Genetic Analysis of Iron Chlorosis Tolerance in *Prunus* rootstocks

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ABSTRACT

The high economic losses caused by the occurrence of iron chlorosis in *Prunus* orchards in the Mediterranean area justifies the implementation of breeding programs to generate high-performance rootstocks for different edaphoclimatic area conditions. For that reason, the genetic control of iron chlorosis tolerance was studied in an F_1 population derived from a three-way interspecific cross between a Myrobalan plum ('P 2175') and an almond x peach hybrid ('Felinem'). Several phenotypic measures were assessed to guarantee an accurate data set for genetic analysis. SPAD values, chlorophyll concentration, and visual diagnostic symptoms were highly correlated with leaf chlorosis in trees. SPAD value was the most reliable measure, since it was an objective, unbiased, and non-destructive method.

Two significant quantitative trait loci (QTLs) involved in SPAD and chlorophyll concentration were identified for 'Felinem' in linkage groups 4 and 6. Both QTLs were detected in four of the six consecutive years of the experiment. For 'P 2175', two of the three putative QTLs identified, *pspad4.1* and *chl4.1,* were placed in linkage group 4. These QTLs were related to the SPAD values and chlorophyll concentration, respectively, and co-localized with QTLs detected in the 'Felinem' map affecting the same traits.

Candidate gene *PFIT*, related to iron metabolism, was localized within the confidence interval of the QTL in linkage group 4. This research suggests an association of this chromosome region with tolerance to iron chlorosis in *Prunus*, and it provides a first approach to localize candidate genes involved in tolerance to this abiotic stress.

Keywords: abiotic stress, QTL analysis, candidate genes, fruit trees breeding

INTRODUCTION

Iron chlorosis is a worldwide problem affecting a wide range of crops in calcareous soils (Marschner 1995). The problem is particularly important in the Mediterranean area, where the lack of iron causes significant economic losses. Fruit trees are among the crops most affected by this nutritional disorder (Tagliavini and Rombolà 2001), and peach is especially sensitive (Almaliotis et al. 1995; Sanz et al. 1992). Iron deficiency chlorosis significantly decreases fruit yield and quality in peach (Álvarez-Fernández et al. 2011, and references therein). One of the best solutions to this abiotic stress lies in the use of tolerant rootstocks to prevent chlorosis (Gogorcena et al. 2000; Gogorcena et al. 2004; Gonzalo et al. 2011; Jiménez et al. 2008; Tagliavini and Rombolà 2001). Thus, breeding programs for *Prunus* rootstocks in different areas have generated complex hybrids to overcome soil and disease problems (Reighard 2002). The objectives of these programs are to generate rootstocks with the maximum number of desirable traits. For that reason, interspecific hybrids were produced to generate high-performance rootstocks for Mediterranean environments (Dirlewanger et al. 2004a; Salesses et al. 1998).

The selection of rootstocks tolerant to iron chlorosis based on their performance in the field under favorable conditions to induce iron chlorosis requires expensive and long-term experiments (Jiménez et al. 2008; Zarrouk et al. 2005). The process can be accelerated using molecular tools such as molecular markers linked to a specific trait, allowing early selection of likely seedlings (Dirlewanger et al. 2006; Etienne et al. 2002). To apply marker assisted selection), markers associated with iron chlorosis tolerance have been identified in soybean (Charlson et al. 2003; 2005). However, the genetic structure of iron chlorosis tolerance in trees is still unknown, even though some evidence in other plant species suggests quantitative control of this nutritional disorder.

In species such as soybean and tomato, quantitative trait loci (QTLs) involved in the response to iron chlorosis have been identified (Charlson et al. 2003; Dasgan et al. 2004; Diers et al. 1992; Lin et al. 1997; 2000). In *Prunus*, since breeding programs have mostly focused on improving fruit organoleptic traits in scions, most QTLs identified in peach are involved in agronomic traits like bloom time, ripening time, fruit size, and fruit set Quilot et al. 2005; Wang et al. 2000), traits related to fruit quality (Eduardo et al. 2011; Etienne et al. 2002; Quilot et al. 2004), and postharvest quality traits (Cantín et al. 2010; Ogundiwin et al. 2007; Peace et al. 2005a; 2006). Other important objectives in fruit tree breeding programs are resistance to pests and diseases. Several QTLs have been described that are associated with biotic stress resistance in different species, including *Prunus* spp. (Asins et al. 2004; Dirlewanger et al. 2004a; Lambert et al. 2007; Marandel et al. 2009; Marguerit et al. 2009; Stoeckli et al. 2009).

Another approach that has been used successfully to find associations between genes involved in relevant metabolic pathways and major genes or QTLs is the use of candidate genes. Candidate genes have been developed in peach mainly for fruit characters (Le Dantec et al. 2010; Ogundiwin et al. 2008; Peace et al. 2005b) and for several resistance genes related to biotic stresses (Bliss et al. 2002). Several candidate genes were placed in similar genomic positions as QTLs involved in the studied characters, suggesting they affect the specific traits of interest (Dirlewanger et al. 2004b).

Genetic studies on tolerance to abiotic stress have been performed in several herbaceous plant species like rice and bean, with the primary objectives mineral uptake or tolerance to drought and/or salinity (Dufey et al. 2009; López-Marín et al. 2009; Xue et al. 2009). However, in woody species, little information on genetic control of abiotic stresses exists. Studies on abiotic stress tolerance are also limited and focus almost

95 exclusively on nutritional issues such as magnesium deficiency in grapevine (Mandl et
96 al. 2006) or calcium uptake in apple (Volz et al. 2006). Modification of plant gene
97 expression occurs in response to iron deficiency to overcome the stress; differential
98 expression of genes involved in iron uptake and transport has been described in several
99 plant species (Buckhout et al. 2009; Mukherjee et al. 2006; Santi and Schmidt, 2009).
100 Likewise, in *Prunus* rootstocks, the response to iron deprivation involved differential
101 expression of *PFRO2, PIRT1,* and *PAHA2:* genes related to reductase activity, iron
102 transport in roots, and proton release (Gonzalo et al. 2011). These genes are good
103 candidates for molecular markers associated with iron chlorosis tolerance that could be
104 applied as a selection tool in breeding programs.
105 This study assesses the genetic basis of iron chlorosis tolerance in *Prunus* species
106 using a progeny derived from a three-way, interspecific hybrid cross. The analysis of
107 tolerance was performed using QTL detection and the candidate genes approach to
108 localize genomic regions involved in traits related to iron chlorosis, since the final
109 objective of this breeding program is to select tolerant rootstocks using molecular
110 markers associated with this abiotic stress.

MATERIALS AND METHODS

Plant Material

A three-way interspecific *Prunus* progeny was produced to associate favorable traits from plum, peach, and almond (Dirlewanger et al. 2004a). Thus, an F_1 population was obtained from a cross between the Myrobalan plum (*P. cerasifera* Ehrh) clone 'P 2175' (Salesses et al. 1998) and the almond-peach hybrid (*P. dulcis* x *P. persica* L. Batsch) 'Felinem', formerly named 'GxN 22' (Felipe 2009). Seventy individuals from the progeny out of 101 used for genetic map generation (Dirlewanger et al. 2004a) were clonally propagated at the INRA of Bordeaux (France) and at the Experimental Station of Aula Dei (CSIC, Zaragoza) to ensure enough replications for iron chlorosis analyses. The individuals of the F_1 population also segregated for other traits such as root–knot nematode resistance (Dirlewanger et al. 2004a) and leaf color (32 red : 66 green), since 'P 2175' displays green leaves and 'Felinem', red ones.

Field trials were established at the Experimental Station of Aula Dei (CSIC) in Zaragoza (northeast Spain). The field is characterized by calcareous soil, with 30 to 35% total calcium carbonate, 7 to 8% active lime, pH 8.0 to 8.5, and clay loam texture (Fernández et al. 2006; Zarrouk et al. 2005). The orchard was managed following local commercial procedures and flood irrigated. The experiment was established in a randomized block design with ten tree replications for each F_1 seedling. The experiment was set up during 2005 and 2006, with the addition of new individuals from the F_1 population during 2007 and 2009 to increase the number of genotypes and replications for genetic analysis.

Phenotypic evaluations

Three different phenotypic measurements of the 'P 2175' x 'Felinem' F_1 individuals and both parents were performed. First, the chlorophyll (Chl) concentration

136 per unit area was estimated in the field using a digital chlorophyll meter named SPAD
137 (Soil and Plant Analyzer Development) model 502 (Minolta Co., Osaka, Japan). Thirty
138 leaves per tree, selected randomly from the middle of bearing shoots located all around
139 the crown, were measured with the SPAD to obtain an average leaf Chl concentration.
140 SPAD readings were taken for the ten individuals of each genotype. Thus, the values
141 reported are the means of the ten trees established for each genotype. Measurements
142 were carried out during six consecutive years (2005 to 2010) at ~ 120 days after full
143 bloom as described (Zarrouk et al. 2005).

144 The leaf colors of the two rootstocks used as parents of the F1 population were
145 different, resulting in progeny which segregated for this trait (Dirlewanger et al. 2004a).
146 This characteristic would affect the chlorophyll content estimation based on the SPAD
147 values. Thus, in order to establish the relationship between Chl concentration per unit
148 leaf area and SPAD readings, 'P 2175' and 'Felinem' plants were subjected to iron-
149 deficient conditions in hydroponic culture to obtain a SPAD values range in both
150 parents (Fig. 1A). Plants were grown hydroponically under controlled conditions as
151 described by Gonzalo et al. (2011). To induce chlorosis iron was not supplied to the
152 nutrient solution during several days.

Fig.1

153 Chl was extracted from leaves with different SPAD values using 100% (v/v)
154 acetone in the presence of sodium ascorbate and measured spectrophotometrically as
155 described (Abadía and Abadía 1993). The chlorophyll concentrations obtained were
156 used to correlate field-determined SPAD values of leaves with their chlorophyll
157 concentration. A linear equation that established the relationship between SPAD values
158 and chlorophyll concentration was calculated for each parent and applied to the F_1
159 population as appropriate based on the progeny leaf color (Fig. 1B). Therefore, the
160 linear equation from 'Felinem' was applied to the F_1 individuals with red leaves, and

161 genotypes with green leaves were transformed using the equation from ‘P2175’. The
162 correlation coefficient between the estimates of chlorophyll concentration and SPAD
163 values was highly significant, with a R^2 of 0.99 and 0.98 for ‘P 2175’ and ‘Felinem’,
164 respectively (Fig. 1B).

165 A visual diagnostic (VD) of iron chlorosis was also performed in the population.
166 This method, though subjective, is simple, economic, and fast. The evaluation was
167 performed based on leaf symptoms, setting up a chlorosis scale. The values assigned
168 were: 0 (no symptoms), 1 (early chlorosis in few apical leaves), 2 (early chlorosis
169 symptoms in all young leaves), 3 (interveinal yellowing in young and old leaves), 4
170 (chlorosis in all leaves of the tree), and 5 (tree defoliation and leaf necrosis) (Lin et al.
171 1997; Sanz and Montañés 1997).

172 **Mineral analysis**

173 Leaf mineral analysis was carried out to assess the nutritional status of the
174 population in the field (Jiménez et al. 2007; Zarrouk et al. 2005). Mineral element
175 concentrations were determined in leaves in 2010, six years after the trial was
176 established in the field. Leaf sampling and SPAD measurements were carried out 120
177 days after full bloom.

178 Leaf samples were collected from the middle part of the bearing shoots of the trees.
179 Thirty leaves from the ten trees per genotype established in the field were selected to
180 have a representative sample . The tissue was dried, and three replicates per genotype
181 were analyzed. The mineral element composition of the dried tissue was measured using
182 published methods (C.I.I. 1969; C.I.I. et al. 1975) as previously reported (Jiménez et al.
183 2008). Total N was determined with the Dumas method by direct combustion
184 (LECO.FP-528); P was analyzed spectrophotometrically by the nitro-vanadate
185 colorimetric method (Thermo Spectronic Helios β); Ca, Mg, Fe, Mn, Cu, and Zn were

186 determined by flame atomic absorption spectrophotometry; and Na and K were
187 determined by flame atomic emission spectrophotometry (Thermo Solaar S4).

188 **Foliar corrector treatment**

189 To guarantee that the measured symptoms were due to lack of iron available in the
190 soil, a foliar treatment was sprayed onto the leaf surface. The application has a re-
191 greening effect associated with increased chlorophyll and Fe concentration. Leaves of
192 trees with different degrees of chlorosis symptoms were sprayed with a solution
193 containing $FeSO_4$ as described (Fernández et al. 2006). Leaf sprays were applied with a
194 commercial hand sprayer until full wetting. The leaf re-greening was estimated using
195 the non-destructive SPAD apparatus three and five days after treatment.

196 **Genotyping and candidate gene mapping**

197 Genotyping of the F_1 population was performed with 93 and 116 SSR markers for
198 'P 2175' and 'Felinem', respectively. Separate genetic linkage maps covering the whole
199 genome were constructed for each parent following the "double pseudo-testcross"
200 (Dirlewanger et al. 2004a).

201 In addition, young leaf tissue from all F_1 genotypes and the parents was collected in
202 the field for total genomic DNA isolation with the DNA-easy kit from Qiagen (Qiagen,
203 Inc., Valencia, CA, USA). The DNA was quantified using a NanoDropTM 2000
204 spectrophotometer (Thermo Scientific) and diluted to 5 ng/µl for PCR amplification
205 reactions. DNA amplification used primer pairs designed from five genes associated
206 with iron metabolism.

207 Three candidate genes, *PFRO2*, *PIRT1,* and *PAHA2* had previously been reported
208 in *Prunus* (Gonzalo et al., 2011) and showed differential expression in the parents of the
209 F_1 population under iron-limiting conditions. Two other genes, *PFIT* and *PNramp,* were
210 described in other plant species as involved in iron chlorosis tolerance (Bauer et al.

211 2007; Schmidt 2003). *PFIT* is associated with the Fe deficiency Induced Transcription
212 Factor and *PNramp* is related to the Natural Resistance Associated Macrophage
213 Proteins.
214 For the new candidate genes, the following primers were designed as described
215 (Gonzalo et al. 2011) (*PFIT*-fw (5'-GCTGCCAGTCTTGTTGAGGT-3'), *PFIT*-rev (5'-
216 TGTCAAGCTAGCCACCACTG-3'); and *PNramp*-fw (5'-
217 AAATTCTGGAGGGCTCTGGT-3'), *PNramp*-rev (5'-
218 GAACACTGCTTCCAGCTTCC-3').
219 Amplification reactions were carried out with DNA of the F_1 population parents as
220 described (Bouhadida et al. 2007). The amplifications were performed on a Gene Amp
221 2700 thermocycler (Applied Biosystems) using the following temperature cycles: 1
222 cycle of 3 min at 95ºC, 35 cycles of 1 min at 94ºC, 45 s at 56-60ºC and 1 min at 72ºC.
223 The last cycle was followed by a final incubation of 7 min at 72ºC and the PCR
224 products were stored at 4ºC before electrophoresis analysis. At least two independent
225 amplification reactions were performed for each DNA sample until two data points were
226 available for each primer pair/genotype combination. The DNA amplification products
227 were loaded on denaturing 5% polyacrilamide gels. The gels were silver-stained as
228 described (Bassam et al. 1983).
229 In the F_1 population parents, the amplicon was re-sequenced to detect
230 polymorphisms. Small insertions and deletions (Indels) were found in only three (*PFIT*,
231 *PFRO*, and *PNramp*) of the five sequences after analysis by "Chromas". To map the
232 candidate genes, the polymorphism detection was performed using polyacrylamide gels.
233 **Statistical analysis and QTL detection**
234 Coefficients of correlation were calculated among years and among methods of
235 chlorosis evaluation. The population distribution was analyzed by Kolmogorov-

236 Smirnov test. The statistical analyses were performed using the software SPSS 17.0
237 (SPSS, Inc, Chicago, IL, USA). Broad sense heritability was calculated for the SPAD
238 readings from the variance components of the progeny and the parents of the F_1
239 population (Falconer 1970).
240 QTL analysis was conducted separately each year by Composite Interval Mapping
241 (Zeng 1993; 1994) using Windows QTL Cartographer v2.5 (Wang et al. 2006).
242 Permutation tests were conducted 1,000 times at a significance level of 0.05 to
243 determine QTL threshold levels (Churchill and Doerge 1994). Additive effects and the
244 fraction of the variance explained by the QTL (R^2) were estimated using Windows QTL
245 Cartographer at highest probability peaks. QTL positions were estimated with a 2-LOD
246 confidence interval surrounding the maximum limit of detection (LOD) peak.

RESULTS

Phenotypic analysis of iron chlorosis tolerance

To differentiate iron chlorosis from other mineral chlorosis in our soil conditions, several genotypes of the F_1 progeny with different degrees of iron chlorosis were sprayed with $FeSO_4$. Re-greening associated with an increased SPAD values was obtained for most individuals. Recovery from chlorosis was evident three days after iron treatment in most individuals, although several showed the biggest differences on the fifth day. However, for some genotypes with severe chlorosis symptoms such as Ind 1, no re-greening was observed after iron treatment (Fig. 2).

Fig.2

To find the best criteria to evaluate chlorosis symptoms or tolerance, SPAD measurements, chlorophyll concentrations, and visual diagnostic were determined in the progeny established in the field under favorable conditions to induce iron chlorosis. These three measures of chlorosis severity were highly correlated throughout the six years of evaluation. The visual diagnostic was negatively correlated with the other two measurements (Table 1), while SPAD values and chlorophyll concentration showed a positive and high correlation. Moreover, the analysis by year exhibited the highest correlations of SPAD values for the last three years of the experiment: 2008, 2009, and 2010, when the more susceptible trees showed severe symptoms of chlorosis (Table 2). Identical results were found for chlorophyll concentration with the highest correlations for the last three years.

Table 1

Table 2

All evaluated traits displayed a normal distribution with continuous variation, typical of quantitative or polygenic inheritance, except for the visual diagnostic. The frequency of distribution for SPAD values and chlorophyll concentration followed a normal distribution (Fig. 3). Values for these traits in the population decreased over the six years of the experiment except for in 2008 and 2010. Chlorosis symptoms observed

Fig.3

in the first two years, 2005 and 2006, were high only for a few individuals that also had low SPAD values and chlorophyll concentrations. Most of the trees established in the field did not show any chlorosis symptoms until the third year of the experiment. After three years, symptoms started to become more visible, and clear differences among genotypes were observed in the last three years of the study. The mean values for both parents were closer to values associated with no chlorosis (see arrow in Fig. 3, Table 3). Mean values for the F_1 progenies decreased each year at the same times as differences among tolerant and sensitive genotypes became greater, except for Chl (Table 3). Values obtained for SPAD and chlorophyll concentration in 2010 were higher than those obtained in 2009 for these two measurements. The visual diagnostic in 2010 showed also less severe chlorosis symptoms.

Table 3

Mineral analysis

The leaf mineral analysis performed the last year of the experiment did not provide additional information about the iron status of the trees (Table 1). In general, trees with iron chlorosis had lower iron concentrations than ones without symptoms, varying from 74 to 117 mg/kg (ppm) Fe. In 2010, the iron concentration was significantly and positively correlated with SPAD values, Chl concentration, and visual diagnosis, with the highest correlation being with the latter.

QTL analysis and candidate gene mapping

Three phenotypic measurements were used to identify QTLs involved in iron chlorosis and place them on the genetic map (Fig. 4). The analysis was performed separately for each parent and for each year. The QTLs identified and their characteristics are represented in Table 4. No QTLs associated with the mineral concentration were identified in this work. The heritability estimated for the SPAD

Fig. 4

Table 4

296 readings oscillated from 50 to 33 % with a mean of 41% for the 6 years the experiment
297 was carried out.

298 *QTLs detected in 'Felinem'*

299 In the 'Felinem' map, QTLs associated with SPAD and chlorophyll concentration
300 were detected in the last four years of the experiment (2007, 2008, 2009, and 2010),
301 when chlorosis symptoms became evident in susceptible trees. Thus, in the Felinem
302 genotype, a QTL affecting SPAD values (*spad4.1*) was identified those four years in
303 linkage group (LG) 4. In all instances, the values overcame the LOD obtained by
304 permutations. The percentage of phenotypic variance explained by this QTL was 24%,
305 19%, 40%, and 27%, respectively, for the four consecutive years. The additive effect of
306 the QTL was negative (Table 4).

307 QTLs affecting chlorophyll concentration in this genotype were also identified.
308 These QTLs were localized in two different genome regions, LG4 and LG6. The QTL
309 located in LG4 (*chl4.1*) was identified in four years: 2007, 2008, 2009, and 2010. This
310 QTL was located in the same region as *spad4.1*. The percentage of variance explained
311 by this QTL was 11%, 9%, 18%, and 17%, respectively. The additive effect was
312 negative, with CCPT005 being the most significant marker (Fig. 4, Table 4). The QTL
313 located in LG6 (*chl6.1*) was identified in 2007, 2008, and 2009, and the phenotypic
314 variance explained by the locus was 30%, 36%, and 12%, respectively. The additive
315 effect associated with the trait was positive.

316 *QTLs detected in 'P 2175'*

317 The analysis for 'P 2175' showed lower LODs than those found in 'Felinem' for all
318 QTLs detected (Table 4). Nevertheless, different putative QTLs associated with each
319 phenotypic measurement were detected (Fig. 4).

For the SPAD analyses, one QTL located in LG1 (*pspad1.1*) was identified in 2007 and 2008. In both years, the QTLs showed a LOD of 2.3, slightly lower than the LODs obtained by permutations. The most significant marker was CPPCT026 and the phenotypic variance explained was 10% and 11% for 2007 and 2008, respectively. In 2008, 2009, and 2010, QTLs associated with SPAD values were identified in LG4 (*pspad4.1*) and LG8 (*pspad8.1*). The LOD for *pspad4.1*was lower than the threshold obtained by permutations, but the QTL was detected in three years and in the same position as the QTL *spad4.1* in 'Felinem'. The percentage of phenotypic variance explained for the QTL *spad4.1* was 6%, 15%, and 11% in 2008, 2009, and 2010, respectively. Finally, a QTL located in LG8 (*pspad8.1*) affecting SPAD values was identified in 2008, 2009, and 2010 with an R^2 of 8%, 13%, and 10%, respectively. The most significant marker associated with *pspad8.1* was CPPCT006 (Table 4, Fig. 4).

In 'P 2175', QTLs associated with chlorophyll concentration were localized in linkage groups LG1, LG4, and LG8, at the same genomic regions as for the SPAD values. The LOD score of these QTLs were close to the threshold obtained by permutations. The percentage of phenotypic variance explained for QTLs affecting chlorophyll concentration was similar those of the QTLs affecting SPAD values.

Finally, QTLs associated with visual leaf diagnostic were localized in LG1 in 2007, 2008, and 2009. This QTL, *pvd1.1*, explained 23%, 17%, and 14% of the phenotypic variance, respectively, and its additive effect was negative (Table 4).

Candidate gene mapping

In addition to these QTLs detected in both genetic maps, two candidate genes associated with iron metabolism, *PFIT* and *PFRO*, were localized on the 'Felinem' map. *PFIT* was positioned in LG4, co-localizing with other QTLs associated with iron chlorosis (*spad4.1* and *chl4.1*) identified in this work (Fig. 4). *PFRO2* was weakly

linked to LG5 with LOD >2 and as a result it has been difficult to determine its exact position in the map (Fig. 4). Finally, no polymorphism was found for the *PIRT1* and *PAHA2* candidate genes, making them impossible to locate on the map at this time. The other candidate gene studied, *PNramp,* amplified in the progeny, but it was difficult to localize it on the maps since its profile was too complex to be read accurately.

DISCUSSION

The identification of factors causing iron chlorosis in plants is critical to evaluating and preventing this problem. For woody plants established in the field, measurements are limited to the aerial part of the plant and diagnosis of the related symptoms becomes particularly important. The principal cause of chlorosis under our experimental field conditions is the lack of iron in the plant. The recovery of SPAD values in leaves after spraying with $FeSO_4$ confirmed that leaf chlorosis symptoms were due to iron deficiency as previously reported (Fernández et al. 2006).

Analysis of the genetic control of complex traits such as tolerance to iron chlorosis requires accurate phenotypic scoring to obtain robust results. In addition, the phenotypic analysis of symptoms should provide a precise data set for quantitative studies. For that reason, three different parameters were used here to determine the occurrence and severity of iron chlorosis. The high correlation found among the three measurementss supports their use as an indicator of iron chlorosis incidence. However, the visual diagnostic, although fast, is a subjective method and the evaluation usually varies from person to person. Furthermore, since the number of genes, the gene action, and the magnitude of the gene controlling chlorosis is unknown, the five-score scale may not be appropriate for evaluating phenotypic segregation in a population (Lin et al. 1997). Leaf chlorophyll concentration is an accepted tool to monitor Fe status in fruit trees (Abadía 1992; Tagliavini and Rombolà 2001) but it is destructive and time-consuming. On the other hand, the SPAD meter provides an unbiased, quantitative measure of the severity of leaf chlorosis associated with Fe deficiency (Peryea and Kammereck 1997). In addition, this methodology provides a quick and nondestructive estimation of the chlorophyll concentration (Yadava 1986). Consequently, this method has been widely

accepted to monitor iron status in fruit trees growing under field conditions (Igartua et al. 2000; Jiménez et al. 2008; Zarrouk et al. 2005).

The frequencies of chlorosis measurements in the progenies showed a normal distribution typical for quantitative traits. The parents of the F_1 population presented high SPAD values and chlorophyll concentrations corresponding to moderate tolerance to iron chlorosis. Few progenies overcame these values, although a high segregation in tolerance to this nutritional disorder was observed in the population. The tolerance to iron chlorosis of the peach-almond 'Felinem', grown under Mediterranean field conditions, is probably due to the influence of its almond pedigree (Felipe 2009). The myrobalan plum 'P 2175', when grown under controlled iron-deficient conditions, has also been described as tolerant to this nutritional disorder (Gonzalo et al. 2011), as are several other plum rootstocks (Socias i Company et al. 1995). The selection of this three-way interspecific cross for this experiment ('P 2175' x 'Felinem') was based on an interest in associating the best favourable traits from almond, peach, and plum to generate new multitolerant rootstocks. Thus, this population has also been used to investigate tolerance to other stresses affecting *Prunus* species (Dirlewanger et al. 2004a; Salesses et al. 1998). The present work evaluated this population to determine the genetic control of tolerance to iron chlorosis.

The heritability of the SPAD values trait was medium according to the scale reported by Falconer et al. (1970), supporting the genetic control found for this trait.

Thus, QTL analysis was performed in the F_1 population obtained from the cross 'P 2175' x 'Felinem'. QTLs associated with tolerance to iron chlorosis were detected for both parents. The different QTLs detected for each parent would be due to the contribution of both to the tolerance or could be the effect of the population size (Lin et al. 1997). The population size can lead to over-estimation of the magnitude of genetic

399 effects associated with QTLs and is one of the most limiting factors in precise QTL
400 localization (Darvasi et al. 1993). Furthermore, management of field trials is more
401 difficult for woody plants than herbaceous ones. The difficulty of establishing a woody
402 plant species in the field, especially under our soil conditions, has limited the number of
403 individuals from the population available for analysis. For that reason, other factors
404 affecting the accuracy of the QTLs detection have been reduced in this experiment. The
405 QTLs analysis was carried out during six consecutive years, the trees were established
406 in a randomized block design, ten trees per genotype were measured and the phenotypic
407 analysis was performed by three different methods.

408 Most of the QTLs were detected in different years by the three evaluation methods
409 used. The QTLs were more easily identified during the later years due to the occurrence
410 of more evident iron chlorosis symptoms in the leaves. At that point of the experiment,
411 the segregation among the F_1 genotypes for chlorosis was higher, suggesting better
412 association between QTLs and chlorosis. The effect of these QTLs in the tolerance to
413 this abiotic stress could be confirmed by the consistency with which they were
414 identified among years (Foulongne et al. 2003). The QTL located on chromosome 4 was
415 detected for SPAD and chlorophyll concentration in both the 'Felinem' and 'P 2175'
416 maps. Also, the additive effect with opposite direction suggested the different
417 contribution of each parent to chlorosis tolerance. Thus, alleles of 'Felinem' in
418 homozygosity could confer tolerance to iron chlorosis on the progeny while, at the same
419 time, 'P 2175' alleles could increase susceptibility to this stress.

420 Chlorosis occurrence in peach has been associated with decreased fruit yield and
421 quality (Almaliotis et al. 1995) and delayed fruit ripening (Álvarez-Fernández et al.
422 2011). In LG 4, some QTLs associated with ripening time, fruit development, and fruit
423 mass were detected in other *Prunus* spp. populations (Cantín et al. 2010; Etienne et al.

2002; Quilot et al. 2004; Wang et al. 2000). The consistency of this QTL in LG4 supports the use of markers associated to the traits to perform marker-assisted selection either for tolerance to chlorosis or for high fruit quality. At present, the new challenge is to demonstrate that the QTLs detected are associated with iron chlorosis tolerance in different populations (Wang et al. 2008), since, in other plant species such as soybean, QTLs involved in iron chlorosis were described as population-specific (Charlson et al. 2003; Lin et al. 1997).

The localization within the confidence interval of the QTLs in LG 4 of the candidate gene *PFIT*, responsible for regulating the expression of genes involved in the response to iron Fe deficiency (Bauer et al. 2007), suggests an association between this molecular marker and iron chlorosis tolerance. Further studies may confirm that this marker can be used in early selection of iron chlorosis-tolerant rootstocks. Likewise, placing *PFRO2* on the genetic map and detecting polymorphism in the other candidate genes (*PIRT1*, *PAHA2*, and *PNramp*) would provide valuable knowledge about the genetic control of iron chlorosis in Prunus.

In Felinem, the QTLs involved in chlorophyll content were localized in LG6 in a region near the Gr gene that is responsible for leaf color (Dirlewanger et al. 2004a). The fact that these QTLs, and those found in LG8, were only for chlorophyll content and not for SPAD readings, point out the convenience to transform values obtained directly from the field to have more accurate results because, as we mentioned before, leaf color affect SPAD readings. Furthermore, the QTLs involved in chlorophyll content that were identified in LG6 in Felinem and in LG8 in P 2175 suggest a different genetic control of this trait. However, a reciprocal translocation had been described in this population among these linkage groups (Dirlewanger et al. 2004a) supporting the idea that could be the same QTL affecting chlorophyll content.

449 It is worthy to mention that the less symptoms observed in 2010 were probably due
450 to the environmental conditions, since it was a rainy year and iron availability in the soil
451 could be affected. This fact was reflected in the lower power of detection of QTLs
452 identified in 2010.
453 This work reports for the first time evidence concerning genetic control of iron
454 chlorosis tolerance in *Prunus* spp. that contributes to the search for solutions against this
455 harmful nutritional disorder. Several QTLs associated with this abiotic stress were
456 identified. QTLs were detected in different years and for different traits in the same
457 genomic region. The data suggest that at least three genomic regions were affecting
458 tolerance to iron chlorosis: one in LG4 with influence from both parents, a second in
459 LG6 from the almond-peach hybrid 'Felinem', and the third in LG8 from the plum 'P
460 2175'. In addition, a candidate gene associated with iron metabolism co-localized with
461 one of the major QTLs identified in this study, confirming the association between the
462 genomic region in LG4 and tolerance to iron chlorosis. The association of a candidate
463 gene to this abiotic stress will accelerate selection of iron chlorosis-tolerant genotypes
464 in breeding programs. Other populations segregating for these traits should be examined
465 to give additional information on the role of this candidate gene in control of this abiotic
466 stress.

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667

668 **Table 1:** Correlation coefficients among the different phenotypic measurements during

669 the 6-year experiment (2005 to 2010).

	SPAD05	Chl05	VD05
SPAD05	1.00	0.87**	-0.84**
Chl05		1.00	-0.66**
VD05			1.00

	SPAD06	Chl06	VD06
SPAD06	1.00	0.90**	-0.98**
Chl06		1.00	-0.89**
VD06			1.00

	SPAD07	Chl07	VD07
SPAD07	1.00	0.86**	-0.96**
Chl07		1.00	-0.81**
VD07			1.00

	SPAD08	Chl08	VD08
SPAD08	1.00	0.84**	-0.96**
Chl08		1.00	-0.82**
VD08			1.00

	SPAD09	Chl09	VD09
SPAD09	1.00	0.86**	-0.94**
Chl09		1.00	-0.77**
VD09			1.00

	SPAD10	Chl10	VD10	Fe
SPAD10	1.00	0.98**	-0.69**	0.23*
Chl10		1.00	-0.70**	0.25*
VD10			1.00	0.59**
Fe				

670

671 The iron (Fe) concentration measured in 2010 was correlated with the other measurements in the

672 corresponding year

673 (*SPAD*= SPAD values; *Chl* (chlorophyll concentration and *VD*= visual diagnostic)

674 * $p < 0.05$ ** $p < 0.01$ represent significant values

675 **Table 2:** Correlation coefficients of SPAD values (SPAD), chlorophyll concentration

676 (Chl), and visual diagnostic (VD) during the 6-year experiment (2005 to 2010).

677

	SPAD05	SPAD06	SPAD07	SPAD08	SPAD09	SPAD10
SPAD05	1.00	0.66**	0.07	0.19	0.42**	0.36*
SPAD06		1.00	0.30*	0.45**	0.34*	0.34*
SPAD07			1.00	0.75**	0.65**	0.57**
SPAD08				1.00	0.77**	0.73**
SPAD09					1.00	0.79**
SPAD10						1.00

	Chl05	Chl06	Chl07	Chl08	Chl09	Chl10
Chl05	1.00	0.82**	0.47**	0.57**	0.77**	0.28*
Chl06		1.00	0.56**	0.67**	0.66**	0.36*
Chl07			1.00	0.84**	0.81**	0.53**
Chl08				1.00	0.85**	0.66**
Chl09					1.00	0.59**
Chl10						1.00

	VD05	VD06	VD07	VD08	VD09	VD10
VD05	1.00	0.69**	0.43**	0.46**	0.56**	0.20
VD06		1.00	0.63**	0.68**	0.71**	0.17
VD07			1.00	0.83**	0.73**	0.50**
VD08				1.00	0.91**	0.45**
VD09					1.00	0.35*
VD10						1.00

678

679 * $p < 0.05$ ** $p < 0.01$ represent significant values

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683 **Table 3**: Mean values for SPAD values, Chlorophyll content (Chl) and visual diagnostic
684 (VD) in P 2175, 'Felinem' and F1 progeny. The range of values in the F_1 population
685 is also represented

	Mean			F_1 range	
Trait	P 2175	Felinem	F_1 progeny	min	max
SPAD05	34	32	31	23	40
SPAD06	36	31	27	16	36
SPAD07	33	26	22	11	31
SPAD08	30	30	24	10	33
SPAD09	23	26	21	11	28
SPAD10	36	31	24	9	34
Chl05	31	37	32	18	49
Chl06	34	36	31	12	41
Chl07	30	30	27	7	36
Chl08	26	34	21	7	38
Chl09	18	29	22	7	32
Chl10	34	36	19	5	40
VD05	0	0	1	0	5
VD06	0	0	1	0	5
VD07	0	1	1	0	5
VD08	0	0	2	0	5
VD09	1	1	2	1	5
VD10	0	0	3	0	5

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689 Table 4: Characteristics of the QTLs detected in different linkage groups for each trait
690 in the 'P 2175' x 'Felinem' progeny. QTLs for each parent are named according to
691 trait abbreviations.
692

Genotype	Linkage group	Trait	Permutation treshold	Locus	[a]Most significant marker	[b]LOD	[c]R^2(%)	[d]Additive
Felinem	4	SPAD07	2.6	*spad4.1*	CPPCT005	4.0	24	-7.1
	4	SPAD08	2.8	*spad4.1*	CPPCT005	3.7	19	-4.6
	4	SPAD09	2.8	*spad4.1*	CPPCT005	7.8	40	-5.3
	4	SPAD10	2.6	*spad4.1*	CPPCT005	3.9	27	-4.9
	4	Chl07	2.6	*chl4.1*	CPPCT005	4.0	11	-5.2
	4	Chl08	2.5	*chl4.1*	CPPCT005	2.8	9	-4.5
	4	Chl09	2.7	*chl4.1*	CPPCT005	7.0	18	-6.1
	4	Chl10	2.5	*chl4.1*	CPPCT005	4.1	17	-3.8
	6	Chl07	2.6	*chl6.1*	BPPCT008	8.0	30	8.7
	6	Chl08	2.5	*chl6.1*	BPPCT008	6.4	36	9.8
	6	Chl09	2.7	*chl6.1*	BPPCT008	4.7	12	9.0
P 2175	1	SPAD07	3.2	*pspad1.1*	CPPCT026	2.3	10	5.9
	1	SPAD08	2.8	*pspad1.1*	CPPCT026	2.3	11	6.9
	4	SPAD08	2.8	*pspad4.1*	AMP110	1.8	6	5.6
	4	SPAD09	2.7	*pspad4.1*	AMP110	2.2	15	3.9
	4	SAPD10	2.8	*pspad4.1*	AMP110	2.7	11	6.0
	8	SPAD08	2.8	*pspad8.1*	CPPCT006	1.8	8	-6.3
	8	SPAD09	2.7	*pspad8.1*	CPPCT006	2.0	13	-7.3
	8	SPAD10	2.8	*pspad8.1*	CPPCT006	2.4	10	-5.6
	1	Chl07	2.5	*pchl1.1*	CPPCT026	2.0	12	6.9
	1	Chl08	2.5	*pchl1.1*	CPPCT026	2.0	11	7.6
	4	Chl09	2.5	*pchl4.1*	AMP110	2.3	17	9.2
	4	Chl10	2.6	*pchl4.1*	AMP110	2.6	11	4.6
	8	Chl09	2.5	*pchl8.1*	CPPCT006	1.8	10	-6.8
	8	Chl10	2.6	*pchl8.1*	CPPCT006	4.0	20	-6.3
	1	VD07	2.4	*pvd1.1*	CPPCT026	2.8	23	-1.4
	1	VD08	2.8	*pvd1.1*	CPPCT026	2.7	17	-1.3
	1	VD09	2.0	*pvd1.1*	CPPCT026	2.0	14	-1.2

[a]Dirlewanger et al., 2004
[b]LOD threshold values for signifcant QTL by 1,000 permutations at $\alpha = 0.05$
[c]Fraction of the phenotypic variance explained by the locus
693 [d]Additive effect. A negative value indicates increase in the tolerance due to the parental and a positive value indicates susceptible
694
695
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Figure 1. A: Gradual iron chlorosis in leaves of both parents of the F_1 population. 'P 2175' (with green leaf color) and 'Felinem' (with red leaf color) leaves are shown after plants were submitted to iron-deficient conditions. The corresponding SPAD values are indicated in a row below the leaves. B: Relationship between chlorophyll content and SPAD values for 'P 2175' and 'Felinem' genotypes.

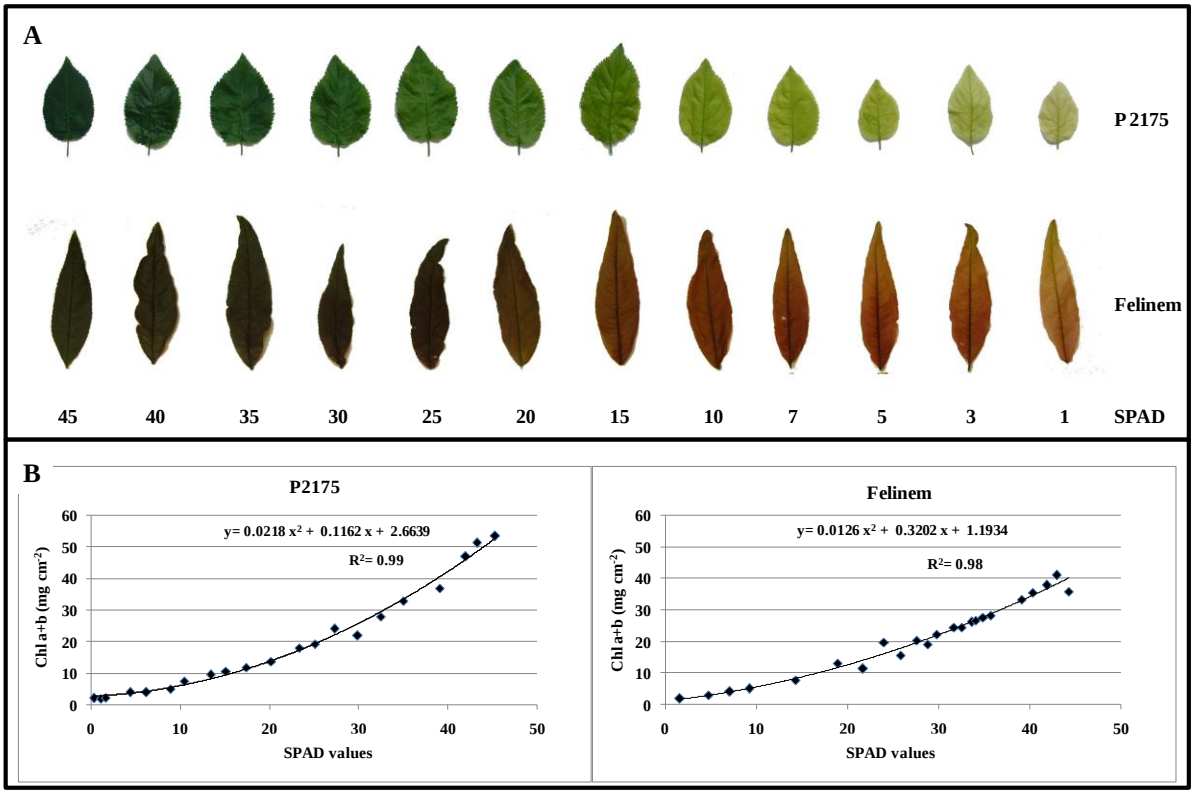


Figure 2. SPAD recovery in a representative sample of eight progeny individuals from the cross ‘P 2175’ x ‘Felinem’ with iron chlorosis symptoms. Trees were sprayed on the leaves with $FeSO_4$. SPAD values were determined before treatment (SPAD 0d) and three (SPAD 3d) and five days (SPAD 5d) after $FeSO_4$ treatment.

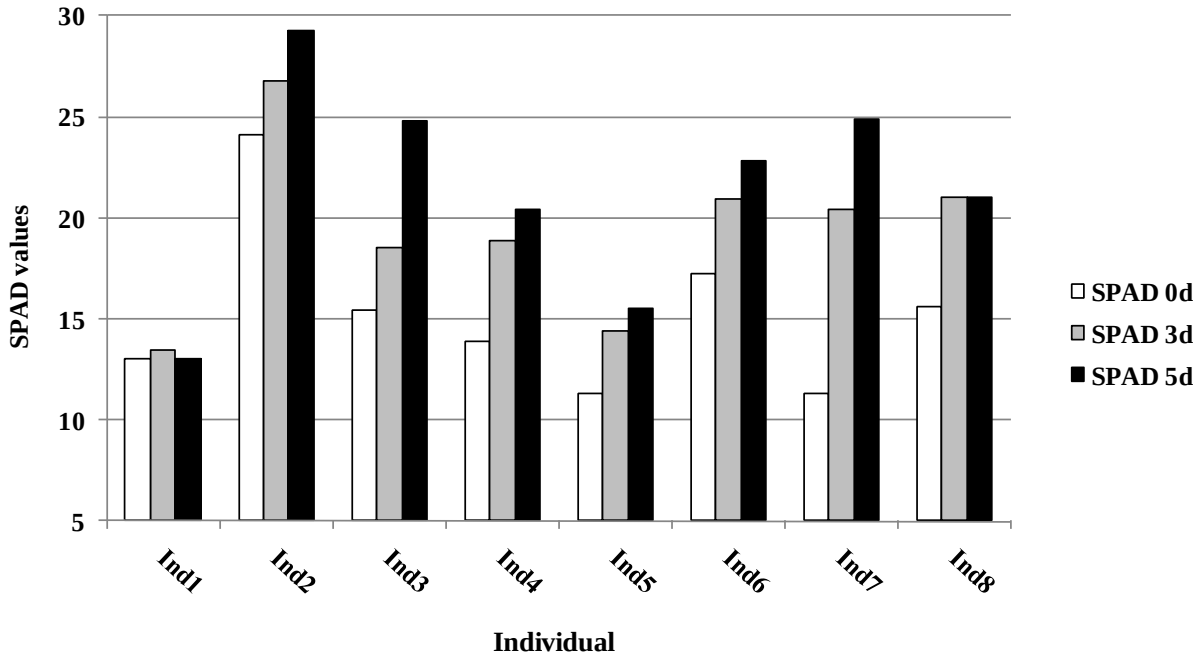


Figure 3: Distribution of the frequencies for SPAD values, chlorophyll concentration, and visual diagnostic of progeny from the cross ‘P 2175’ x ‘Felinem’. Values for both parents are indicated by an arrow (⇩: P 2175; ⇩: Felinem; ⇩: when both parents are in the same interval).

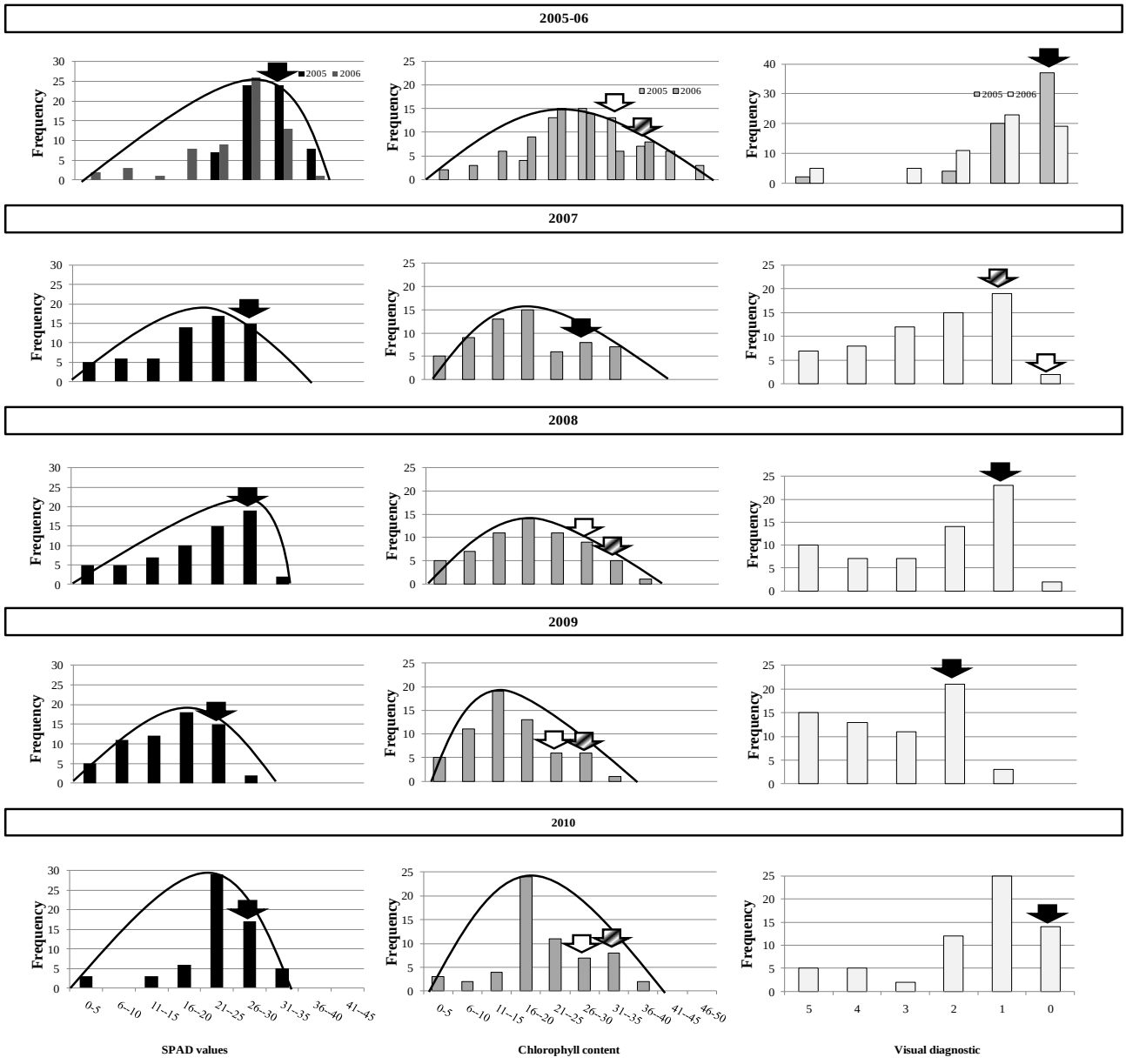
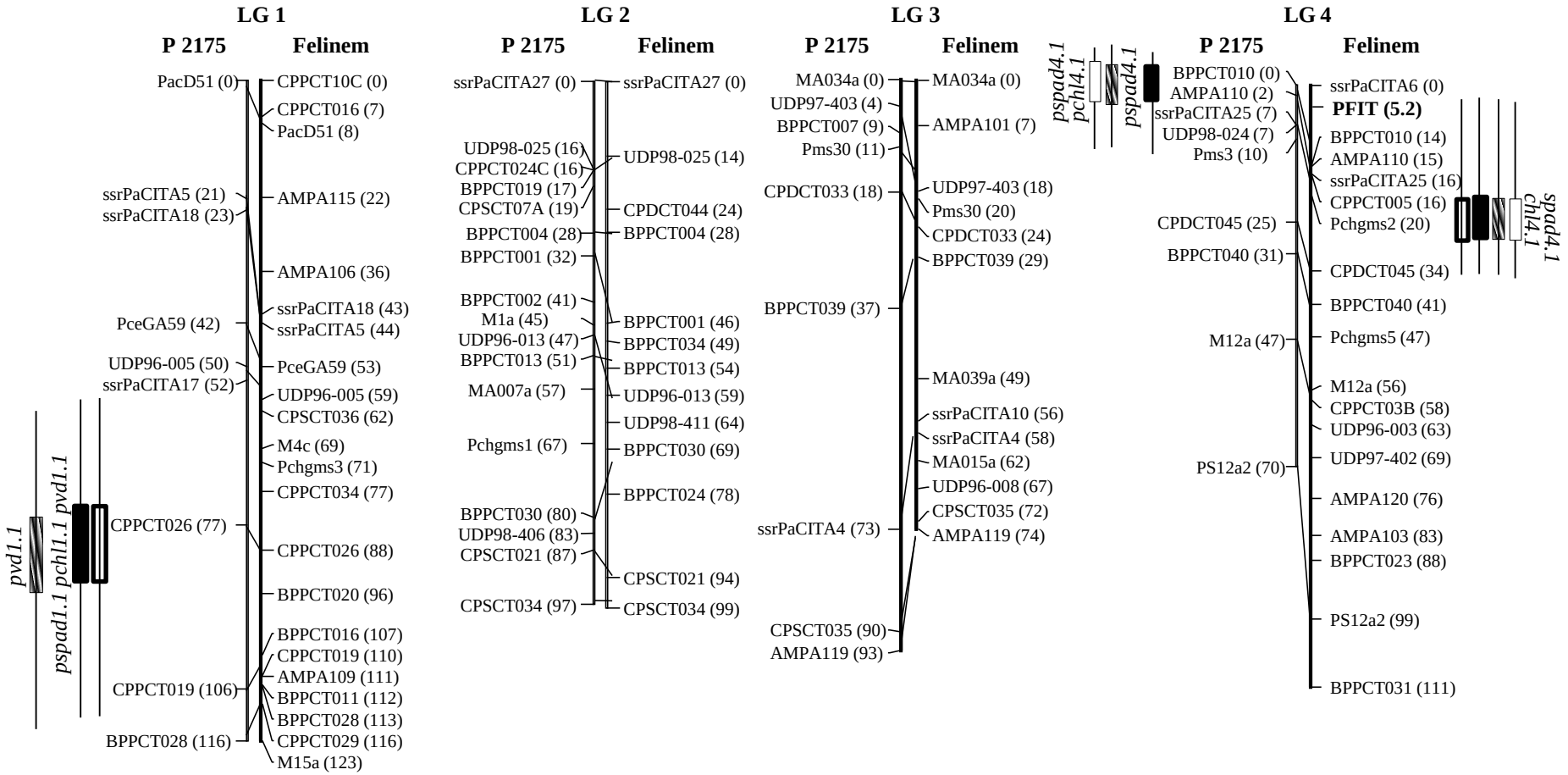
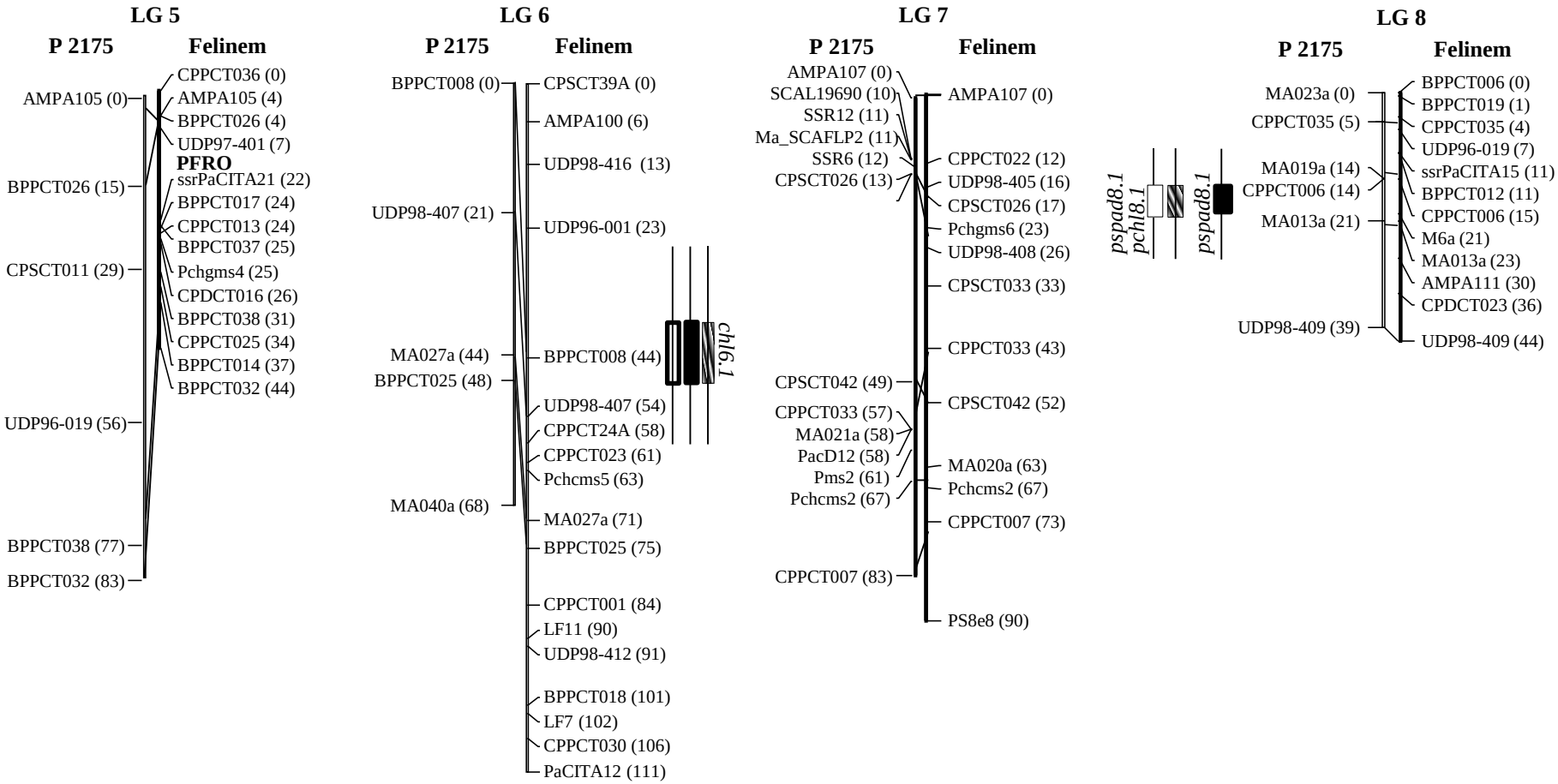


Fig. 4 QTLs associated with iron chlorosis localized in the P 2175 and Felinem maps. Intervals of confidence for each QTL are identified by a *vertical line*. The most significant region, where the most significant marker is localized, is represented by a *rectangular box*, different for each year the QTL has been detected (2007; 2008; 2009; 2010). The candidate genes *PFIT* and *PFRO* were localized in their most probable position in linkage groups 4 and 5, respectively. Marker names can be checked in Dirlewanger et al. (2004a).




LG 5
P 2175
Felinem
AMPA105 (0)
BPPCT026 (15)
CPSCT011 (29)
UDP96-019 (56)
BPPCT038 (77)
BPPCT032 (83)
CPPCT036 (0)
AMPA105 (4)
BPPCT026 (4)
UDP97-401 (7)
PFRO
ssrPaCITA21 (22)
BPPCT017 (24)
CPPCT013 (24)
BPPCT037 (25)
Pchgms4 (25)
CPDCT016 (26)
BPPCT038 (31)
CPPCT025 (34)
BPPCT014 (37)
BPPCT032 (44)
LG 6
P 2175
Felinem
BPPCT008 (0)
UDP98-407 (21)
MA027a (44)
BPPCT025 (48)
MA040a (68)
CPSCT39A (0)
AMPA100 (6)
UDP98-416 (13)
UDP96-001 (23)
BPPCT008 (44)
chl6.1
UDP98-407 (54)
CPPCT24A (58)
CPPCT023 (61)
Pchcms5 (63)
MA027a (71)
BPPCT025 (75)
CPPCT001 (84)
LF11 (90)
UDP98-412 (91)
BPPCT018 (101)
LF7 (102)
CPPCT030 (106)
PaCITA12 (111)
LG 7
P 2175
Felinem
AMPA107 (0)
SCAL19690 (10)
SSR12 (11)
Ma_SCAFLP2 (11)
SSR6 (12)
CPSCT026 (13)
CPSCT042 (49)
CPPCT033 (57)
MA021a (58)
PacD12 (58)
Pms2 (61)
Pchcms2 (67)
CPPCT007 (83)
AMPA107 (0)
CPPCT022 (12)
UDP98-405 (16)
CPSCT026 (17)
Pchgms6 (23)
UDP98-408 (26)
CPSCT033 (33)
CPPCT033 (43)
CPSCT042 (52)
MA020a (63)
Pchcms2 (67)
CPPCT007 (73)
PS8e8 (90)
LG 8
P 2175
Felinem
pspad8.1
pchl8.1
pspad8.1
MA023a (0)
CPPCT035 (5)
MA019a (14)
CPPCT006 (14)
MA013a (21)
UDP98-409 (39)
BPPCT006 (0)
BPPCT019 (1)
CPPCT035 (4)
UDP96-019 (7)
ssrPaCITA15 (11)
BPPCT012 (11)
CPPCT006 (15)
M6a (21)
MA013a (23)
AMPA111 (30)
CPDCT023 (36)
UDP98-409 (44)