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Mapping loci for chlorosis associated with chlorophyll *b* deficiency in potato

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Abstract About 30% of the potato plants from a (*Solanum tuberosum* × *S. berthaultii*) × *S. tuberosum* backcross population had chlorotic, malformed leaves; but a gradation in symptom severity suggested regulation by more than one gene. The study was undertaken to determine whether this was the case, whether any genes previously reported to control chlorosis in potato were involved, and to see how symptoms were related to effects on chlorophyll content. Testing for quantitative trait loci indicated major control by a single recessive gene on chromosome 1, close to one or more loci that have been reported to produce chlorosis in tomato, but distinct from similar genes previously identified in potato. The proposed symbol for the potato gene that confers phenotype with chlorotic and malformed leaves is *cml* (chlorotic and malformed leaves). The effects of this gene appeared to be accentuated by a second gene, located on chromosome 12. Chlorotic plants showed a 50% decrease in chlorophyll *b* level in the affected parts of leaves. It is concluded that *cml* is different from previously reported genes for chlorosis in potato, that at least one other gene modifies the intensity of symptom expression, and that the observed chlorosis is produced through effects on chlorophyll *b* level.

Keywords Chlorophyll · Chlorosis · Malformed leaves · Potato · QTL mapping

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Abbreviations

allele B	An allele, originating from *S. berthaultii*, on the linkage map for segregation from the hybrid parent
allele T^R	An allele, originating from the recurrent *S. tuberosum*, on the linkage map for segregation from the recurrent parent
CIM	Composite interval mapping
DW	Dry weight
LRS	Likelihood ratio statistics
SIM	Simple interval mapping

Introduction

Cultivated potato (*Solanum tuberosum*) is a vegetatively propagated autotetraploid species ($2n = 4x = 48$) with complex polysomic inheritance. It has been

pointed out that autotetraploidy associated with vegetative propagation enables potato cultivars to maintain a high level of heterozygosity and to store a large number of deleterious recessive mutant genes (Hermsen et al. 1978). Included among such recessive genes are chlorotic mutants, several of which have been described in potato.

In a (*Solanum tuberosum* × *S. berthaultii*) × *S. tuberosum* backcross population that we have used to map a variety of quantitative traits, some genotypes showed reduced vigor, associated with malformed, chlorotic leaves; but there was a wide range in the severity of symptoms. The gradation in symptoms from severe to mild to undetectable suggested polygenic inheritance, and we therefore examined the population for quantitative trait loci (QTLs) associated with the trait. The objectives of our study were (1) to map QTLs for chlorosis in the backcross mapping population, (2) to determine if the chlorosis is controlled by a major gene with modifying genes, or several minor-effect genes, and (3) to find out if observed chlorosis was associated with changes in chlorophyll *a* or *b* levels.

Materials and methods

The population studied was created by backcrossing a haploid *Solanum tuberosum* (HH1-9) to a hybrid of haploid *S. tuberosum* (USW2230) × diploid *S. berthaultii* (PI 473331), where the hybrid clone was the female. Bonierbale et al. (1994) performed RFLP linkage mapping on this population, and it is the same population that was used to find QTLs for tuberization (Van den Berg et al. 1996b; Ewing et al. 2004), dormancy (Van den Berg et al. 1996a), polyamines in leaves (Davies et al. 1999), levels of abscisic acid in tubers (Simko et al. 1997), sugars in phloem sap (Simko et al. 1999), and resistance to *Phytophthora infestans* (Ewing et al. 2000; Simko 2002; Rauscher et al. 2006) and *Verticillium albo-atrum* (Simko et al. 2004a). The genotypes in the population were maintained as in vitro plants.

Because of the heterozygous nature of the parental clones, segregating alleles from both parents contributed to the genetic variation of progenies in this population. Two molecular linkage maps were constructed by Bonierbale et al. (1994)—one based on segregation from the hybrid parent (*S. berthaultii* alleles, B) and the other based on segregation from the recurrent parent (*S. tuberosum* alleles, T^R). In the framework map that is based on segregation from the hybrid parent there are 81 loci at average intervals of 10 cM. There are 35 markers in the map based on recombination from the recurrent parent, and they are not as uniformly distributed as are the 81 markers; chromosomes 1, 7, and 12 have only one marker each. The integrated molecular linkage map that combines the two framework maps was produced using the JoinMap version 3.0 (Stam 1993) computer software.

Plants of 158 genotypes, along with the original parents, the hybrid used in constructing the backcross population, and siblings of the hybrid were grown in a plastic greenhouse under ambient summer temperatures at Cornell University. The light intensity in the greenhouse varied from 250 μmol m^{-2} s^{-1} on a cloudy day to 1,500 μmol m^{-2} s^{-1} on a sunny day. Temperatures during the day varied between 20°C and 35°C; night temperatures varied from 15°C to 20°C. The experiment was repeated over 2 years, with five plants per genotype the first year and four plants the second.

Plants were rated for chlorosis and leaf malformation approximately 7 weeks after transplanting. There was a close association between the two traits, and a single rating was made to represent both. The rating scale was from 0 to 2: 0 indicated no symptoms (Fig. 1a); 1, mild to moderate chlorosis and malformation (Fig. 1b); and 2, severe chlorosis and malformation (Fig. 1c). The second year, ratings were made twice, a week apart. Data for the five plants were averaged for the first year, and data for the four plants (two ratings per plant) were averaged for the second year. Thus for each of the 158 genotypes there were two mean values, one for each year. The mean values for the 2 years were averaged, and the resulting means were used for QTL analysis.

Chlorophyll was extracted from whole expanded leaves of similar age and size in 80% acetone solution. Concentrations of chlorophyll *a* and *b* were calculated according to the formula of Wellburn (1994).

Statistical analyses of the linkage between RFLP markers and chlorosis rating were performed by MapManager QTX software (Manly et al. 2001). The software generates likelihood ratio statistics (LRS) as a measure of significance of possible QTLs (Haley and Knott 1992). The LRS has been converted to the conventional base-10 LOD score by dividing it by 4.61.

Fig. 1 Comparison of the normal phenotype (**a**) with mildly (**b**) and severely (**c**) affected chlorotic phenotypes. The three leaves (**d**) were taken from the three plants shown in a–c, respectively, left to right. Although not visible in the photograph, the center leaf was faintly mosaic

For main effects, a threshold value of LOD = 2.70 was set for declaring a marker significant in a QTL model. The appropriate threshold value was determined from 10,000 random permutations that take into account the trait distribution in the mapping population (Churchill and Doerge 1994). Simple interval mapping (SIM) was used to find a significant marker-trait association. SIM was followed by composite interval mapping (CIM), which can locate a QTL while controlling for the effect of other QTLs (Jansen 1993; Zeng 1993). Interactions between pairs of loci were tested in a two-stage test as suggested in the MapManager QTX manual. First, the total effect of the two loci must have a *P*-value less than 10^{-5}. Second, the interaction effect itself must have a LOD score more than 6.40 (estimated from 1,000 random permutations). This two-stage test is used because the interaction significance effect by itself cannot be reliably tested if there is the possibility of strong main effects (MapManager QTX manual) (Manly et al. 2001).

Results

Occurrence of the defect

No chlorosis was detected in either parent used in the original cross, in any of the 20 hybrids resulting from that cross, or in the haploid *S. tuberosum* used for the backcross. Fifty of the 158 genotypes in the backcross population showed at least some degree of chlorosis and malformation. The intensity of chlorosis was highly variable among genotypes (Fig. 1); and the milder forms (Fig. 1b) were affected with mosaic too faint to be distinguished in our photographs, although malformed leaflets are evident (Fig. 1d, center). We have grown the plants in subsequent years and found that those originally classified as chlorotic continued to exhibit symptoms consistent with the original classifications (data not shown). Representative affected plants gave negative ELISA tests for the viruses PLRV, PVY, PVX, PVA, and PVM (data not shown).

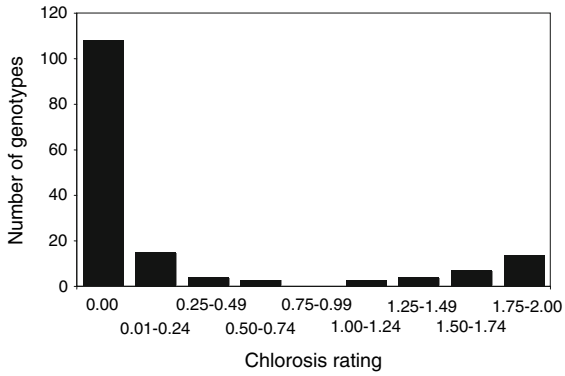

Fig. 2 Frequency distribution of the chlorosis rating (0, no symptoms; 1, mild to moderate chlorosis and malformation; and 2, severe chlorosis and malformation) on 158 genotypes from the backcross mapping population

The frequency distribution of mean ratings for the chlorotic defect was bimodal, weighted toward what would be expected from a monogenic trait, but with a significant number of intermediate values (Fig. 2). This would be consistent with segregation of one gene with a major effect, accompanied by the segregation of one or more genes with lesser effects.

Major effect QTLs

For main effects, linkage analysis with B alleles segregating from the hybrid parent yielded a highly significant QTL on chromosome 1 (SIM LOD = 6.43, CIM LOD = 8.72), between the markers *TG70* and *TG71* (Fig. 3). Through linkage analyses with T^R alleles segregating from the recurrent parent we also found one main effect QTL. It was linked to marker *TG116* (SIM LOD = 3.13, CIM LOD = 4.90), the only marker on chromosome 1 that had segregating T^R alleles.

In view of the proximity of *TG116* to *TG71* (11.4 cM, Fig. 3), it is likely that the analyses with segregating T^R alleles and segregating B alleles tagged the same QTL. To examine this possibility, we tested for interaction between the two apparent QTLs. The interaction between markers *TG71* and *TG116* was highly significant (LOD = 7.67). The total association (main effect plus interaction) of the two loci with chlorosis is equivalent to an LOD of 19.72. Chlorosis was absent or at a very low level on most of the genotypes when the B allele from *TG71* or the T^R allele from *TG116* was present (mean values were

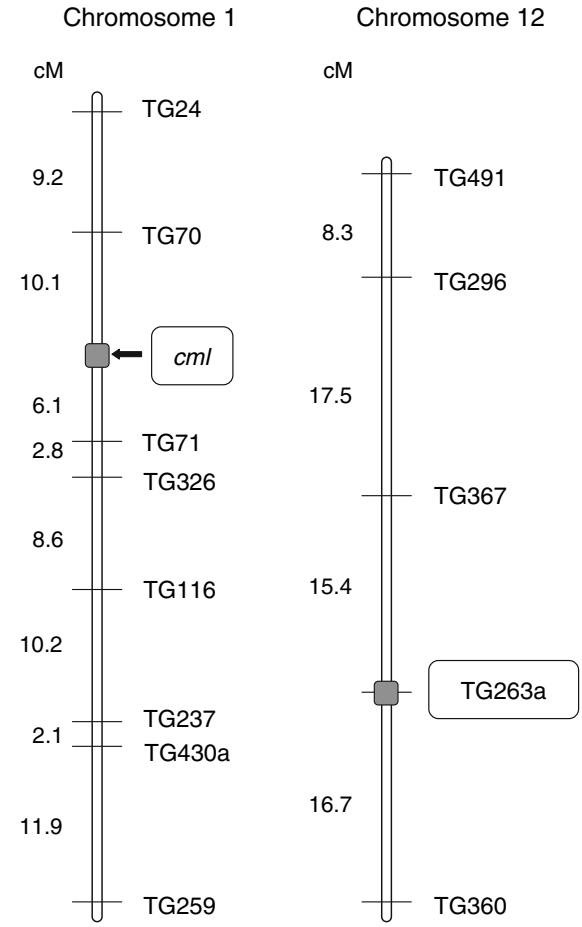

Fig. 3 Map showing the position of the chlorosis *cml* locus on chromosome 1, and the chlorosis-accentuating locus on chromosome 12. The integrated molecular linkage map combines two framework maps based upon the segregation of 81 B alleles and 35 T^R alleles, respectively. Distances on the left are in centiMorgans (cM)

from 0.06 to 0.25) (Table 1). However, with both these alleles absent, severe chlorosis was observed on most of the genotypes (mean value of 1.24), indicating that the chlorosis allele is recessive.

Minor effect QTL

When the markers from chromosome 1 that were significantly associated with chlorosis were used as 'background' loci in the CIM approach, a new minor effect QTL (CIM LOD = 3.00) appeared on chromosome 12 linked to the marker locus *TG263a*. This QTL was not detected by the SIM approach (SIM LOD = 0.65), indicating that its effect was masked by the strong effect of the major QTL on chromosome 1.

Table 1 Intralocus interaction between a B allele at *TG71* and a T^R allele at *TG116* on chromosome 1

Allele status		Number of genotypes[a]	Qualitative rating[b]		Quantitative rating
B allele at *TG71*	T^R allele at *TG116*		No chlorosis or very mild chlorosis (mean value <1)	Intense chlorosis (mean value ≥1)	Average chlorosis rating score ± s.e.
+	+	41	41	0	0.06 ± 0.02
+	–	41	39	2	0.09 ± 0.06
–	+	36	31	5	0.25 ± 0.10
–	–	25	8	17	1.24 ± 0.16

[a] Only genotypes analyzed with both molecular markers are shown in the table, thus reducing the number from 158 to 143

[b] The Fisher's exact test of independence of the two markers and the chlorosis was significant at $P = 3 \times 10^{-13}$

As in the case of the QTL at *TG71*, chlorosis is associated with the absence of a B allele at *TG263a*. No other significant QTL was detected through main effect or interaction tests.

Relationship of chlorosis to chlorophyll content

To determine whether the chlorotic symptoms were related to differences in chlorophyll content we analyzed the amount of chlorophyll *a* and *b* in 142 genotypes. Content of chlorophyll *a* ranged from 5.7 to 11.6 (mg g^{-1} DW) with a mean value of 7.4 (mg g^{-1} DW), while chlorophyll *b* ranged from 1.9 to 4.2 (mg g^{-1} DW) with a mean value of 2.6 (mg g^{-1} DW). The data from individual genotypes were used in QTL mapping as indicated under Materials and methods. No significant QTL was detected for chlorophyll *a* or *b* content, or for the ratio of the two.

We also compared the chlorophyll content of chlorotic areas of the leaves with nearby areas that showed no symptoms and with similar areas on leaves from non-chlorotic genotypes. Tissues were not significantly different in chlorophyll *a* content, but there was a 50% reduction ($P = 5 \times 10^{-5}$) in the chlorophyll *b* content excised from lighter green/yellowish blotches when compared to the green parts of the chlorotic leaves or to the asymptomatic leaves (Table 2).

Table 2 Content of chlorophyll *a* and *b* in chlorotic and non-chlorotic plants

Analyzed tissue	Chlorophyll *a* (mg g^{-1} DW) ± s.e.	Chlorophyll *b* (mg g^{-1} DW) ± s.e.
Non-chlorotic leaves	7.41 ± 0.08	2.61 ± 0.04
Chlorotic leaves		
Green areas	7.41 ± 0.09	2.56 ± 0.19
Yellowish areas	7.35 ± 0.13	1.29 ± 0.17

Discussion

The chlorosis rating for plants that lacked both a B allele linked to *TG71* and a T^R allele linked to *TG116* was on average 1 point higher than the rating of other plants in the population (Table 1). Chlorosis was not detected in the parents of the hybrid, the backcross parent, in the hybrid used to make the backcross, or in siblings of the hybrid used to make the backcross. A likely explanation is that a recessive allele from USW2230, the *S. tuberosum* parent used to make the hybrid, was the source of the allele for chlorosis linked to *TG71*. Since USW2230, which was derived from the tetraploid cultivar Saco (Akeley et al. 1955), did not show severe chlorotic symptoms, we infer that USW2230 was heterozygous for the chlorotic gene, and that it contributed the recessive allele to the hybrid. Likewise, under this assumption the backcross parent (HH1-9) was heterozygous for the chlorotic gene.

The proposed gene symbol *(cml)* for this mutation stands for chlorotic and malformed leaves. It would then follow that plants homozygous for the recessive *cml* allele showed the chlorotic symptoms.

The low ratings of eight genotypes (Table 1) that lacked both a B allele at *TG71* and a T^R allele at *TG116* can likely be explained by crossovers, since the *cml* locus does not appear to be exactly at either of these markers. Because the locus is closer to *TG71*

(6.1 cM) than to *TG116* (17.5 cM), we can expect the apparent effect of a B allele at *TG71* to be slightly greater in reducing chlorosis than that of a T^R allele at *TG116*. The data in Table 1 tend in this direction, although the difference is small in comparison to the experimental variation.

Eleven of the 12 genotypes with the most severe chlorosis (scores 1.85–2.00, see Fig. 2) had both B absent at *TG71* and T^R absent at *TG116*. (The single exception lacked a T^R allele at *TG116*, but had a B allele at *TG71*; however, a B allele was absent at *TG70*, so a crossover may have occurred between *TG71* and *cml*—see Fig. 3). All 12 of these genotypes also had a B allele absent from *TG263a*.The absence of a B allele at *TG263a* by itself did not have a detectable effect on chlorosis; genotypes having a B allele absent showed about the same level of chlorosis as genotypes with a B allele present (0.09 and 0.07, respectively) in the presence of both a B allele at *TG71* and a T^R allele at *TG116*. However, the absence of a B allele at *TG263a* intensified chlorosis (1.30 when absent versus 1.05 when present) when both a B allele at *TG71* and a T^R allele at *TG116* were absent.

Relationship of chlorosis to chlorophyll content

Since the chlorotic blotches comprised only a small portion (<10%) of the whole leaf, we assume that the 50% reduction in chlorophyll *b* content of blotches was not reflected in the whole leaf analysis because it was masked by genotype-to-genotype and other variation. For example, if 10% of the leaf area on a chlorotic plant was affected by chlorosis, and if the affected area had a 50% decrease in chlorophyll *b* content, the whole leaf analysis would show only a 5% decrease in chlorophyll *b* content—a difference probably too small to be detected by the methods used. The mosaic pattern observed in the chlorotic leaves suggests that the gene causing this effect acts during leaf development, affecting some, but not all parts.

Other genes for chlorosis

At least four other chlorosis mutations have been reported in potato: *virescens*, *yellow margin*, *green*, and *light green*. Inheritance studies indicate that all of these mutations are conferred by single recessive genes. *Virescens* (*v*) is characterized by its light green coloration and retardation in early growth (Hermsen et al. 1978). Hermsen (1978) found a linkage between *virescens* and an *S*-bearing translocation that controls the incompatibility reaction in potato. This *S*-locus is not linked with the incompatibility *S*-locus that is located on chromosome 1 (Hermsen 1978; Eijlander et al. 2000). By means of trisomic analysis both the *S*-translocation and the *v* locus that confers *virescens* chlorophyll deficiency mutation were mapped to chromosome 12 (Hermsen et al. 1973). Unfortunately, the exact position of the *v* gene on the molecular map is not known, and therefore we cannot compare its location to the minor effect QTL found at *TG263a*. Another light green mutation, similar to *virescens*, was described by De Jong et al. (1998) and named *light green (lg)*. The *light green* is closely linked with a gene that is lethal in the homozygous recessive state. The *lg* locus is located on chromosome 6, between RFLP markers *CP18* and *GP24* (De Jong et al. 1998) and therefore different both from the major and minor effect loci found in our backcross population.

The *yellow margin (ym)* mutation is characterized by small roundish leaflets with yellow or reddish margins (Simmonds 1965; Hermsen et al. 1978). Though the *ym* gene was originally mapped on chromosome 12 by means of trisomic analysis (Wagenvoort 1982), later mapping with molecular markers put the gene on chromosome 5 (Jacobs et al. 1995). Neither a gene location on chromosome 5 nor the description of the *ym* phenotype match the chlorosis observed in the present work.

Yet another chlorophyll mutation that is controlled by a single recessive gene was described by Jones et al. (1963) and named *green (g)*. Plants that are homozygous recessive at this locus have reduced chlorophyll levels and affected iron metabolism. The *g* locus is closely linked to a locus controlling production or expression of anthocyanin pigment in potato (Plaisted and Peterson 1967). Since none of the loci known to control anthocyanin are present on chromosomes 1 or 12 (Van Eck et al. 1993; Van Eck et al. 1994; Jacobs et al. 1995; Jung et al. 2005; Simko, unpublished) the *g* gene appears to be different from the *cml* gene or the QTL that accentuated chlorosis in our studies.

Because the molecular maps of potato and tomato are highly colinear (Bonierbale et al. 1988; Tanksley

et al. 1992), we compared the locations of the potato chlorosis genes to those residing on tomato chromosomes 1 and 12. Rick (1980) lists at least seven genes on chromosome 12 that are chlorosis related: *albescent (alb)*—strong white or light green vegetation; *auroid (aud)*—uniform yellow foliage; *flecked dwarf (fd)*—retarded plant with light green flecked leaves; *multifurcata (mua)*—dull green interveinal chlorosis and multibranched first inflorescense; and three genes for *yellow-green (yg-2, yg-3, yg-4)*—foliage uniformly yellow-green. Moreover, the *Cab-5* gene for the chlorophyll *a/b* binding peptide is also located on this chromosome (Pichersky et al. 1987; Tanksley et al. 1992).

Chromosome 1 harbors another eight genes that might be chlorosis related: *aurea (au)*—bright yellow foliage, *dilatata (dt)*—yellowish leaves with darker veins; *flavescens (fla)*—light green leaves with only a few segments; *imbecilla (imb)*—weak plant that has yellowish leaves with a few branches; *indiga (ind)*—small plant with dainty, gray-green leaves; *jaundiced (jau)*—plant with retarded growth and dull yellow green foliage; *sufflaminata (sfa)*—smaller chlorotic plant with concave pinnae; and *viroid (vrd)*—plant with very distorted leaves that show white-speckled chlorosis (Rick 1980). Van Tuinen et al. (1996) showed that the chlorophyll deficiency observed in *aurea* mutants is due to a disturbance in the biosynthesis of phytochrome chromophore. Levels of both chlorophyll *a* and *b* are reduced in these tomato mutants (Koornneef et al. 1985), whereas for our potato mutant we detected a reduction only in the level of chlorophyll *b*.

Of the symptoms listed above, those described for *viroid* bear close resemblance to what we observed in the most severely affected plants of our population. Expression of both *vrd* and *cml* appear to be environmentally sensitive. In our mapping population, several genotypes showed mild or even medium chlorotic symptoms in some replications but no chlorosis in others. Similarly the *viroid* mutation is reported to be environmentally sensitive (Rick and Zobel 1969). Comparison of the potato and tomato (Bonierbale et al. 1988; Tanksley et al. 1992) linkage maps indicates that *cml* is located in a similar chromosomal area to that of *vrd*. All this suggests that *cml* might be orthologous to the tomato *vrd* gene, although much stronger evidence would be needed to establish such a connection.

In addition to the QTL we observed at *TG263a* it is of course possible that still other genes in our population affected the severity with which *cml* is expressed. For example, genes for chlorosis orthologous to the ones known to occur on tomato chromosome 1 would have been difficult to detect in our analysis, especially if close to *TG71* or if segregating from the recurrent parent. Finer mapping or cloning would be needed to resolve such a question.

Occurrence of *cml* in breeding populations

The recessive *cml* allele that is associated with the chlorotic mutation in the homozygous state may be common in potato germplasm, at least in North America. The HH1-9 recurrent parent, one of the *cml*-allele donors in our mapping population, was selected from an inter-mated population of 800 *S. tuberosum* haploids originating from several tetraploid cultivars (Sanford and Hanneman 1982). The other *cml*-allele donor is USW2230, a dihaploid derived from the tetraploid cultivar Saco (Bonierbale et al. 1994). Cultivar Saco originated from a cross between two USDA breeding lines (USDA X96-56 and USDA 41956) (Akeley et al. 1955), one of which was likely a carrier of the *cml* gene. Both parents of Saco were frequently used in the potato-breeding programs leading to development of several cultivars grown in the USA and Canada. In our previous study (Simko et al. 2004b) we found that line USDA X96-56 is present in pedigrees of at least 25 cultivars including Kennebec, Superior, and Early Gem, and a large number of breeding lines. The line USDA 41956 was less frequently used in crosses, but still could be traced in pedigrees of at least three cultivars and several breeding lines (Simko et al. 2004b).

Concluding remarks

It is difficult to assess the practical implications of the presence of *cml* in potato breeding populations, except that offspring homozygous recessive for the trait are clearly defective. We do not know whether *cml* has harmful effects when heterozygous, especially in tetraploid populations. There is evidence that the recessive mutant *ym* even bestows a beneficial effect in the heterozygous condition (Dodds and Paxman 1962); conceivably, the same could be true for one or more other chlorotic mutants. Knowledge

about the chromosome locations for *cml* and other chlorotic genes should facilitate investigation of such questions.

Chlorotic mutants such as *cml* and the modifying locus on chromosome 12 could prove to be useful tools in the study of chlorophyll formation. For example, it would be interesting to know why *cml* affects only levels of chlorophyll *b*, whereas levels of both chlorophyll *a* and *b* are reduced in *aurea* tomato mutants (Koornneef et al. 1985).

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