177200 encoding AADS4323.1; Espineda et al. 1999). The B. distachyon gene Bradi2g61500.1 is annotated to be orthologous to AtCAO. Rice (Oryza sativa) has two isoforms of chlorophyllide a oxygenase. OsCAO1 (LOC_Os10g41760) is induced under dark conditions whereas OsCAO2 (LOC_Os10g41760) is induced under light, and is expressed mainly in non-photosynthetic tissues (Lee et al. 2005). A BLASTN search of the HarvEST database ‘Barley 2943 Mapped SNPs’ with the coding sequence of rice OsCAO1 yielded unigene HAR35_2495 with 83% identity. In order to investigate the possible presence of two CAO genes in barley, a TBLASTN search of the NCBI ‘est other’ database, limited by ‘Hordeum’, was done using OsCAO1 and OsCAO2 as probes. Both queries resulted in the same expressed sequence tags (ESTs) (scores down to 200 were compared), hinting at one isoform of HvCAO.

A consensus sequence for HvCAO was constructed from the ESTs, and gene-specific primers were designed according to it (Table 2). HvCAO cDNA was sequenced in the three cultivars Sejets Tron, Svalófs Bonus and Ackermanns Donaria. The sequences were entered into GenBank with the accessions JQ627619 (Tron), JQ627620 (Bonus) and JQ627621 (Donaria). The cDNA sequences of Tron and Bonus were identical. Donaria differed from them in single nucleotides (Table 3). However, the deduced chlorophyllide a oxygenase polypeptides of the three cultivars were identical. The Tron sequences will be referred to as the ‘wild-type barley’ sequences in the following.

Recently, the sequences of >24,000 full-length cDNAs from barley were published (Matsumoto et al. 2011). GenBank accession AK359686.1 and AK248476.1 are two HvCAO sequences from the cultivar Haruna Nijo. Alignment of the sequences to the HvCAO sequences determined in this study showed Haruna Nijo AK359686.1 to have the same SNP alleles as Donaria (Table 3), except for a unique C to T exchange at position 1037. Interestingly, this exchange is not found in AK248476.1 or any barley EST in the database. This base exchange also leads to a change in the primary sequence of the resulting polypeptide: Pro346 in Tron is replaced by leucine in Haruna Nijo (BAJ90895.1; Fig. 3).

Barley chlorophyllide a oxygenase consists of 550 amino acid residues. Alignment of the barley chlorophyllide a oxygenase polypeptide with those encoded by AtCAO, OsCAO1, OsCAO2.

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**Table 2** PCR primers used in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>HvCAO</td>
<td>up1</td>
<td>5’-CCC TCT TTT CCC GCT GCT CGA CG-3’</td>
<td>–51 to –29</td>
</tr>
<tr>
<td></td>
<td>up2</td>
<td>5’-GCC CAG CCA GGG TAG GTC CGT CC-3’</td>
<td>–20 to –1</td>
</tr>
<tr>
<td></td>
<td>up3</td>
<td>5’-CCA GAC CTT CGC TCT CCC-3’</td>
<td>5 to 25</td>
</tr>
<tr>
<td></td>
<td>up4</td>
<td>5’-GCT CAC TCT CCA CGG TCG GTC-3’</td>
<td>584 to 607</td>
</tr>
<tr>
<td></td>
<td>lo5</td>
<td>5’-CCA TGA GAG TGG GTC GTA CGA ACT CC-3’</td>
<td>584 to 607</td>
</tr>
<tr>
<td></td>
<td>lo7</td>
<td>5’-GCT TAA CGG TAA CCG ATC GAC GCC-3’</td>
<td>1242 to 1264</td>
</tr>
<tr>
<td></td>
<td>lo8</td>
<td>5’-GCT TAA CGG TAA CCG ATC GAC GCC-3’</td>
<td>1606 to 1629</td>
</tr>
<tr>
<td></td>
<td>lo10</td>
<td>5’-GCT TGG GTA TCC CCA TGA GCC-3’</td>
<td>1707 to 1729</td>
</tr>
<tr>
<td>chlH</td>
<td>UJ20</td>
<td>5’-TAC TTC TCC CAG ACC AAG TT-3’</td>
<td>2761 to 2780</td>
</tr>
<tr>
<td></td>
<td>UJ21</td>
<td>5’-TAC TTC TCC CAG ACC AAG TT-3’</td>
<td>4088 to 4107</td>
</tr>
<tr>
<td>dvr</td>
<td>up6</td>
<td>5’-GCC AAG GTC TGC CGG GTG G-3’</td>
<td>640 to 661</td>
</tr>
<tr>
<td></td>
<td>lo9</td>
<td>5’-GCC TCA CTT ACA AGC GGG ACG GCC-3’</td>
<td>855 to 878</td>
</tr>
<tr>
<td>por</td>
<td>up5</td>
<td>5’-GCT CAG GCT GAT CCA CCT CGG-3’</td>
<td>564 to 584</td>
</tr>
<tr>
<td></td>
<td>lo7</td>
<td>5’-GCA GCG TCT GGC TGA GGT CC-3’</td>
<td>1155 to 1177</td>
</tr>
</tbody>
</table>

HvCAO encodes barley chlorophyllide a oxygenase, chlH encodes the H-subunit of magnesium chelatase, dvr encodes 3,8-divinyl protoporphyrinogen a 8-vinyl reductase, and por encodes NADPH:protochlorophyllide a oxidoreductase. The ‘lo’ primers are reversely complementary to the coding sequence. Positional information is for the coding sequence with the translation start at +1.
and BdCAO showed 67, 87, 78 and 94% identity, respectively. In the alignment in Fig. 3, the domains of chlorophyllide a oxygenase (Nagata et al. 2004) as well as conserved and functional sites of the Arabidopsis sequence (Espineda et al. 1999, Sakuraba et al. 2009) are indicated.

### Sequencing of HvCAO in the fch2 and clo-f2 mutants

Analysis of genetic mapping data had indicated that fch2 might be HvCAO. To investigate this further, the coding region of HvCAO was sequenced in the fch2 and clo-f2 mutants to see if chlorophyllide a oxygenase is defective in these plants. Mutations were found in all available lines, with the exception of clo-f2.2807. The location of the mutations in the gene and the changes to the resulting polypeptide are given in Table 4 and Fig. 4.

In six of the mutants, frameshift insertion or deletion or nonsense mutations were found, which all led to truncated proteins: the deletion in clo-f2.101 and the insertion in the original fch2 mutant eliminate all catalytic domains in the result- ing polypeptide; in the nonsense mutant clo-f2.108 and the deletion mutant clo-f2.105, the truncation is after the Rieske FeS-binding site. In the deletion mutants clo-f2.2800 and clo-f2.3613, the conserved sequence unique to chlorophyllide a oxygenases is missing or incomplete, respectively.

In eight clo-f2 mutants, missense mutations were discovered in HvCAO. These led to changes of amino acid residues that are conserved between the chlorophyllide a oxygenases of barley, rice, B. distachyon and A. thaliana (Fig. 3). While domain A is the one differing most between the chlorophyllide a oxygenases, it contains many conserved residues, particularly in and around the degron sequence. Alanine at position 94 is one of these bordering residues and it is changed to a threonine in the mutants clo-f2.109 and clo-f2.123 (Figs. 3, 4, Table 4). The six remaining missense mutations led to changes of conserved residues in the catalytic C domain (Figs. 3, 4, Table 4): in clo-f2.103 a conserved glycine of the Rieske FeS site is changed to an aspartate, in clo-f2.122 the cysteine at position 320 between the Rieske and the mononuclear iron site is changed to a tyrosine. The mutations in clo-f2.181 and clo-f2.133 change amino acids in the mononuclear iron-binding site itself, a leu- cine to a phenylalanine and an alanine to a valine, respectively. The change of proline at 419 to leucine in clo-f2.107 is between the mononuclear iron site and the unique conserved site, the aspartate to asparagine (at position 495) in clo-f2.102 is C-terminal of the latter site in a conserved region of previously unnoticed function.

The Tron-derived line clo-106 had previously been reported to be Chl b deficient, yet non-allelic with fch2 (Simpson et al. 1985), and was included in the present study as a control. Surprisingly, the sequencing chromatograms showed equally high signals for C and T at position 1071 (Tron has C at position 1071), suggesting a mutation in HvCAO in part of the seeds in the clo-106 stock. In order to better understand the situation in this mutant, the cDNA from six individual leaves was sequenced: three showed equally intense C and T signals at position 1071, two leaves had a T and one a C. This supported the assumption that the seeds in the present clo-106 stock were a mixture of wild-type, homozygous and heterozygous genotypes concerning the mutation in HvCAO. However, all plants originating from the old clo-106 seed batch showed a light-green phenotype indistinguishable by eye. To obtain a true clo-106 line with the wild-type allele of HvCAO, 28 plants of the present clo-106 seeds were grown, harvested and geno- typed. Of these, 10 were clo-106 (C 1071), nine were heterozygous for the mutation in HvCAO, and nine constituted a new line named clo-f2.181 (T at position 1071). This new fch2 allele was described together with the other mutants above (Figs. 3, 4, Table 4). Analysis of the pigments (Table 1) showed that clo-f2.181 leaves contained Chl a and b in a ratio of 15 (wild-type Tron had 3.8). In clo-106, the amount of Chl was reduced compared with the wild type (74% of the Chl a of the wild type), while the Chl a/b ratio was higher in this line (3.4).

In mutant clo-f2.2807, HvCAO could not be amplified from cDNA initially, while in wild-type barley and the other fch2 and clo-f2 lines HvCAO amplicons were as abundant as those of genes encoding other enzymes of the Chl biosynthetic pathway [H-subunit of magnesium chelatase (chlH), 3,8-divinyl-protoclorophyllide a 8-vinyl reductase (dvr) and NADPH: protoclorophyllide a oxidoreductase (por); Fig. 5B]. The result of two different amplicons of HvCAO of clo-f2.2807 is shown in Fig. 5B (highlighted with asterisks). If a 5-fold higher amount of clo-f2.2807 RNA was used in the production of first-strand cDNA, faint bands of amplicons of HvCAO were detected. This observation suggests that the level of transcript of HvCAO in clo-f2.2807 is lower in the wild type.

### Discussion

Identification of multiple independent alleles is the most effect- ive approach to validate a candidate gene. In the original fch2 line and in 13 allelic clo-f2 genetic stocks, mutations in the
Fig. 3 Multiple ClustalW 2.1 alignment of chlorophyllide a oxygenase from barley wild-type Tron (this study), B. distachyon (Bradi2g61500.1), O. sativa CAO1 (LOC_Os10g41780) and A. thaliana (AAD54323.1). Triangle 1 indicates the transit peptide cleavage site in Tron, Brachypodium and rice. The cleavage site for the Arabidopsis sequence is marked by triangle 2. Domain A is underlaid light gray and the degron sequence (Sakuraba et al. 2009) within it is highlighted in yellow. Linker domain B is underlaid dark gray (Nagata et al. 2004). The Rieske FeS site is in blue, the mononuclear Fe site is white on red, and the chlorophyllide a oxygenase unique conserved site is in green (Espineda et al. 1999). The residues affected by missense mutations in the clo-f2 mutants are white on black in the barley sequence: Ala94Thr in clo-f2.109 and clo-f2.123; Gly280Asp in clo-f2.103; Cys320Tyr in clo-f2.122; Leu373Phe in clo-f2.181; Ala376Val in clo-f2.133; Asp495Asn in clo-f2.102. The Tron residue Pro346, which is exchanged to leucine in Haruna Nijo, is boxed (GenBank BAJ90895.1; Matsumoto et al. 2011). 

Barley fch2 encodes chlorophyllide a oxygenase
coding sequence of *HvCAO* were found. The lines with nonsense and frameshift mutations are devoid of Chl *b*. The changes to the chlorophyllide *a* oxygenase polypeptide resulting from missense mutations are reflected in the observed phenotypes of Chl *b* deficiency in the mutants, as will be discussed below, and allow insights into the mechanism and regulation of chlorophyllide *a* oxygenase in barley. We are therefore confident that the Chl *b* deficiency in the *fch2* and *clo-f2* mutants is due to mutations in *HvCAO* and that *fch2* and *HvCAO* are identical.

Missense mutants in the regulatory A domain

A mutation in the regulatory A domain of chlorophyllide *a* oxygenase is found in both *clo-f2.109* and *clo-f2.123*. Finding the same mutation in these two lines is interesting as the mutants were isolated after mutagenic treatment of two different mother cultivars, Tron in the case of *clo-f2.109* and Bonus in the case of *clo-f2.123* (Simpson et al. 1985). In the mutants, the Chl *a/b* ratio is increased to 9.5 and 9.3, respectively; the proportion of Chl *b* of total Chl is about half of that found in the wild type. The mutation results in the exchange of an alanine residue by a threonine. The residue in position 94 is conserved and it is close to the degron (Q97DLLTIMILH106 in Arabidopsis; Fig. 3; Sakuraba et al. 2009). This protease degradation signal was suggested to become accessible upon accumulation of Chl *b* in the chloroplast and in this way to be involved in regulation of chlorophyllide *a* oxygenase activity by proteolysis (Sakuraba et al. 2009). In Arabidopsis, the removal of the degron caused increased chlorophyllide *a* oxygenase stability and Chl *b* formation. Likewise, deletion of the entire A domain led to decreases

### Table 4 Allelic mutations in the *fch2* and *clo-f2* mutants

<table>
<thead>
<tr>
<th>Line</th>
<th>Mother cultivar</th>
<th>Mutation</th>
<th>Polypeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>fch2</em></td>
<td>(Lion × Manchuria) × California</td>
<td>Insertion G 791-CCTGG-A 792</td>
<td>264 native residues, followed by LDVSRIHVLT EPALLLVQ(283)</td>
</tr>
<tr>
<td><em>clo-f2.101</em></td>
<td>Tron</td>
<td>Deletion C 427–G 446</td>
<td>142 native residues, followed by SVVGP(147)</td>
</tr>
<tr>
<td><em>clo-f2.102</em></td>
<td>Tron</td>
<td>G 1483 A</td>
<td>Asp495Asn</td>
</tr>
<tr>
<td><em>clo-f2.103</em></td>
<td>Tron</td>
<td>G 839 A</td>
<td>Gly280Asp</td>
</tr>
<tr>
<td><em>clo-f2.105</em></td>
<td>Tron</td>
<td>Deletion A 1061–T 1064</td>
<td>354 native residues</td>
</tr>
<tr>
<td><em>clo-f2.107</em></td>
<td>Tron</td>
<td>C 1256 T</td>
<td>Pro419Leu</td>
</tr>
<tr>
<td><em>clo-f2.108</em></td>
<td>Tron</td>
<td>G 990 A</td>
<td>329 native residues</td>
</tr>
<tr>
<td><em>clo-f2.109</em></td>
<td>Tron</td>
<td>G 280 A</td>
<td>Ala94Thr</td>
</tr>
<tr>
<td><em>clo-f2.122</em></td>
<td>Bonus</td>
<td>G 959 A</td>
<td>Cys320Tyr</td>
</tr>
<tr>
<td><em>clo-f2.123</em></td>
<td>Bonus</td>
<td>G 280 A</td>
<td>Ala94Thr</td>
</tr>
<tr>
<td><em>clo-f2.133</em></td>
<td>Bonus</td>
<td>C 1127 T</td>
<td>Ala376Val</td>
</tr>
<tr>
<td><em>clo-f2.181</em></td>
<td>Tron</td>
<td>C 1071 T</td>
<td>Leu373Phe</td>
</tr>
<tr>
<td><em>clo-f2.2800</em></td>
<td>Donaria</td>
<td>Deletion A 1240–T 1241</td>
<td>413 native residues, followed by GVPWTHGVLKHILHQLQAWKA RREEHTAMCNASPPACMLAFIYE(457)</td>
</tr>
<tr>
<td><em>clo-f2.2807</em></td>
<td>Donaria</td>
<td>Transcript deficiency</td>
<td></td>
</tr>
<tr>
<td><em>clo-f2.3613</em></td>
<td>Donaria</td>
<td>Deletion T 1362–T 1363</td>
<td>454 native residues, followed by IYE(457)</td>
</tr>
</tbody>
</table>

The one-letter amino acid code is used to describe longer changes to the sequence of the polypeptide resulting from the mutation.

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**Fig. 4** The locations of the mutations identified in the *HvCAO* gene in relation to functional domains in the polypeptide. Missense mutations are shown with gray vertical bars. Frameshift deletions, insertions and nonsense mutations that result in truncated polypeptides are shown by red vertical bars. The domains (TP, transit peptide; A, B and C, domains) and conserved sites (FeS, Rieske FeS-binding site; Fe, mononuclear iron site; ucs, unique conserved site) of chlorophyllide *a* oxygenase are shown.
in Chl $a/b$ ratios (Yamasato et al. 2008). In addition, random missense mutations in the A domain were shown to enhance the stability of the chlorophyllide $a$ oxygenase polypeptide (Sakuraba et al. 2009). The missense mutation found in clo-f2.109 and clo-f2.123, in contrast, decreases the amount of Chl $b$ and thus probably does not stabilize chlorophyllide $a$ oxygenase. Barley residue Ala94 corresponds to Ala95 in Arabidopsis, which was not exchanged in the random mutagenesis of the A domain (Sakuraba et al. 2009). We propose that in clo-f2.109 and clo-f2.123, the exchange of the conserved alanine adjacent to the degron disrupts the structure so that the chlorophyllide $a$ oxygenase degron is exposed to protease attack. The resulting degradation of the polypeptide results in Chl $b$ deficiency.

### Structural model for the catalytic C domain

Four of the residues affected by missense mutations in HvCAO are predicted to be in the Rieske FeS- or in the mononuclear iron-binding site. In this connection it is instructive to relate the exchanges to known protein structures containing these sites. A PSI-BLAST (one repeat) of chlorophyllide $a$ oxygenase against the Protein Data Bank (PDB) found 3,6-dichloro-2-methoxybenzoic acid monooxygenase [Dicamba-MO; PDB accession 3GKE; substrate-bound form 3GL2; Dumitru et al. 2009; **Supplementary Fig. S1**] as one model structure (Expect $= 10^{-17}$). Other significant BLAST hits (Expect $< 3 \times 10^{-6}$) were the Rieske FeS and mononuclear iron site containing carboxyl 1,9a-dioxigenase (PDB accession 3GKQ) and 3-ketosteroid-9-$\alpha$-hydroxylase (PDB accession 2ZYL). Comparing the structures of these three polypeptides illustrates structural similarity and conservation of binding residues in the Rieske FeS and mononuclear iron sites (**Supplementary Fig. S2**). Dicamba-MO catalyzes a somewhat similar reaction to chlorophyllide $a$ oxygenase: Where chlorophyllide $a$ oxygenase oxidizes a chlorophyllide $a$ chlorin ring methyl substituent, Dicamba-MO is involved in the oxidation of the O-methyl substituent of the aromatic ring in the herbicide 3,6-dichloro-2-methoxybenzoic acid. The O-hydroxymethyl group is subsequently removed (Herman et al. 2005, Dumitru et al. 2009). The alignment of Dicamba-MO with the chlorophyllide $a$ oxygenases from barley, the evolutionarily distantly related prochlorophyte *P. hollandica* and the moss *Physcomitrella patens* is shown in **Fig. 6**.

The residues binding the FeS cluster [C-x-H-x(16)-C-x(2)-H] and the mononuclear iron [D-x(2)-H-x(4)-H-x(n)-D] in the Dicamba-MO structure are identical in the four proteins (**Figs. 6, 7**). The conservation of the metal-binding and surrounding residues suggests that the Dicamba-MO structure can be used as a model for barley chlorophyllide $a$ oxygenase in the following discussion.

### Mononuclear iron site mutant clo-f2.102

The alignment in **Fig. 6** shows the conservation of a C-terminal aspartate residue (position 294 in Dicamba-MO) in the evolutionarily disparate sequences. Although separated by $> 100$ residues from the nearest other mononuclear iron ligand (histidine at position 165), the structure (**Fig. 7**) shows its involvement in the mononuclear iron site in Dicamba-MO. The mutation in the clo-f2.102 mutant affects this C-terminal, conserved iron-liganding aspartate and exchanges it to asparagine. In barley chlorophyllide $a$ oxygenase, aspartate at position 495 is separated by 114 residues from the nearest other mononuclear iron ligand. The result of this mutation is total Chl $b$ deficiency (**Table 1**).

### Electron transfer inhibition in clo-f2.103, clo-f2.181 and clo-f2.133

In the Dicamba-MO monomer, the Rieske FeS cluster and the mononuclear iron site are far apart from each other (**Supplementary Fig. S1A**). The formation of a trimer results in three intersubunit Rieske FeS/mononuclear iron sites.

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**Fig. 5** Mutant clo-f2.2807 is reduced in HvCAO mRNA. (A) Total RNA preparations from Donaria (lane 1) and clo-f2.2807 (lanes 2 and 3). In lane 2, the mass of nucleotides loaded is five times that in lane 3. (B) PCR-amplified cDNA of the Chl biosynthesis loci chlH (lanes 1), dvr (lanes 2) and por (lanes 3) as controls. Amplicons with HvCAO primers up1 and Io7 are in lanes 4 (highlighted with *) and those with primers up2 and Io10 are in lanes 5 (**). In the reactions labeled ‘clo-f2.2807 (5×)’, the amount of RNA used in cDNA first-strand synthesis was five times that in the cDNA syntheses for the reactions ‘Donaria’ and ‘clo-f2.2807’.
The crystal structure (Fig. 7) illustrates how electron transport occurs between the Rieske FeS cluster of one subunit of the protein and the mononuclear iron of an adjacent subunit where it reductively activates \( \text{O}_2 \), enabling substrate oxidation (D’Ordine et al. 2009). Functional oligomers of chlorophyllide \( \text{a} \) oxygenase have not been described to our knowledge, but the similarity to Dicamba-MO indicates oligomerization. In the following we show how the electron transport between the Rieske FeS and mononuclear iron sites (dashed line in Fig. 7) in Dicamba-MO is likely to be inhibited by three missense mutations corresponding to those identified in \( \text{HvCAO} \) in the \( \text{clo-f2} \) lines. This explains how the ability to synthesize Chl \( \text{b} \) is affected in the barley mutants.

The mutation in the conserved Rieske FeS site in the line \( \text{clo-f2.103} \) results in total Chl \( \text{b} \) deficiency. The amino acid changed is glycine (position 280) which in Dicamba-MO (Gly59) is at the end of a hairpin of \( \beta \)-sheet connecting the two C-x(-x)-H motifs which bind the two irons of the Rieske FeS center (Gly59 is dark blue in Fig. 7). This glycine is conserved in the three evolutionarily divergent sequences of barley, \( \text{P. patens} \) and \( \text{P. hollandica} \) chlorophyllide \( \text{a} \) oxygenases and also in 

(Supplementary Fig. S1B; Dumitru et al. 2009). The crystal structure (Fig. 7) illustrates how electron transport occurs between the Rieske FeS cluster of one subunit of the protein and the mononuclear iron of an adjacent subunit where it reductively activates \( \text{O}_2 \), enabling substrate oxidation (D’Ordine et al. 2009). Functional oligomers of chlorophyllide \( \text{a} \) oxygenase have not been described to our knowledge, but the similarity to Dicamba-MO indicates oligomerization. In the following we show how the electron transport between the Rieske FeS and mononuclear iron sites (dashed line in Fig. 7) in Dicamba-MO is likely to be inhibited by three missense mutations corresponding to those identified in \( \text{HvCAO} \) in the \( \text{clo-f2} \) lines. This explains how the ability to synthesize Chl \( \text{b} \) is affected in the barley mutants.

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residue is identical in Dicamba-MO and the three chlorophyllide \( a \) oxygenases (Fig. 6). In Dicamba-MO, this alanine (position 161) is the neighbor of the mononuclear iron-binding ligand His160 which is suggested to be an electron transport component (Fig. 7; D’Ordine et al. 2009). The presence of a valine residue which has a 50% greater molecular volume than alanine (Wang et al. 1999) next to the mononuclear iron site might negatively affect chlorophyllide \( a \) oxygenase activity and lead to the observed deficiency in Chl \( b \).

**Missense mutant clo-f2.122**

The residue changed by the missense mutation in clo-f2.122 (Cys320 is replaced by tyrosine) is between the Rieske FeS and mononuclear iron-binding sites. The alignment of barley, rice, \( B. \ dystachyon \) and \( A. \ thaliana \) chlorophyllide \( a \) oxygenases (Fig. 3) shows that Cys320 is conserved, and also in \( P. \ patens \) chlorophyllide \( a \) oxygenase (Fig. 6). In \( P. \ hollandica \), the orthologous residue is a valine (Fig. 6). Interestingly, when \( P. \ hollandica \) chlorophyllide \( a \) oxygenase was overexpressed in a Chl \( b \)-less Arabidopsis mutant, the Chl \( a/b \) ratio observed was extremely low and it decreased from 1.1 to 0.9 in high light conditions. In contrast, the Chl \( a/b \) ratio in wild-type Arabidopsis increased in high light (Hirashima et al. 2006). In agreement with this, the effect of light intensity on the prochlorophyte has been reported to be opposite to the effect on plants: in \( P. \ hollandica \), the Chl \( a/b \) ratio increases in low light and decreases in high light (Burger-Wiersma and Post 1989). A redox- and/or light intensity-dependent regulatory system has been demonstrated for many chloroplast enzymes (Montrichard et al. 2009). If the conserved cysteine in position 320 in barley is involved in such regulation, the presence of a valine in this position in \( P. \ hollandica \) might explain the different behavior toward light intensities. The involvement of Cys320 in redox regulation of chlorophyllide \( a \) oxygenase might be through the formation of a disulfide bridge. However, it has been suggested that chlorophyllide \( a \) oxygenase is not regulated via the thioredoxin system that was shown to regulate phytohormone \( a \) oxygenase (Bartsch et al. 2008). It should be noted that this result considers only a certain group of thioredoxin targets (C-x-x-C) where an intramolecular disulfide bridge is reduced by thioredoxin. Immunoprecipitates of \( Chlamydomonas \) chlorophyllide \( a \) oxygenase suggest the co-precipitation of a thioredoxin (Eggink et al. 2004). It will be very interesting to see if chlorophyllide \( a \) oxygenase is regulated by thioredoxin and the formation of intermolecular disulfide bridges in addition to regulation by protein degradation (Sakuraba et al. 2009) and gene expression (Tanaka and Tanaka 2005).

**Missense mutant clo-f2.107**

In missense mutant clo-f2.107, the proline residue in position 419 is changed to a leucine. This affects one of a pair of proline residues in a strongly conserved stretch between the mononuclear iron site and the chlorophyllide \( a \) oxygenase unique site. This mutant has the highest Chl \( b \) content of all fch2 and clo-f2.
mutations (Chl a/b is 5.7). Interestingly, the analysis of pigments showed more Chl a in clo-f2.107 than in the wild type (Table 1). The alignment of evolutionarily diverse sequences (Fig. 6) shows that the Pro–Pro pair is conserved in barley, P. hollandoica and P. patens. Of the Pro–Pro sequences in the PISCES-culled PDB database (Wang and Dunbrack 2003), 56% were found to adopt a polyproline helix structure (Rai et al. 2006). Polyproline stretches are considered to be important for protein–protein interactions (Mansiaux et al. 2011). The exchange of the proline residue at position 419 might therefore impair the inter-action of chlorophyllide a oxygenase with a possible other component of the reaction such as ferredoxin (Oster et al. 2000).

Nonsense and frameshift mutations

In the present study, two nonsense mutations, three mutants with deletions and one with an insertion were identified. All of these lead to premature stop codons and eventually polypeptides that are truncated, without conserved sites or entire domains (Fig. 4; Espineda et al. 1999, Nagata et al. 2004) of chlorophyllide a oxygenase, and thus without catalytic function. The deletions in clo-f2.2800 and clo-f2.3613 result in polypeptides lacking the unique conserved site. The translation products in the mutants clo-f2.108 and clo-f2.105 are truncated before the mononuclear iron-binding site, whereas the inser-tion in mutant fch2 leads to an omission of the C-terminus including the Rieske FeS-binding site. The mutation in clo-f2.101 is even more severe, allowing translation of the N-terminal first quarter of chlorophyllide a oxygenase only. Given the severe mutations in these lines, it is understandable that no Chl b could be detected in either of them (Table 1).

It is possible that truncated Chl a oxygenase polypeptides are not present in these mutants in vivo. Transcript instability by the mechanism of nonsense-mediated decay has been observed in barley xantha mutants (Gadjieva et al. 2004). In addition, premature stop codons or other modifications leading to a reduction in structural stability of the polypeptide have been suggested to increase degradation of polypeptides in other barley mutants (Olsson et al. 2004).

The transcript-deficient mutant clo-f2.2807

No mutation was detected in the coding region of HvCAO of clo-f2.2807. The decreased amount of transcript suggests a mu-tation in a regulatory element of HvCAO. In the two other mutants induced by X-ray irradiation of seeds, clo-f2.2800 and clo-f2.3613 (Machold et al. 1977), base deletions were detected (Table 4). The same type of mutation might be expected in clo-f2.2807. The transcript reduction must critically affect chlorophyllide a oxygenase levels since Chl b cannot be detected in clo-f2.2807 (Table 1).

The mutations that were identified in this study are distrib-uted all over the HvCAO gene (Fig. 4). This will be utilized in future work focusing on the chlorophyllide a oxygenase protein and the function of the various sites. An expression system to produce chlorophyllide a oxygenase recombinantly will allow in vitro analyses, including in vitro activity measurements of the mutated versions of chlorophyllide a oxygenase identified in the present study. The availability of the mutations of HvCAO in viable and well characterized fch2 and clo-f2 mutant lines (Highkin 1950, Sagromsky 1974, Simpson et al. 1985) will allow additional in vivo studies that provide an important comple-ment to the in vitro studies. This is of particular interest as transformation of barley—and thus directed modification of polypeptides in vivo—is only possible with certain genotypes (Harwood 2012) at present. By correlating detailed knowledge of the seven different missense mutations and the ability of the mutants to make Chl b, it will be possible to test the ideas about the catalytic mechanism of chlorophyllide a oxygenase detailed above.

In the leaky mutants with detectable amounts of Chl b, the presence of Chl b implies that chlorophyllide a oxygenase is only partly active, but present. In the mutants with no detect-able Chl b, in contrast, the phenotype could also be explained by a lack of the protein as a consequence of the mutation. Future studies of the chlorophyllide a oxygenase protein will also analyze for the actual presence of the truncated or mis-sense mutated versions of chlorophyllide a oxygenase in the fch2 and clo-f2 plants.

Mutants clo-106 and clo-f2.181

We showed that the stock clo-106 was a mixture of seeds with wild-type, heterozygous and homozygous mutant genotypes for the replacement of C by T at position 1071 in HvCAO. However, all plants, irrespective of their genotype, showed a light-green phenotype, indicating the presence of another mu-tation in that batch. By analyzing the SNP at position 1071, a line with the wild-type allele of HvCAO (homozygous for C at position 1071) was isolated which keeps the name clo-106. The pigment determination showed no Chl b deficiency in this non-fch2 mutant; the Chl a/b ratio was 3.4. The chlorina phenotype of clo-106 is due to an unknown mutation conferring a reduc-tion in total Chl; it contains 74% of the amount of Chl a in Tron.

In the same manner, a line homozygous for T at position 1071 was isolated which we designate chlorina-f2.181 (clo-f2.181; since chlorina 180 is the highest numbered chlorina-mutant mentioned in Simpson and von Wettstein 1992). It should be noted that clo-f2.181 carries the unknown clo-106 mutation in addition to the mutation in HvCAO. The new line clo-f2.181 has a low Chl b content; the Chl a/b ratio is 15 (Table 1).

Pigment determinations

Comparing the Chl a contents of the fch2 and clo-f2 mutants, most are in a range between 60 and 90% of that of the wild type. Exceptions to this are clo-f2.103 and clo-f2.123 with 38 and 30%, respectively. The reductions in Chl a content in the fch2 and clo-f2 lines were ascribed to the different composition of the photosynthetic apparatus resulting from the lack of Chl b.
Barley fch2 encodes chlorophyllide a oxygenase

Barley fch2 en LA0 encoder chlorophyllide a oxygenase
the pigments in this solution by HPLC, the protocol of Hobe et al. (2000) was modified (personal communication Dr. Hobe). A 20 µl sample was analyzed using a Chromolith SpeedROD RP-18 50–4.6 mm column (Merck) and a linear gradient from 70 to 100% acetone over 3.5 min at a flow rate of 1.5 ml min$^{-1}$. Total run time was 6.5 min. The diluent water for the acetone was buffered with 0.2 mM Tris–HCl, pH 7.0. The eluting pigments were identified by their absorption properties and quantified based on the absorption at 440 nm. Average retention times (± SD; 12 samples) were: neoxanthin 1.48 min (±0.08 min), violaxanthin 1.93 min (±0.09 min), lutein 2.82 min (±0.07 min). Chl b 3.80 min (±0.07 min), Chl a 3.96 min (±0.07 min) and β-carotene 4.53 min (±0.10 min).

Bioinformatics

The GrainGenes database (wheat.pw.usda.gov), HarvEST (harvest.ucr.edu) and the Triticaceae Mapped EST DataBase (trimedb.psc.riken.jp) were used for barley marker and EST sequences. Rice sequences were from db.brachypodium.org. Accession numbers were from NCBI BLAST searches. Rice data was from rice.plantbiology.msu.edu; psc.riken.jp) were used for barley marker and EST sequences. Rice genome sequences were from Arabidopsis thaliana

Supplementary data

Supplementary Data are available at PCP online.

Funding

This work was supported by The Danish Council for Independent Research Natural Sciences [to M.H.]; the Deutsche Forschungsgemeinschaft [DO14821/1-1 to C.D.].

Acknowledgments

The authors are grateful to Lisbeth Faldborg for technical assistance. We are also grateful to Dr. Stephan Hobe for support in determining pigment content.

References


Barley fch2 encodes chlorophyllide a oxygenase

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