



(86) Date de dépôt PCT/PCT Filing Date: 2001/07/17

(87) Date publication PCT/PCT Publication Date: 2002/02/14

(45) Date de délivrance/Issue Date: 2010/10/12

(85) Entrée phase nationale/National Entry: 2003/01/17

(86) N° demande PCT/PCT Application No.: CA 2001/001028

(87) N° publication PCT/PCT Publication No.: 2002/012551

(30) Priorité/Priority: 2000/07/17 (US60/218,765)

(51) Cl.Int./Int.Cl. *C12Q 1/68* (2006.01)

(72) Inventeurs/Inventors:

CASTONGUAY, YVES, CA;
O'DONOUGHUE, LOUISE S., CA;
LANDRY, BENOIT S., CA;
LABERGE, SERGE, CA;
MONROY, ANTONIO F., CA;
VEZINA, LOUIS P., CA

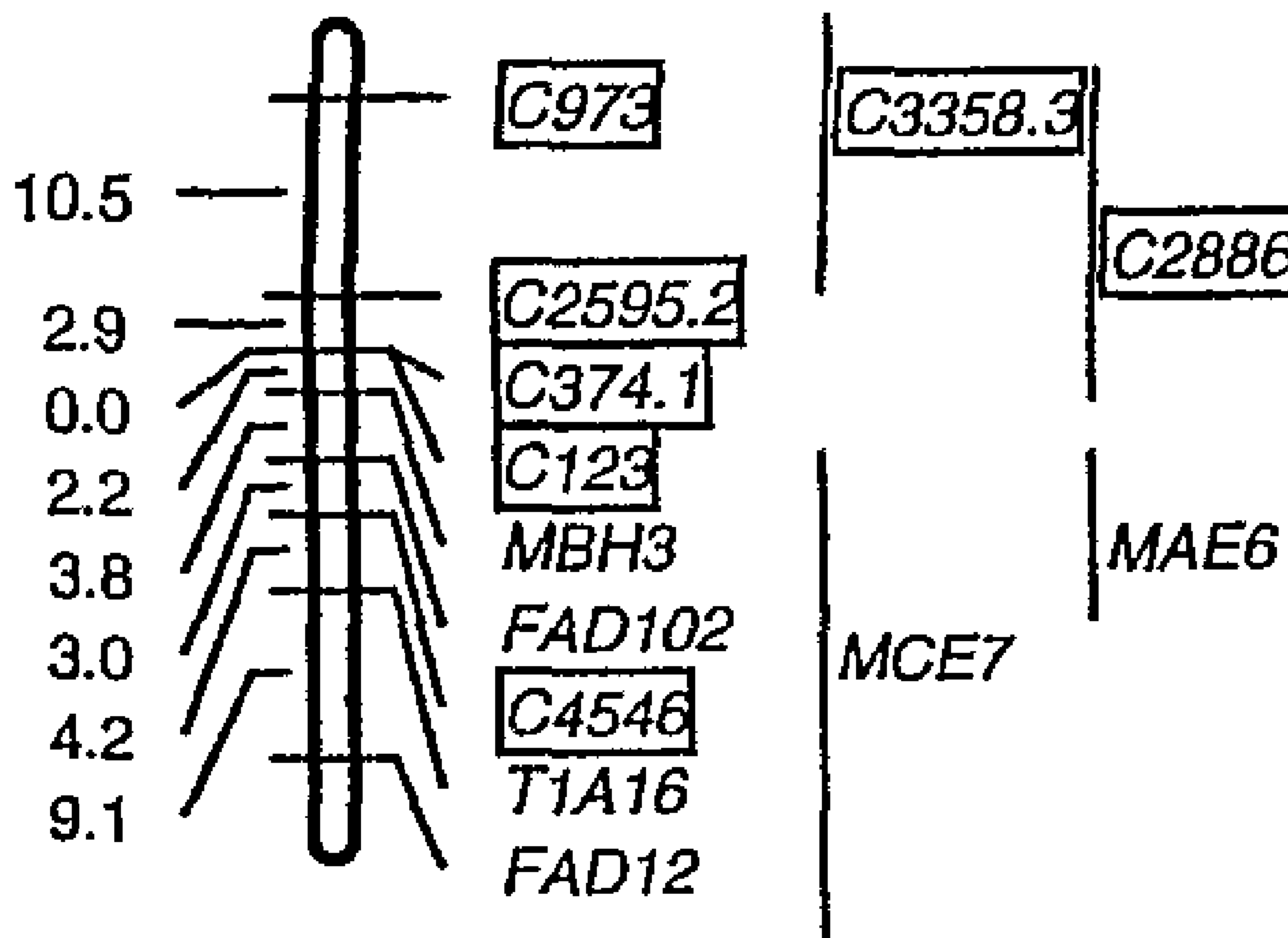
(73) Propriétaires/Owners:

HER MAJESTY THE QUEEN IN RIGHT OF CANADA AS
REPRESENTED BY THE MINISTER OF
AGRICULTURE AND AGRI-FOOD, CA;
DNA LANDMARKS INC., CA

(74) Agent: GOWLING LAFLEUR HENDERSON LLP

(54) Titre : PROCEDE CARTOGRAPHIQUE D'EXTRACTION GENOMIQUE POUR L'IDENTIFICATION DE SITES DE
REGULATION DU NIVEAU DE TRANSCRITS ET PRODUITS GENETIQUES

(54) Title: MAP-BASED GENOME MINING METHOD FOR IDENTIFYING REGULATORY LOCI CONTROLLING THE
LEVEL OF GENE TRANSCRIPTS AND PRODUCTS



(57) Abrégé/Abstract:

The invention pertains to a method for identifying one or more regions within a genome of an organism of interest that mediate the expression of one or more genes of interest. The method comprises identifying a first and a second organism of interest, the first



(57) Abrégé(suite)/Abstract(continued):

organism of interest is characterized by exhibiting a measurable response to an environmental stimulus, or otherwise exhibiting a phenotype associated with differential gene expression associated with a process of interest. The second organism of interest is characterized by lacking or not exhibit as strong a response to the stimulus as that observed within the first organism of interest, or it exhibits a different phenotype compared with that of the first organism of interest, wherein the different phenotype is associated with the process of interest, or it exhibits a phenotype of interest that segregates when compared with the first organism of interest, or a combination thereof. The first and second organisms of interest are crossed to produce a population of segregated progeny and RNA is extracted from each of the segregated progeny. The level of gene expression for one or more genes of interest that are associated with the response to an environmental stimulus, or process of interest is quantified.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
14 February 2002 (14.02.2002)

PCT

(10) International Publication Number
WO 02/012551 A3

(51) International Patent Classification⁷: **C12Q 1/68**

(21) International Application Number: PCT/CA01/01028

(22) International Filing Date: 17 July 2001 (17.07.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/218,765 17 July 2000 (17.07.2000) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/218,765 (CIP)
Filed on 17 July 2000 (17.07.2000)

(71) Applicants (for all designated States except US): **HER MAJESTY THE QUEEN IN RIGHT OF CANADA** as represented by **THE MINISTER OF AGRICULTURE AND AGRI-FOOD** [CA/CA]; Soils and Crops Research and Development Centre, 2560 Hochelaga Blvd., Sainte-Foy, Quebec G1V 2J3 (CA). **DNA LANDMARKS INC.** [CA/CA]; P.O. Box 6, St.-Jean-sur-Richelieu, Quebec J3B 6Z1 (CA).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **CASTONGUAY, Yves** [CA/CA]; 3828 Alfred Bessette, Sainte-Foy, Quebec G1X 4T4 (CA). **O'DONOUGHUE, Louise, S.** [CA/CA]; 524 Dollard des Ormeaux, Mont St.-Hilaire G3H 2S5 (CA). **LANDRY, Benoit, S.** [CA/CA]; 134 Allée des Cigales, L'Acadie, Quebec J2Y 1B3 (CA). **LABERGE, Serge** [CA/CA]; 1421 avenue Oak, Sillery, Quebec G1T

1Z5 (CA). **MONROY, Antonio, F.** [BO/CA]; 6372 Trans Island, Montréal, Quebec H3W 3B6 (CA). **VEZINA, Louis, P.** [CA/CA]; 206 Route 138, Neuville, Quebec G0A 2R0 (CA).

(74) Agents: **SECHLEY, Konrad, A. et al.**; Gowling Lafleur Henderson LLP, Suite 2600, 160 Elgin Street, Ottawa, Ontario K1P 1C3 (CA).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CI, CG, CL, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

with international search report

(88) Date of publication of the international search report:
2 October 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/012551 A3

(54) Title: MAP-BASED GENOME MINING METHOD FOR IDENTIFYING GENE REGULATORY LOCI

(57) Abstract: The invention pertains to a method for identifying one or more regions within a genome of an organism of interest that mediate the expression of one or more genes of interest. The method comprises identifying a first and a second organism of interest, the first organism of interest is characterized by exhibiting a measurable response to an environmental stimulus, or otherwise exhibiting a phenotype associated with differential gene expression associated with a process of interest. The second organism of interest is characterized by lacking or not exhibit as strong a response to the stimulus as that observed within the first organism of interest, or it exhibits a different phenotype compared with that of the first organism of interest, wherein the different phenotype is associated with the process of interest, or it exhibits a phenotype of interest that segregates when compared with the first organism of interest, or a combination thereof. The first and second organisms of interest are crossed to produce a population of segregated progeny and RNA is extracted from each of the segregated progeny. The level of gene expression for one or more genes of interest that are associated with the response to an environmental stimulus, or process of interest is quantified.

**MAP-BASED GENOME MINING METHOD FOR IDENTIFYING REGULATORY LOCI
CONTROLLING THE LEVEL OF GENE TRANSCRIPTS AND PRODUCTS**

The present invention relates to a method for the identification of regulatory
5 loci that control gene expression within any organism. More specifically, the present
invention relates to the use of mRNA transcripts, and variation in transcript levels for
the identification of quantitative trait loci pertaining to a desired trait within an
organism.

10 BACKGROUND OF THE INVENTION

The selection of desired plant traits within plant breeding programs is typically
based upon selection of one or more phenotypic traits. However, many important
agronomic traits are complex, dramatically influenced by the environment, and are
15 under polygenic control where a phenotypic trait is regulated by a plurality of genes,
rather than by a single locus control. In traits under polygenic, or multigene control,
the expression of alleles at many loci may contribute to the phenotype of interest.

Repeatedly, it has been shown that many genes fail to be expressed in
20 response to developmental or environmental cues. An understanding of the factors
that control the expression of these genes is important especially within transgenic
organisms, for example, upon the introduction of a foreign gene to control its
transcript level in a developmental, tissue or stress dependant manner. Such studies
reveal the complexity and multi-level redundancy of controls that exist in the
25 expression of genes. Mechanisms of control of gene expression can vary
considerably between genes (see for example Hirt. H. 1999 Trends Plant Sci. 4: 7-8).
Under a given set of environmental or developmental conditions, genes involved in a
given process do not always respond in a similar manner, nor do they accumulate in
the same cell types or tissues indicating that they respond to different control or signal
30 mechanisms.

Using current techniques, it is difficult to sort out key regulatory genes that
control the expression of genes of interest among the whole cascade of events.

Differentially expressed genes have been identified using approaches such as differential screening of cDNA libraries, genome sequencing combined with homology searches in gene banks, gene knock-out and complementation, mutation of homeotic genes, high throughput screening of ESTs using high density arrays of genes. These approaches, even though laborious, have occasionally been useful to identify genes involved in the regulation of gene expression but are not specifically designed for that purpose. Gene knock-out approaches (mutant variation) for the identification of factors controlling gene expression are suitable to small genome species such as *Arabidopsis*. Such techniques are also laborious, unspecific and often lead to undetectable or lethal phenotypes. Combination of techniques such as differential display of mRNA species, subtractive or normalized libraries and gene array can be used to single out regulatory genes, however these techniques are not specific to the analysis of the expression of a single gene and are time consuming. Moreover, with these techniques the transcripts of the regulatory genes of interest must be present in samples used to generate the libraries in order for the transcripts to be identified. The task can be overwhelming when multiple regulatory genes, with unsynchronised expression are involved.

A quantitative trait locus (QTL) is a region of the genome that codes for one or more proteins and that explains a significant proportion of the variability of a given phenotype that may be controlled by multiple genes. Typically, one or more genetic markers have been used to identify a desired QTL. To date, most QTL studies of plant species have looked at gross morphological or agronomic phenotypes (e.g. yield, disease and stress resistance, time to flowering etc.). For instance, in WO 2000/18963 soybean plants comprising QTL associated with enhanced yields, and methods for selecting and breeding those plants is disclosed. This method involved the use of a specific marker nucleic acid capable of hybridizing to a second nucleic acid molecule that maps to specific region of *Glycine soja* and that is associated with enhanced yield. In US 5,948,953 a QTL associated with brown stem rot (BSR) resistance in a soybean plant was identified. The QTL associated with BSR resistance may be used for plant selection using marker assisted selection. WO 99/31964 discloses the use of a marker nucleic acid that is genetically linked to a set of 63 specified loci for plant selection. The identified polymorphisms may be used in DNA fingerprinting and for

mapping genes or QTLs associated with pest or disease resistance. Several recent QTL studies have explored the relationship between quantitative variation in specific metabolic changes including metabolite accumulation or changes in enzyme activities. For example, Byrne et al (Byrne, P.F., McCullen, M.D., Snook, M.E., Musket, T.A., Theuri, J.M., Widstrom, N.W., Wiseman, B.R. and E.H. Coe. 1996. Proc. Natl. Acad. Sci. 93: 8820-8825) disclose the mapping of a QTL accounting for 58% of the variance of the concentration of maysin, a flavone acting as a host-plant resistance factor against the corn earworm, to a locus encoding a transcription activator for portions of the flavonoid pathway. Prioul et. al. (Prioul, J.-L., Quarrie, S., Causse, M. and D. de Vienne. 1997. J. Exp. Bot. 48: 1151-1163; Prioul, J.-L., Pelleschi, S., Séne M., Thévenot, C., Causse, M., de Vienne, D. and A. Leonardi. 1999. J. Exp. Bot. 50: 1281-1288) used enzyme activities, substrate and product levels of known biosynthetic pathway as quantitative traits in QTL analysis. Similarly, Pelleschi et al. (Pelleschi, S., Guy, S., Kim, J.-Y., Pointe, C., Mahé, A., Barthes, L., Leonardi, A., and J.-L. Prioul. 1999. Plant Mol. Biol. 39: 373-380) disclose the use of invertase activity as a marker for the identification of candidate genes for QTLs associated with variation in invertase activity in maize. Damerval et al. (Damerval C., Maurice, A., Josse, J.M. and D. de Vienne. 1994. Genetics 137: 289-201) discloses the use of peptides on a 2D-gel as a quantitative trait for QTL mapping.

None of the above approaches demonstrate or suggest that a QTL mapping approach can be used to identify genes involved in the regulation of expression of single genes or cascades of genes, nor do they suggest the use of using mRNA transcripts as the phenotypic trait for QTL mapping.

Dumas et al. (Dumas p., Sun Y., Corbeil G., Tremblay S., Pausova Z., Kren V., Krenova D., Pravenec M., Hamet P., and J. Tremblay 2000, J. Hypertens 18:545-551) disclose the use of mRNA as a phenotypic marker to map QTL associated with stress gene expression in rat. A related approach, using differential gene expression to map QTL associated with blood pressure in rats, was proposed by Cicila and Lee (Cicila G T. and S J Lee 1998 Hypertens Res 21: 289-296). Neither of these papers suggests a QTL approach for the identification of genes that are involved in the regulation of single genes or complex regulatory cascades responsible for controlling

4

transcript levels. Furthermore, there is no discussion of QTL mapping of differential gene expression in organisms characterized as having a ploidy level greater than diploid, nor is there any discussion of identifying QTLs associated with differential gene expression in non-animal hosts.

5

The present invention is directed to the identification of genomic regions involved in the genetic regulation of the expression of one or more genes of interest associated with a desired trait. By using a QTL map-based analysis of the genome, loci for regulatory genes associated with the differential accumulation of transcripts or gene products are identified.

10

It is an object of the invention to overcome disadvantages of the prior art.

The above object is met by the combinations of features of the main claims, the sub-claims disclose further advantageous embodiments of the invention.

15

SUMMARY OF THE INVENTION

The present invention relates to the use of mRNA transcripts, and variation in transcript levels for the identification of quantitative trait loci pertaining to a desired trait within an organism. More specifically, the present invention relates to a method for the identification of loci for regulatory genes that control gene expression for a given trait within any organism. Finally, the present invention relates to a method for the identification of genes and gene sequences that regulate expression of genes and regulatory cascades controlling expressional traits within the organism.

According to the present invention there is provided a method for identifying one or more regions within a genome of an organism of interest that mediate the expression of one or more genes of interest comprising:

- i) identifying a first organism of interest characterized in that the first organism of interest exhibits a measurable response to an environmental stimulus, or otherwise exhibits a phenotype associated with differential gene expression associated with a process of interest;
- ii) identifying a second organism of interest characterized in that the second organism of interest:
 - a) lacks or does not exhibit as strong a response to the stimulus as that of the first organism of interest;
 - b) exhibits a different phenotype compared with that of the first organism of interest, the different phenotype associated with the process of interest;
 - c) exhibits a phenotype of interest that segregates in a population resulting from a cross with the first organism of interest;
 - d) or a combination of two or more of a), b) c) and d);
- iii) crossing the first and second organisms of interest to produce a population of segregated progeny;
- iv) extracting RNA from each of the segregated progeny and quantifying the level of gene expression of one or more genes of interest, the one or more genes of

interest associated with the response to an environmental stimulus, or the process of interest;

- v) preparing a linkage map of the segregated progeny using one or more markers;
- vi) determining a relationship between the one or more markers on the linkage map and the gene expression of the one or more genes of interest and identifying one or more quantitative trait loci (QTL).

The present invention also includes the method as defined as above, wherein after the step of crossing (step iii)), and prior to the step of extracting RNA, the segregated progeny are subjected to a desired environmental stimulus or are characterized as being at a specific developmental stage.

The method of the present invention as described above may be used for the identification of one or more QTLs corresponding to a transcription factor or any factor controlling the expression of the one or more genes of interest.

Furthermore, this invention pertains to the method as described above wherein after the step of determining (step vi)), one or more genes located at the one or more QTL are isolated and characterized.

The present invention is also directed to the method as described above wherein in the step of determining (step vi)), a marker is identified at the QTL. Furthermore, the marker may be used for:

- tracing progeny of an organism;
- determining hybridity of an organism;
- identifying a variation of linked phenotypic trait, expression trait, or both phenotypic trait and expression trait;
- construction of a genetic map;
- identifying individual progeny from a cross;
- isolating a genomic DNA sequence surrounding a gene-coding or non-coding DNA sequence; and

- marker-assisted selection, map-based cloning, hybrid certification, fingerprinting, genotyping, as an allele specific marker, or a combination thereof.

5 The present invention also embraces a method (B) for identifying a regulatory gene that mediates the expression of a gene of interest comprising:

- 10 i) identifying one or more Quantitative Trait Loci (QTLs) that explain a significant proportion of variation of expression of one or more genes of interest;
- ii) mapping the one or more regulatory genes;
- iii) determining whether the one or more regulatory genes map within the one or more QTLs;
- iv) isolating one or more regulatory genes within the one or more QTLs.
- 15 v) sequencing the one or more regulatory genes isolated in step iv).

 This invention also includes a method of mediating the response of an organism of interest to an environmental stimulus comprising, transforming the organism of interest with a regulatory gene identified using the method (B) as
20 described above.

 Further, this invention realtes a method of mediating development of the organism of interest comprising, transforming the organism of interest with a regulatory gene identified using the method (B) as described above.

25

 The present invention also embraces a method (C) for identifying a regulatory sequence that mediates the expression of a gene of interest comprising:

- 30 i) identifying one or more Quantitative Trait Loci (QTL) that explain a significant proportion of variation of expression of one or more genes of interest;
- ii) mapping the one or more regulatory genes;

- iii) determining whether the one or more regulatory genes maps within the one or more QTL;
- iv) isolating one or more regulatory genes within the one or more QTL; and
- v) sequencing the regulatory sequence of the one or more regulatory genes isolated in step iv).

This invention pertains to a method of mediating the response of an organism of interest to an environmental stimulus comprising, transforming the organism of interest with a regulatory sequence fused to a gene of interest, the regulatory sequence being identified using the method (C) as described above.

Further, this invention includes a method of mediating development of the organism of interest comprising, transforming the organism of interest with a regulatory sequence fused to a gene of interest, the regulatory sequence being identified using the method (C) as described above.

The present method allows the identification of genes that share common regulatory loci or that are under the same regulation. This may have important implications for many claimed applications, for example, but not limited to marker-assisted selection, phenotyping, pathway mapping cascade analysis, gene regulation interaction, gene flux analysis etc.

Genomic regions associated with the level of expression of genes can be identified using QTL mapping analysis. This approach, using gene expression level as a quantitative trait permits identifying gene expression control mechanisms by narrowing the search for such regulatory genes and regulatory sequences to specific genomic regions. The significant QTLs identified herein, and the unexpected high level of variance that they explain indicate the potential of using QTL analysis of gene expression to locate important regulatory factors responsible for stress-induced gene expression. Comparative QTL analysis of multiple genes induced by the same external stimulus further permits the elucidation of complex pathways regulating gene expression and to regroup genes that share regulatory factors.

The technique of the present invention is applicable to genes of interest whose expression is regulated by any factors using a population segregating for levels of expression of such genes. It will allow for the identification of genomic regions. Once identified, these genomic regions can be used to characterize a given genotype
5 without environmental or developmental interferences inherent to techniques based on gene expression.

By identifying one or more QTLs for polygenic traits that are induced by environmental stimulus (for example, but not limited to, temperature stress) or that are
10 related to a given process (for example but not limited to yield determination), the technique of the present invention may also identify one or more QTLs that are commonly related to the phenotypic trait being analysed and the expression of genes of important adaptative value for that given trait. Regulatory genes and regulatory sequences found within these loci are likely to be of important value for the
15 improvement of the polygenic trait.

It is to be understood that the technique of the present invention is applicable to any host organism, however, there are several organisms, for example, but not limited to, unicellular organisms or plants that offer unique advantages over other
20 eukaryotic systems with regard to the application of the technique of the present invention. For example, in some plant species, the generation of doubled haploids from pollen in which every locus is homozygous, the selfing or backcrossing to one or other parental lines of F1s to generate segregating F2 or backcross populations, the possibility to repeatedly self-pollinate individual F2 plants for several generations
25 (F6-F10) to produce a series of recombinant inbred lines, the possibility to multiply every genotype by clonal propagation allows multiple destructive analyses of the same genetic material, and the possibility to submit genetically identical material to various environmental stimuli or other process. Furthermore, these types of clonal material can be replicated in various environmental conditions where genotype,
30 environment, or both genotype and environment, interaction effect on the phenotype can be studied.

This summary of the invention does not necessarily describe all necessary features of the invention but that the invention may also reside in a sub-combination of the described features.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the
5 following description in which reference is made to the appended drawings wherein:

FIGURE 1 shows a linkage map of diploid alfalfa. Loci are listed on the right, and
recombination distances (cM) are listed on the left of each linkage group. Bars
on the right of linkage groups indicate the approximate position of loci. Cold-
10 regulated (msaCI) genes are double framed and genes with homology to
genes with regulatory function are framed. Genes that are members of
multigene families are numerically distinguished, for example C3358.1 and
C3358.3.

FIGURE 2 Position and statistical significance (LOD scores) of QTLs for LT₅₀ and
cold regulated msaCI gene expression. msaCI genes are double framed and
genes with homology to genes with regulatory function are framed. When
gene expression QTLs are detected in both crown and leaf tissue, the graph for
LOD scores of expression in crown is shown at the top and the graph for LOD
20 scores of expression in leaves is shown at the bottom. LT₅₀ QTLs apply to
whole plants and are independent of tissue type. Genes that are members of
multigene families are numerically distinguished for example C3358.1 and
C3358.3.

FIGURE 3 shows one example of a dot blot analysis of the expression of a gene of
interest, in this case the msaCIA gene, in segregating F₂ progeny of diploid
alfalfa. Each dot corresponds to a unique genotype except for the last column
where a control is dotted in triplicate. Level of transcripts are quantified by
densitometry and used as a quantitative trait for QTL analysis. Optical density
30 value is indicated for each genotype and the control.

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to a method for the identification of regulatory loci that control gene expression within any organism. More specifically, the present invention relates to the use of mRNA transcripts, and variation in transcript levels for the identification of quantitative trait loci pertaining to a desired trait within an organism.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

A quantitative trait locus (QTL) is a region of the genome that explains a significant proportion of the variability of a given phenotype controlled by multiple genes, for example, but not limited to, traits associated with increased yield, freezing tolerance, drought tolerance and the like. Within these regions are located one or more genes coding for factors that have a significant effect on the phenotype of the organism.

As described herein, a linkage map constructed using any type of marker such as morphological, isozyme or DNA based markers may be used to locate each gene of interest to a given location on the map. The expression of a single gene of interest is considered as a quantitative trait. Without wishing to be bound by theory, if the variability of the expression of the gene of interest is the result of differences in the gene itself, for example, due to various allelic forms of the gene, then a major quantitative trait locus should be located close to the location of the structural gene. However, as outlined below, it is observed that the variance of the expression of the gene of interest is highly, and significantly explained by QTLs located elsewhere in the genome (see Figure 1). Therefore, genomic regions located at a distance from the gene of interest are controlling, or regulating the expression of the gene of interest. These genomic regions may be identified using the linkage map and the regulatory genes that encode factors that control the expression of the gene of interest are then identified using techniques as known to one of skill in the art.

By “gene of interest”, it is meant one or more known genes that may be used as a quantitative trait that is being characterized using the method of the present invention, and may include upstream and down stream regulatory regions, introns, and exons. A gene of interest is typically, but not always, expressed under conditions used to determine the effect of regulatory genes on the expression, or suppression, of the gene of interest. The level of expression of the gene of interest may be determined using any methods known in the art, for example, Northern analysis, RNase protection, array analysis, PCR and the like. The gene of interest or the level of its transcripts, is a quantitative trait that is used for further identification of one or more QTLs associated with the expression of the gene of interest. One or more genes of interest may be used within the method of the present invention for the analysis of a response to an environmental stimulus or other process of interest as defined herein. By increasing the number of genes of interest used within the analysis, it is possible to identify one or more QTLs that are responsible for the regulation of several genes of interest associated with the response to the environmental stimulus or other process of interest within the plant, possibly highlighting the importance of the QTLs. Such an approach is disclosed below and in the Examples.

By “regulatory gene”, it is meant a gene whose product directly or indirectly effects the expression of a gene of interest, and includes the coding region, upstream (5') and downstream (3') non-coding and regulatory regions, and introns. Typically, a regulatory gene is differentially expressed, in response to a stimulus, for example but not limited to, an environmental stimulus such as heat shock or temperature stress.

An example of a regulatory gene includes, but is not limited to, a gene that encodes a transcriptional factor, or a gene that encodes another protein factor that in some manner regulates the expression of another gene. The product encoded by a regulatory gene may directly effect the expression of a gene of interest, for example which is not intended to be limiting in any manner, by binding regulatory regions upstream (5') or downstream (3') of the coding region of the gene of interest, or within introns, as is known in the art, and enhancing or silencing the expression of the gene of interest. A regulatory gene may also encode a product that effects either the stability of the transcript of the gene of interest, the rate or stability of translation or

the transcript synthesized from the gene of interest, or both post transcriptional and post translational events pertaining to the expression of the gene of interest. It is also contemplated that a regulatory gene may effect the differential expression of a gene of interest indirectly, by mediating the expression of one or more secondary genes, whose products may then interact with the expression of the gene of interest. It is also contemplated that one or more regulatory genes may act synergistically to mediate the differential expression of a gene of interest.

The method of the present invention allows for the identification of genes that share common regulatory loci or that are under the same regulation. This may have important implications for many applications, for example, but not limited to marker assisted selection, phenotyping, pathway mapping cascade analysis, gene regulation interaction, gene flux analysis, or the identification of regulatory genes having significant effects on polygenic traits

15

By "environmental stimulus" it is meant any stimulus effecting, and producing a measurable response within an organism of interest. For example, which is not intended to be limiting in any manner, the organism of interest may be a plant, plant organ, tissue or cell, and the environmental stimulus may be, for example but not limited to, biotic stimuli including infestation by a virus, bacteria, fungi, insect, nematode or other herbivore, or an abiotic stimuli, for example but not limited to, a stress associated with chilling, freezing, water, drought, osmotic, heat, salt, oxidative, or pollutant. An environmental stimulus may also include the effects of mineral nutrition, light, and endogenous or exogenous chemicals on organism of interest or an organ, tissue or cell thereof.

25

By "process of interest" it is meant any process that produces a measurable effect within an organism of interest or an organ, tissue, or cell thereof, for example, which is not to be considered limiting in any manner, a plant. A process of interest may include but is not limited to, developmental, chemical or environmental control of gene expression during, in the non-limiting case of a plant, embryogenesis, flower, seed, root or leaf development, organogenesis, or circadian, ultradian and other internal rhythms. Other processes of interest may include but are not limited to,

30

harvestable yield, photosynthate translocation, sink and source, leaf area index, root shoot ratio, nutritive value of harvested material, plant morphology, rate of cell cycle, rate of cell differentiation, cell size, plant life cycle, senescence, maturation, dormancy, germination, or genomic rearrangements, for example, due to transposon
5 activation. However, it is to be understood that analogous or other unique processes may be characterized using the methods described herein, within any organism of interest.

By, "an organism of interest" it is meant any organism within which one or
10 more QTLs are to be identified and characterized, for example but not limited to a plant (algae, bryophytes, ferns, angiosperm, or gymnosperm), an animal, a unicellular organism, bacteria, phytoplankton, yeast, fungi, and also includes cell or tissue cultures of these organisms.

15 The method of the present invention may be used to identify the level of transcripts of a gene that maps to a specific location in the genome and to determine whether the accumulation of the encoded transcript, or protein, is regulated by genes located at loci elsewhere in the genome. Using a QTL (quantitative trait loci) map-based analysis of the genome, it is possible to identify loci associated with the
20 differential accumulation of transcripts or gene products. Therefore genes found within these QTLs could encode for members of multigene families under different transcriptional controls (for example, harbouring important cis-regulating sequences which in turn influence the regulation of transcription or act on upstream or downstream sequences required for enhanced translation or mRNA stability),
25 transcription factors (including enhancer and repressors), signal transduction pathway proteins (for example, protein kinases, protein phosphatases, 14-3-3 proteins etc.), external signals receptors, proteins involved in the regulation of secondary messenger levels (Ca^{2+} , IP_3 , cAMP, etc.), nucleases involved in the degradation of transcripts, proteases and proteases inhibitors, novel transcription regulating factors.

30

An example of the approach used for identifying regulatory genes that control, mediate, or influence, the expression of one or more genes of interest, for example, but not limited to, differential expression in response to an environmental stimulus, or

any event within an organism of interest that results in differential gene expression, involves:

- i) identifying a first parent organism of interest, that exhibits a response to an environmental stimulus, or otherwise exhibits a phenotype associated with differential gene expression associated with a process of interest, and a second parent organism of interest that lacks or that does not exhibit as strong a response to the stimulus, or exhibits a different phenotype associated with differential gene expression associated with the process of interest, whether or not this variation is found in nature or induced by gene mutation, gene disruption, or gene insertion. It is desired, but not necessary, that the first and second parents differ in their response to the stimulus, or in differential gene expression associated with the process of interest as long as the response to the stimulus, or in differential gene expression, segregates in the progeny;
- ii) crossing the first and second parents to produce a population of segregating progeny. Population types can include, but are not restricted to, F1 (from heterozygous parents), F2, or F3 families, Recombinant Inbred lines, Doubled Haploids, Backcross lines, Testcross lines, and gymnosperm segregating megagametophytes;
- iii) if required, subjecting the progeny of this cross to a desired environmental stimulus;
- iv) extracting RNA from one or more organs, tissues or cell type, for example, in the case of a plant as an organism of interest, RNA may be obtained from flower, primordia, leaf, stem or root tissues, or cells;
- v) measuring the transcript levels of one or more genes of interest using any suitable method, for example but not limited to, dot blot or Northern hybridization, array analysis, or quantitative PCR, array analysis and quantifying the level of gene expression for each progeny;
- vi) preparing a genetic linkage map of the segregating population using any appropriate method, for example but not limited to, the use of DNA based markers such as, RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), microsatellites, IMP (Inter MITE polymorphism), or

SNPs (Single Nucleotide Polymorphism), protein markers, or morphological markers to characterize the individuals of the mapping population and
subjecting the data obtained from one or more of these techniques to obtain
two-point and multi-point linkage analysis for the construction of the linkage
map;

vii) determining the relationship between markers on the linkage map and the
expression of the one or more genes of interest in response to the
environmental stimulus, or associated with a process of interest, to identify
statistically significant QTLs. Such a relationship may be determined using
any method known to one of skill in the art, for example, but not limited to,
single point ANOVAs, simple regression, Interval mapping, Composite
interval mapping. Such an analysis may be performed using
MAPMAKER/QTL, MQTL, QTL Cartographer, or other similar software.
The statistical significance and percent of variance for each QTL are also
calculated.

The method as described herein provides an effective method for the
exhaustive identification of many, if not all, the regulatory factors (genes) involved in
the control of the expression of a gene of interest, and provides an estimate of the
relative contribution of each factors to the observed variability of expression of the
gene of interest, as well as identifying the regulatory gene's genomic location and
associated markers which can be used in traditional plant breeding. In addition,
“master switch” regulatory genes affecting the expression of many genes, for example
within a cascade, can be identified when multiple genes induced by a given
environmental stimulus or that are related to a given process are analysed
simultaneously.

Once one or more QTLs have been identified that are significantly associated
with the expression of the gene of interest, then each of these loci and linked markers
may be used either directly as markers, for example, but not limited to breeding and
screening purposes, including plant breeding, or further characterized to determine the
gene or genes involved with the expression of the gene of interest, using map-based
cloning methods as would be known to one of skill in the art. For example one or

more known regulatory genes can be mapped to determine if the genetic location of these genes coincide with the QTLs controlling mRNA expression of the gene of interest. Confirmation that such a coinciding regulatory gene is effecting the expression of one or more genes of interest can be obtained using standard techniques in the art, for example, but not limited to, genetic transformation, gene complementation or gene knock-out techniques, or overexpression. The genetic linkage map can also be used to isolate the regulatory gene, including any novel regulatory genes, via map-based cloning approaches that are known within the art whereby the markers positioned at the QTL are used to walk to the gene of interest using contigs of large insert genomic clones. Positional cloning is one such a method that may be used to isolate one or more regulatory genes as described in Martin et. al. (Martin, G.B., Brommonschenkel, S.H., Chungwongse, J., Frary, A., Ganai, M.W., Spivey, R., Wu, T. Earle, E.D. and S.D. Tanksley, 1993, Science 262: 1432-1436; which is incorporated herein by reference), however, other suitable methods may also be used as recognized by one of skill in the art. Again confirmation that such a coinciding regulatory gene is effecting the expression of one or more genes of interest can be obtained via genetic transformation and complementation or via knock-out techniques described below.

Therefore, the present invention is directed to identifying regions within the genome of a plant that are involved in mediating the expression of one or more genes of interest in response to an environmental stimulus, or a process associated with differential gene expression within a process of interest. The present invention is also directed to characterizing the identified QTL to identify the one or more regulatory genes and regulatory sequences at these loci.

The method of the present invention may also be used to identify loci corresponding to factors (encoded by regulatory genes) associated with the control or mediation of expression of a gene of interest in an organism of interest. This method can be used for the identification of regulatory genes that control and modulate the level of mRNA transcripts of one or more genes of interest, whether or not this variation is found in nature or induced by gene mutation, gene disruption, or gene

insertion, for example, but not limited to, genes of interest that are regulated by factors involved in:

- biotic stresses, for example but not limited to virus, bacteria, fungi, insects, or nematodes;
- 5 - abiotic stresses including but not limited to chilling, freezing, water, drought, osmotic, heat, salt, oxidative, pollutants;
- developmentally, chemically and environmentally controlled gene expression;
- embryogenesis, for example, but not limited to tissue, or organ development, which in the case of a plant may include seed development, organogenesis,
- 10 floral development;
- mineral nutrition, including both macro and micro nutrients;
- light (irradiance level and light quality);
- circadian, ultradian and other internal rhythms;
- chemical inducers including natural growth regulators, for example, in the
- 15 case of a plant being the organism of interest, auxins, gibberellins, ABA, cytokinins, ethylene, and their analogs, synthetic hormones, herbicides, salicylic acid, jasmonic acid;

or genes of interest that have a significant impact on biological processes such as:

- 20 - the determination of yield, for example which is not to be considered limiting, in plants, determination of harvestable yield, through the effects of regulatory genes on biomass, seed setting (number and size), photosynthate translocation, sink and source, leaf area index, root shoot ratio;
- nutritive value of biomass, for example but not limited to, harvested material
- 25 and associated effects on the levels of quality factors such as cofactors, vitamins, proteins, antioxidants, highly digestible fibers;
- morphology, for example, but not limited, in plants, to the effect of regulatory genes on flower color (e.g. genes involved in anthocyanins synthesis), plant height (e.g. genes involved in gibberellins synthesis), internode length, leaf
- 30 insertion;
- rate of cell cycle, for example but not limited to cyclins;
- rate of cell differentiation, for example but not limited to homeodomain proteins;

- cell size, for example but not limited to, in plants, to genes involved in auxin synthesis, auxin receptors, auxin-induced genes;
- organism life cycle, for example through the effect of regulatory genes on senescence, maturation, dormancy, germination;
- 5 - genomic rearrangements, for example, transposon activation;

In addition to using the method of the present invention to identify loci corresponding to transcription, or any factors controlling the expression of genes in an organism of interest for various desired traits, the markers located in these QTL may also be used in applied breeding. For example, identified polymorphisms associated with one or more identified genetic markers located at a locus (a QTL) may be used: for genome-based diagnostic and selection techniques :

- for tracing progeny of an organism;
- to determine hybridity of an organism;
- 15 - to identify variation of linked phenotypic traits, mRNA expression traits, or both phenotypic and mRNA expression traits;
- as genetic markers for constructing genetic linkage maps;
- to identify individual progeny from a cross wherein the progeny have a desired genetic contribution from a parental donor, recipient parent, or both parental donor and recipient parent;
- 20 - to isolate genomic DNA sequence surrounding a gene-coding or non-coding DNA sequence, for example, but not limited to a promoter or a regulatory sequence;
- in marker-assisted selection, map-based cloning, hybrid certification, fingerprinting, genotyping and allele specific marker; and
- 25 - as a marker in an organism of interest.

Using a QTL map-based analysis described herein, regulatory loci associated with the expression of a gene of interest, at the whole organism, organ, tissue or cell specific level, in response to an environmental stimulus, or a phenotype associated with differential gene expression associated with a process of interest may also be identified. For example, which is not to be considered limiting in any manner,

candidate regulatory genes that modulate the expression of mRNA, such as (also see Table 2 Example 2):

- Transcription factors (zinc-finger and AP2 proteins);
 - Mitogen-activated protein kinases (MMK4, MMK3, MMK2, MMK1);
 - 5 - Calcium-dependent protein kinases ;
 - Serine-threonine protein kinase;
 - Ca^{2+} transporting ATPase;
 - GTP binding proteins;
 - RNA binding proteins;
 - 10 - Protein phosphatases (type 2A and 2C);
 - Ca^{2+} -binding proteins, for example Calmodulin;
 - 14-3-3 proteins;
 - GTPase activating proteins;
 - Adenylyl cyclase protein;
 - 15 - Phospholipase C;
 - Lipoxygenase;
 - Histone deacetylase;
 - Receptor kinase; or
 - Phosphatidyl inositol 3-kinase
- 20 may be evaluated to determine if they map to regions of the genome (QTL) that explain a large proportion of the expression of cold-regulated genes in a plant, for example but not limited to, alfalfa (*Medicago falcata* L.).

The method as described herein may be used to complement high throughput
 25 genome analysis studies based on DNA chips and grid arrays, or any other system capable of measuring gene expression. Using segregating populations, it is possible to locate QTLs regulating the transcripts level of genes which expressions is related to phenotypes of interest (e.g stress and disease resistance, biochemical production, morphological variations etc.). Mapping of candidate genes from EST collections
 30 establishes potential co-location to important QTLs and allows for the identification of allelic polymorphism and the development of allele-specific markers. The identification of these QTLs, and the associated regulatory genes, may be used for genome-based diagnostic and selection techniques for assessment of gene expression

potential under given developmental or environmental conditions and the coordination of gene expression in a developmental, tissue, or environmental manner.

Genomic regions associated with the level of expression of genes, for
5 example, but not limited to those induced by low temperature, can be identified using
QTL mapping analysis. This approach, using gene expression level as a quantitative
trait permits identifying gene expression control mechanisms by narrowing the search
for such regulatory genes to specific genomic regions. DNA sequences responsible
for variation in gene expression are also likely to be found within these regions. The
10 gene identification method described herein will become easier and more rapid with
the availability of whole genome sequences and the routine integration of increasingly
large number of ESTs onto high density maps or by taking advantage of the extensive
collinearity of blocks of genes along chromosomes of related species in comparative
genetic mapping.

15

The very significant QTLs identified within the Examples herein, and the
unexpected high level of variance that they explain indicates the potential of using
QTL analysis of gene expression to locate important regulating factors responsible for
stress-induced gene expression. Comparative QTL analysis of multiple genes induced
20 by the same environmental stimulus or that are involved in a given process permits
the elucidation of complex pathways regulating gene expression and to regroup genes
that share the similar regulation factors.

The technique of the present invention will be applicable to genes which
25 expression is regulated by any factors using a population segregating for levels of
expression of such genes. It will allow for the identification of genomic regions that
can be used to characterize a given genotype without environmental or developmental
interferences inherent to techniques based on gene expression.

30 In order to identify regulatory genes that may be localized at one or more
QTLs that explain a significant proportion of the variation of the expression of one or
more genes of interest, probes are obtained from known regulatory genes, for
example, but not limited to the regulatory genes listed above, and in Table 2 of

Example 2, and these regulatory genes, or their expression products are mapped on a linkage map. These probes may include nucleotide fragments or full-length genes for the detection of DNA or RNA, or antibodies for the detection of the expression products of a regulatory gene. It is to be understood that unknown regulatory genes
5 located at one or more QTLs may also be identified using methods as known to one of skill in the art, for example, but not limited to, positional cloning and mapping portions of the sequenced DNA to determine if the cloned DNA co-locates (maps) with the one or more identified QTLs. Preferably, the location of the candidate regulatory genes at one or more QTLs is confirmed, for example by mapping
10 upstream or downstream regions from each putative regulatory gene on the linkage map. In this manner, only regulatory genes that are characterized by having 5' or 3' regions that co-locate with the same QTLs are used for further characterization.

Regulatory genes that are located within one or more QTLs that are associated
15 with the expression of one or more genes of interest may then be isolated and characterized, and their expression modulated within a transgenic plant, for example but not limited to alfalfa, in order to assess their effect on expression of the gene of interest, and if desired, their effect on the response of the organism of interest to an environmental stress. Such analysis may include, but is not limited to,
20 complementation or gene knockout, and gene activation, studies involving transformation of the organism of interest with a sense or antisense construct of the regulatory gene or enhancer sequences, respectively. An organism of interest that lacks or exhibits a weak response to an environmental stimulus, or a process of interest, is transformed with a sense construct of the regulatory gene, while an
25 organism of interest that exhibits a strong response, or scores positively for process of interest, is transformed with an antisense construct of the regulatory gene, or disrupted by insertion of a foreign DNA such as T-DNA into the coding region of the gene, or activated by insertion of an enhancer sequence 5' or 3' of the gene of interest. Expression of one or more genes of interest, and the regulatory gene may then be
30 monitored to determine the effect of the sense, antisense, knockout, or enhancer insertion regulatory gene on the organisms response to an environmental stimulus, or on process of interest. The effect of the sense, antisense, knockout, or enhancer

insertion constructs on a physiological response to an environmental stress may also be determined.

It is also contemplated that a regulatory gene located at a specific QTL, or groups of QTLs, may also be used as a marker for the one or more QTLs within breeding programs.

Therefore this invention also pertains to a method for identifying a regulatory gene that mediates the expression of a gene of interest comprising:

- i) preparing a linkage map of the organism of interest;
- ii) identifying one or more QTLs that explain a significant proportion of the variation of the expression of one or more genes of interest; and
- iii) isolating and mapping one or more regulatory genes to determine if the regulatory gene maps within the identified one or more QTLs.

The present invention is also directed to mediating a response of an organism of interest to an environmental stress, or characterizing the *in vivo* effect of an identified and isolated regulatory gene, by introducing the regulatory gene into the organism of interest and, optionally, determining the effect of the regulatory gene on the expression of one or more genes of interest, or its effect on the response of an organism of interest to an environmental stimulus.

The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

Example 1: Identifying QTLs associated with cold-regulated gene expression

General description of the experimental approach

Expression of cold-regulated (*msaCI*) genes and LT_{50} phenotypes are used as an example of quantitative traits to be characterized within clonal propagules of F_2 genotypes of diploid alfalfa. The segregation patterns of the phenotypes (*msaCI* genes expression, and LT_{50}) are used to search for quantitative trait loci (QTL) on a linkage map of diploid alfalfa genome.

Plant Material

An F_1 population is constructed by intercrossing diploid parents exhibiting contrasting cold tolerance (*M. falcata* x *M. sativa*). The F_1 line is produced by a cross-pollination between a cold sensitive *M. sativa* genotype from the cultivated alfalfa at the diploid level group (Bingham, E.T. and T.J. McCoy. 1979. Crop Sci. 19: 97-100) as the female parent with one hardy diploid genotype from *M. falcata* cv. Anik (Pankiw, P. and Siemens, B. 1976. Can. J. Plant Sci. 56: 203-205) as the male parent. Flowers are emasculated and hand pollinated. One randomly selected genotype of the F_1 is selfed by hand pollination and the F_2 progeny from this cross is used to produce a linkage map and QTL analysis.

Growth conditions

Cuttings from 117 F_2 genotypes are clonally propagated and transplanted in deep pot inserts after root initiation. Clonal propagules are then grown under environmentally-controlled conditions at 21°C and 17 °C (day, night respectively) temperatures, with a 16 hour photoperiod at an irradiance of approximately 225 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density.

Cold acclimation conditions

Plants are cold acclimated two weeks in a growth chamber at 2°C, with an 8h photoperiod and an irradiance of approximately 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density.

LT₅₀ determination

Clonal propagules of each genotype from the segregating population and parental genotypes are submitted to a freezing test as described by Castonguay et al (Castonguay, Y., Nadeau, P., and S. Laberge 1993. Plant Cell Physiol. 34: 31-38). Plants are subjected to a stepwise decrease in ambient temperature during which time individual plants are retrieved at intervals and allowed to regrow under initial growth conditions (21/17°C, day night temperatures, respectively) for three weeks before assessment of plant survival. The 50% killing temperature (LT₅₀) is computed by the SASTM Probit procedure (Statistical Analysis System, Cary, NC). Three replicates of an incomplete block design with cyclic permutations (12 blocks) are performed in order to statistically compare the large number of ecotypes used in the study.

Description of msaCI genes

15

Seven msaCI genes isolated from a cDNA library from cold-acclimated crowns of tetraploid alfalfa (*M. sativa* cv. Apica; Laberge, S, Castonguay, Y. and Vézina, L.-P. 1993. Plant Physiol 101, 1411-1412; Castonguay, Y., S. Laberge, P. Nadeau, and L.-P. Vézina. 1997. p. 175-202. In B.D. McKersie and D.W. Brown (ed.). Biotechnology and the improvement of forage legumes. CAB International, Wallingford, UK) are used in this study:

msaCIA	Glycine-rich	Laberge et al. (1993)*
msaCIB	Putative nuclear protein	Monroy et al. (1993)**
25 msaCIC	Bimodular protein	Castonguay et al. (1994)***
msaCID	Pathogenesis-related protein	Unpublished
msaCIE	Glyceraldehyde-3P-dehydrogenase	Unpublished
msaCIF	Galactinol synthase	Unpublished
msaCIG	Dehydrin-like	Unpublished

30

*Laberge, S, Castonguay, Y. and Vézina, L.-P. 1993. Plant Physiol. 101, 1411-1412.

**Monroy, A.F., Castonguay, Y., Laberge, S., Sarhan, F., Vézina, L.-P. and Dhindsa, R.S. 1993. Plant Physiol. 102, 873-879.

***Castonguay, Y., Laberge, S., Nadeau, P. and Vézina, L.-P. 1994. Plant Mol. Biol. 24, 799-804.

5 RNA extraction

Approximately 0.5 g (fresh weight) leaf tissue from each cold acclimated genotype is ground to a fine powder in liquid N₂ using a mortar and a pestle, and total RNA is extracted using standard methods as previously described (Castonguay et al. 1994. Plant Mol. Biol. 24, 799-804). Total RNA is quantitated by UV absorption at 260 nm (Fourney, R.M., J. Miyakoshi, R.S. Day III, and M.C. Paterson. 1988. Focus 10:1).

Dot blot quantification

15

For each parental genotype and each genotype of the segregating population, five µg of total RNA are vacuum-transferred to a nylon membrane (Hybond N⁺, Amersham Pharmacia Biotech, Oakville, ON) using a Bio-DotTM apparatus (Bio-Rad, Mississauga, ON) and immobilized by cross-linking under UV light for 3 minutes. Membranes are hybridized overnight at 68°C in 2X SSC, 0.25% (W/V) low-fat powder milk, 1% SDS with [³²P]dCTP-labeled probe prepared from purified inserts of cold-regulated genes according to standard protocols (Sambrook, J., Maniatis, T. and Fritsch. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Membranes are exposed with Kodak X-Omat AR5 X-ray film at -80°C and transcripts level is quantitated by densitometric analysis of autoradiographs using OneD ScanTM software (Scanalytics inc., Billerica, MA). See Figure 3 for an example of one such dot blot obtained using msaCIA gene as a probe. The mean transcript levels of all samples on each membrane is used to standardize hybridization signal across membranes.

30

Linkage map

A genetic linkage map is constructed based on AFLP and RFLP. DNA is extracted from 1-2 g of fresh leaf tissue from 4-6 week-old clonal propagules. The method for extraction is essentially as described by Doyle and Doyle (Doyle, J.J., and Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. FOCUS 12, 13-15),
5 except that the leaf tissue is ground without liquid nitrogen at room temperature.

To uncover RFLP markers, DNA purified from 169 F₂ genotypes is separately digested with restriction enzymes *DraI* and *EcoRV*. Each digested DNA sample (6 µg per genotype is separated on horizontal gels (0.9 % agarose, 1 XTAE), and the
10 separated fragments transferred to nylon membranes (Hybond N⁺, Amersham Pharmacia Biotech, Oakville, ON) by capillary blotting. Ten duplicate copies of membranes are made for simultaneous segregation analyses with multiple probes. Membranes are hybridized overnight at 65°C with probes labelled with [³²P]-dCTP. DNA probes consist of randomly selected cDNAs and cDNAs selected according to
15 their homology to regulatory genes both obtained from *Medicago sativa* cDNA libraries, and the 7 *msaCI* cDNAs described above. cDNA inserts are first specifically amplified by PCR and labelled by random hexamer labelling using the T7 QuickPrimeTM Kit (Amersham Pharmacia Biotech, Oakville, ON). Prehybridization, hybridization, and probe labeling are performed according to standard protocols
20 (Sambrook, J., Maniatis, T. and Fritsch. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Col Spring Harbor, New York).

To identify AFLP markers, F₂ DNAs are digested with *EcoRI* and *MseI* restriction enzymes, ligated to *Eco*- and *Mse*-specific primer adapters, then amplified
25 by pre-selective *Eco* and *Mse* PCR primers (Vos. P., Hogers, R., Bleeker, M., Reijans, M., van der Lee, T., Hornes, M., Frijters, A., Pot, J., Pelerman, J., Kuiper, M, and Zabeau, M. 1995, Nucleic Acids Res. 23, 4407-4414). Pre-selective amplicons are diluted 20-fold to be used as material for selective amplification. Both *Eco* and *Mse* primers, used for selective amplification, contained 3 selective nucleotides at the
30 3' end. The *Eco* primer is labelled with IRD dyes, which permit the detection of amplicons by the infrared laser of a LI-COR automated sequencer (LI-COR, Inc. Lincoln, Neb, USA). Selective amplification is carried out according to the "touch down" protocol described by Vos et al. (1995, Nucleic Acids Res. 23, 4407-4414).

After selective amplification, amplicons were separated on 25 cm gels composed of 7% Long Ranger acrylamide, 7 M urea, and 0.6 X TBE, using a LI-COR automated DNA sequencer model 4000L.

5 The genetic map of the diploid cross is constructed using the segregation data from 112 RFLP and 117 AFLP segregating markers. Co-segregation analyses are performed using MAPMAKER/EXP v 3.0 software (Lander, E.S., Green, P., Albertson, J., Barlow, A., Daly, M.J., Lincoln, S.E., and Newburg, L 1987. Genomics 1, 174-181) to determine the linkage group assignment of each marker and the
10 location of each molecular marker within each linkage group. Individual linkage groups are obtained using two-point analysis with a minimum LOD score of 15 and maximum recombination level of 30 %. Multiple point analysis with an LOD threshold of 2.0 is then used to order the loci within the linkage groups. The resulting map (Figure 1) covers approximately 1400 cM and has an average marker interval of
15 approximately 8 cM. Fifteen markers remained unlinked.

Identification of QTL associated with msaCI genes expression and freezing tolerance

20 QTLs involved in msaCI gene expression are identified with MAPMAKER/QTL v 1.1. (Lander, E.S., and Boststein, D. 1989, Genetics 121, 185-199). The linkage map is scanned for the presence of QTLs in specific regions of the genome using the expression of each gene as a quantitative trait. The action of each QTL in relation to each other and the percent of the variance of msaCI gene
25 expression explained by each QTL is determined. An LOD threshold of 2.0 is used for the identification of QTL.

Analysis of cold-regulated accumulation of transcripts for each of the 7 msaCI genes reveals a large genotypic variability for their expression with, in some cases, 10
30 to 40 fold differences in expression between genotypes (for example see Fig. 3).

QTLs related to the variability of msaCI gene transcript levels observed among genotypes are found for all 7 msaCI genes, and for the LT₅₀ phenotype

(Table 1). Genes that play an important regulatory role in the expression of msaCI genes are found within these QTLs. The number of QTLs associated with expression of msaCI genes varied from 1 (msaCIF) up to 5 (msaCIC). In most cases their LODs were highly significant. The phenotypic variation explained by these QTLs varied from 10 to 60%. In most cases the increase in expression and frost tolerance, respectively, was due to the allele from the cold tolerant *M. falcata* parent.

Three QTLs relating to freezing tolerance (LT₅₀) are identified. Two of these QTLs are located in linkage group E in a genomic region associated also with the expression of msaCIB and msaCID.

QTLs for the expression of msaCI genes are found associated with the position of the corresponding structural gene for msaCIA (see linkage group D, Figure 2) msaCID (see linkage group E, Figure 2) and msaCIG (linkage group F, Figure 2).

15

In the case of msaCID the RFLP locus coincides with expression QTLs for three genes; the msaCID gene itself, msaCIA and msaCIB (see Linkage Group E, Figure 2). This suggests that either msaCID is a regulatory gene or a regulatory gene is located in its vicinity.

20

QTLs for the expression in crown and leaf tissues that are located in the same genomic region were observed for msaCIA (between 120-130 cm, Figure 2, Linkage Group E) and msaCID (see linkage group E, Figure 2). Otherwise the loci affecting the expression in each tissue type were different. QTLs controlling the expression of several msaCI genes are located in linkage groups B and E suggesting a common regulatory mechanism. A particularly striking example is a QTL on linkage group B which has an effect on the expression in leaf tissue of five of the msaCI genes studied (Figure 2, Table 1).

25

TABLE 1: QTL associated with the mRNA expression of cold-regulated genes and freezing tolerance in diploid alfalfa. Linkage groups associated with gene expression in leaves or crowns or to freezing tolerance (LT₅₀) along with LOD scores are presented. Regulatory genes that map to these genomic regions are identified. The highlighted genes C4494 and C2186 show homology to the CBF1-gene from Arabidopsis and the SCOF-1 gene from soybean, respectively, for which an involvement in cold tolerance has been demonstrated. (* data not shown in Figure 2).

Gene/trait	Linkage group		LOD		Candidate		Homology to genes coding for regulatory gene
	Crown	Leaf	Crown	Leaf	Crown	Leaf	
msaCIA		B		3		C2784	Serine/threonine-protein kinase
D			11		msaCIA		
D			9		C2314		Phosphatase 2C
D			9		C5047		Leucine rich repeat (LRR) receptor-like protein kinase
D			9		C2250		GTP binding protein (Ras-related)
E			4		msaCID		
E	E	E	4	5	C4320	C4320	Mitogen-activated protein kinase
		E		4		C271	Serine protein kinase
		E		4		C948	Casein kinase I
		E		4		C3749	CDC2+/CDC28-related protein kinase R2
msaCIB		B		3		-	
E			4		msaCID		
E			4		C3283		Ethanolamine kinase

Gene/trait	Linkage group		LOD		Candidate		Homology to genes coding for
	Crown	Leaf	Crown	Leaf	Crown	Leaf	
msaCIC	A	F		5		C4494	AP2 domain transcription factor (CBF1 homolog)
			4		C4546		Mitogen activated protein kinase (MAP3K)
		A		5		C2595	Calreticulin
		A		5		C374	Peptidyl-Prolyl cis-trans isomerase
		A				C3358	Adenosine kinase
		A				C2886	Cyclase-associated protein (CAP)
	B		6		-	-	
		B		3			
	G		6		C168		Transcription factor
	G		5		C2420		Ca ²⁺ -transporting ATPase-like protein
	G		5		C374		Peptidyl-Prolyl cis-trans isomerase
msaCID	I*		5				
	J*		6				
		K*		5			
		B		5		C2784	Serine/threonine-protein kinase
	E	E	14	8	msaCID	msaCID	
	E	E	10	5	C5237	C5237	Cell division cycle protein 48
	E	E	12	8	C3346	C3346	Ethylene responsive element transcription factor

Gene/trait	Linkage group		LOD		Candidate		Homology to genes coding for
	Crown	Leaf	Crown	Leaf	Crown	Leaf	
msaCIE	E	E	7	4	C3283	C3283	Ethanolamine kinase
	J*		5				
	L*		6				
	H*	B	5	8		C2784	Serine/threonine-protein kinase
msaCIF		I*		3			
msaCIG		B		4		C2784	Serine/threonine-protein kinase
LT 50	F		6		msaCIG		
	C		6		C2456		GTP-binding nuclear protein (RAN1A)
	C		4		C2186		Zinc finger transcription factor (SCOF-1 homolog)
	E		4		C5237		Cell division cycle protein 48
	E		4		C3283		Ethanolamine kinase

Example 2: Isolating regulatory genes and regulatory sequences associated with QTLs of interest

General description of the experimental approach

5

The detection of QTLs that explain a significant proportion of the variation of the expression of *msaCI* genes indicates possible localization of regulatory genes at these loci. A number of expressed sequence tags (EST) from cold-acclimated alfalfa encoding genes with known functions in the regulation of gene expression are mapped.

10

Candidate regulatory genes that are located within a QTL associated with the expression of *msaCI* genes are isolated and characterized, and their expression examined within transgenic alfalfa in order to assess their effect on cold-regulated gene expression.

15

Description of candidate regulatory genes

A series of ESTs showing homology to genes with known regulatory functions are isolated from a λ gt10 cDNA library prepared from mRNA isolated from crowns of tetraploid alfalfa (*M. sativa* L. cv. Apica; R. Michaud, C. Richard, C. Willemot and H. Gasser 1983. Can. J. Plant Sci. 63: 547-549):

20

Transcription factors (zinc-finger and AP2 proteins)

Mitogen-activated protein kinases (MMK4, MMK3, MMK2, MMK1)

Calcium-dependent protein kinases

25

Serine-threonine protein kinase

Ca^{2+} transporting ATPase

GTP binding proteins

RNA binding proteins

Protein phosphatases (type 2A and 2C)

30

Calmodulin

14-3-3 proteins

GTPase activating proteins

Adenylyl cyclase protein

Phospholipase C
 Lipoxygenase
 Ca²⁺-binding proteins
 Histone deacetylase
 5 Receptor kinase
 Phosphatidyl inositol3-kinase

Genetic mapping of candidate regulatory genes

10 Probes for specific regulatory genes listed in table 2 are prepared by PCR
 amplification, purified on Sephacryl S-200 MicroSpin Columns (Amersham
 Pharmacia Biotech, Oakville, ON) and stored at -20°C. These probes are used for
 Southern hybridization to restricted DNA from F₂ diploid genotypes of the progeny of
 the cross described in Example 1. Purified probes are radiolabeled to high specific
 15 activity by random hexamer labelling and hybridized to DNA blots according to
 standard protocols for RFLPs as described in Example 1 (Sambrook, J., Maniatis, T.
 and Fritsch. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor
 Laboratory Press, Col Spring Harbor, New York). Regulatory genes that are
 polymorphic and that are being mapped along with their putative functions based on
 20 homologies with sequences in GENBANK/EMBL data bases are presented in Table
 2.

TABLE 2 Polymorphic regulatory gene and their putative functions. Highlighted
 clones indicate potential candidate genes involved in the regulation of msaCI genes or
 25 freezing tolerance (LT₅₀).

Clone	Homology to
C102	Defender against apoptotic death-1
C103	Pumilio RNA binding protein
C113	Nucleolin
C123	EF-hand calcium binding protein

C127	MADS transcriptional factor
C157	ABA-induced protein
B166	GTP binding protein
A168	Transcription factor
C183	GTP binding protein
B187	G-box binding protein
A207	Protein kinase C (substrate)
C221	Tyrosine phosphatase
A271	Serine protein kinase
C343	Homeobox protein (knotted-1)
C374	Peptidyl-Prolyl cis-trans isomerase
C389	ABA response enhancer
C532	Helix-loop-helix transcription factor
C571	GTP binding protein
C834	P72 DEAD box protein
C948	Casein kinase
C973	Mitogen activated protein kinase (MMK4)
C2105	WRKY3 DNA binding protein
C2186	Zinc finger transcription factor (SCOF-1 homolog)
C2250	GTP binding protein (Ras-related)
C2314	Phosphatase 2C
C2352	Progesterone binding homolog
C2420	C²⁺ transporting ATPase-like protein
C2426	Ethylene responsive small GTP binding protein
C2456	GTP binding protein (RAN1A)
C2595	Calreticulin
C2658	Calmodulin domain protein kinase (CDPK)
C2784	Serine/threonine protein kinase
C2886	Cyclase-associated protein (CAP)
C2939	AP2 domain transcription factor (TINY-like)
C2988	Leucine rich repeat (LRR) receptor-like protein

	kinase
C3050	Protein kinase
C3125	Nucleic acid binding protein
C3253	Receptor kinase
C3268	Serine-threonine protein kinase
C3283	Ethanolamine kinase
C3291	IAA-induced ARG-2 protein
C3346	Ethylene responsive transcription factor
C3358	Adenosine kinase
C3367	RNA binding protein
C3506	Protein kinase
C3520	Nucleotide binding protein
C3601	Ring-H2 finger protein
C3604	Transcription factor
C3626	DNA binding protein
C3749	CDC2+/CDC28-related protein kinase R2
C3843	Phosphatidyl inositol 3-kinase
C3844	GTP binding protein (RAB-type)
C4050	Serine/threonine phosphatase type 2A
C4266	RAC-like protein (RHO homolog)
C4320	Mitogen activated protein kinase
C4438	14-3-3 protein
C4494	AP2 domain transcription factor (CBF1 homolog)
C4546	Mitogen activated protein kinase (MAP3K)
C4622	Calmodulin domain protein kinase (CDPK)
C5047	Leucine rich repeat (LRR) receptor-like protein kinase
C5206	Homeobox protein (Knotted-1)
C5237	Cell division cycle protein 48

EST sequences are also used to design primers for the amplification of the identified loci as STS (Sequence Tagged Sites) from DNA of the individuals of the segregating population. When the resulting PCR products are monomorphic among the individuals of the population, the PCR products are restricted with various enzymes to
5 detect polymorphism. If no polymorphism is detected the PCR products obtained from the two parents are individually sequenced and SNPs (Single Nucleotide Polymorphisms) are searched.

The candidate regulatory genes described above are mapped as RFLPs within
10 the F₂ progeny as described in Example 1 in order to assess whether they are localized within QTLs associated with variation in cold-induced expression of msaCI genes as identified in Example 1.

All genes listed in Table 1 and highlighted in table 2 are located in genomic
15 regions containing QTLs and are therefore putative candidate regulatory genes for the expression of msaCI genes, the LT₅₀ trait, or a combination thereof. Two of these genes C4494 and C2186 show homology to the CBF1 gene from Arabidopsis and the SCOF-1 gene from soybean, respectively, for which an involvement in cold tolerance has been demonstrated (Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G.,
20 Schabenberger, O., and Thomashow, M.F. 1998. *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. Science 280: 104-106. Kim, J.C., Lee, S.H., Chsong, Y.H., Yoo, C.-M., Lee, S.I., Chun, H.J., Yun, D.J., Hong, J.C., Lee, S.Y., Lim, C.O., and Cho, M.J., 2001. A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. The
25 Plant Journal 25: 247-259). C4494 (CBF1 homolog) is located at a QTL associated with the expression of msaCIB in leaves (linkage group F, Figure 2), whereas C2186(SCOF-1 homolog) is mapping to a region containing a QTL for LT₅₀ (linkage group C, Figure 2). These genes encode cold induced transcription factors that were unequivocally shown to drive the constitutive expression of COR (msaCI) genes in
30 transgenic plants and to increase freezing tolerance under non-acclimated conditions.

The results also indicate a number of interesting novel candidate regulatory genes involved in the expression of cold tolerance and msaCI gene expression (Table

1, Figure 2). For example the clone C2784, which shows homology to a serine/threonine protein kinase, maps to a location on linkage group B where QTLs for the expression of msaCIA, msaCID, msaCIE and msacIG in leaves are found. In alfalfa cells, osmotic stress led to the rapid activation of two protein kinases. One of these kinases appears to be a homologue of the ASK1 serine/threonine kinase 1 from *Arabidopsis* (Munnik, T. and H.J.G. Meijer. 2001. FEBS Letters 498: 172-178). Confirmation of the roles of such candidate regulatory genes may be obtained using methodologies outlined below.

10 Sequencing of genomic clones of candidate regulatory genes and candidate regulatory sequences

ESTs of candidate regulatory genes that map to one or more QTLs associated with variation of the expression of msaCI genes are isolated from a genomic library constructed in EMBL3 phage (Sambrook, J., Maniatis, T. and Fritsch. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), using DNA from the two parents identified above. Each candidate regulatory gene is sequenced in its entirety including upstream and downstream regions of the coding region using the dideoxynucleotide chain termination method (Sambrook, J., Maniatis, T. and Fritsch. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Comparisons between allelic forms from each parent is also made to identify mutations responsible for the QTL. Confirmation of the localization of candidate regulatory genes is carried out by mapping upstream or downstream regions of each regulatory gene on the linkage map generated above. Only regulatory genes that are characterized by having 5' or 3' regions co-mapping with the same one or more QTLs are used for further characterization in complementation or gene knockout studies.

30 Altered expression of candidate genes in transgenic plants (complementation and knockout studies)

Alfalfa transformation is performed using *Agrobacterium tumefaciens* as previously described by Desgagnés et al. (Desgagnés R., S. Laberge, G. Allard, H. Khoudi, Y. Castonguay, J. Lapointe, R. Michaud and L.-P. Vézina. 1994. Plant Cell Tissue Organ Cult 42: 129-140). Two approaches are taken; 1) Constitutive
5 expression of the candidate gene using a gene fusion to a constitutive promoter, for example, but not to be limited 35S; and inducible expression. The coding region of the candidate gene is fused in frame to a full-length 35S promoter and the resulting construct is cloned into the binary expression vector pGA482. Full length candidate regulatory genes (i.e. candidate regulatory genes that, with surrounding 5' and 3'
10 regions, map to one or more QTLs identified above) along with approximately 1kb of 5' and 1 kb of 3' non-coding regions, are cloned into the binary expression vector pGA482. Constructs for both constitutive and inducible expression in the sense orientation are used to transform plants that lacks the allele at the QTL corresponding to expression of the trait of interest (for example, but not to be limited to msaCI gene
15 expression), and the antisense orientation constructs are used to transform plants that harbor the allele at the QTL. The transgenics are then grown and subject to cold acclimation conditions, as needed to determine the effect of the introduced sense or antisense gene on both expression of one or more msaCI genes and the LT₅₀, thereby validating the candidate gene as the regulatory factor controlling mRNA expression of
20 the msaCI genes at the QTL.

The present invention has been described with regard to preferred
25 embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

**THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE
PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:**

1. A method for identifying one or more regulatory genes mediating expression of a plurality of genes of interest in an organism comprising:
 - i) identifying a first organism of interest characterized in that said first organism of interest exhibits a measurable response to an environmental stimulus, or otherwise exhibits a first phenotype associated with differential gene expression associated with a process of interest;
 - ii) identifying a second organism of interest characterized in that said second organism of interest:
 - a) lacks or does not exhibit as strong said measurable response to said environmental stimulus when compared with said first organism of interest;
 - b) exhibits a second phenotype compared with said first phenotype, said second phenotype being associated with said process of interest and different from said first phenotype;
 - c) exhibits a phenotype of interest segregating in a population resulting from a cross with said first organism of interest; or
 - d) a combination of two or more of a, b and c ;
 - iii) crossing said first and second organisms of interest to produce a population of segregated progeny;
 - iv) extracting RNA from each of said population of segregated progeny, and quantifying the level of said expression of said plurality of genes of interest, said plurality of genes of interest associated with said response to an environmental stimulus, or said process of interest;

- v) identifying a single Quantitative Trait Locus (QTL) using a marker, or set of markers, that comprise one or more regulatory genes, the single QTL related to a trait induced by said environmental stimulus or other process of interest of step i, and related to said expression of said plurality of genes of interest;
 - vi) identifying, isolating and characterizing one or more regulatory genes located at said single QTL; and
 - vii) confirming said one or more regulatory gene is involved in said expression of said plurality of genes of interest under conditions of step i resulting from:
 - a) said measurable response to said environmental stimulus; or
 - b) said differential gene expression associated with said process of interest.
2. The method of claim 1, wherein after said step of crossing (step iii), and prior to said step of extracting RNA (step iv), said segregated progeny are subjected to a desired environmental stimulus or are characterized as being at a specific developmental stage.
3. The method of claim 1, wherein said method is used for the identification of said single QTL corresponding to a transcription factor or any factor controlling the expression of said plurality of genes of interest.
4. The method of claim 1, wherein in said steps of identifying (steps i and ii), said environmental stimulus, or said process of interest is selected from the group consisting of:
- a biotic stress;
 - an abiotic stress;
 - an alteration in mineral nutrition;
 - an alteration in light quality or flux;
 - a response arising from an addition of one or more exogenous chemicals;
 - a stress leading to an alteration in levels of endogenous chemicals within a plant;
 - a developmental, chemical or environmental control of gene expression during embryogenesis;
 - organogenesis;

- senescence;
- maturation;
- dormancy;
- germination;
- circadian, ultradian and other internal rhythms;
- harvestable yield;
- seed set;
- photosynthate translocation;
- an alteration in sink and source capacities within a plant;
- an alteration of leaf area index;
- an alteration of root shoot ratio;
- an alteration in nutritive value of harvested material;
- an alteration in plant morphology;
- a change in rate of cell cycle;
- an alteration in rate of cell differentiation;
- an alteration in cell size;
- an alteration in plant life cycle; and
- genomic rearrangements.

5. The method of claim 1, wherein in said step of identifying (step v), a marker, or a set of markers is identified at said single QTL.
6. The method of claim 1, wherein in said step of identifying, isolating and characterizing (step vi), said plurality of genes are isolated and sequenced.
7. The method of claim 1, wherein the identifying, isolating and characterizing step further comprises the steps of:

mapping one or more candidate regulatory genes;

identifying said one or more regulatory genes mapping with said single QTL, said one or more regulatory genes being a subset of said one or more candidate regulatory genes;

isolating said one or more regulatory genes within said single QTL resulting in one or more isolated regulatory genes; and

sequencing said one or more isolated regulatory genes.

8. The method of claim 7, wherein the sequencing step results in one or more sequenced regulatory genes further comprising the step of :

confirming said one or more sequenced regulatory genes are involved in said expression of said plurality of genes for said trait under conditions of:

a) said environmental stimulus; or

b) said other process of interest.

9. The method of claim 1, wherein said step of identifying, isolating and characterizing (step vi), further comprises the step of obtaining said one or more regulatory genes using positional cloning.

10. The method of claim 1, wherein genetic transformation, gene complementation, gene knock-out techniques, or gene overexpression is used in said step of confirming (step vii), to confirm said one or more regulatory genes are involved in said expression of said plurality of genes of interest.

11. The method of one of claims 1 and 7-10, wherein the organism is a plant.

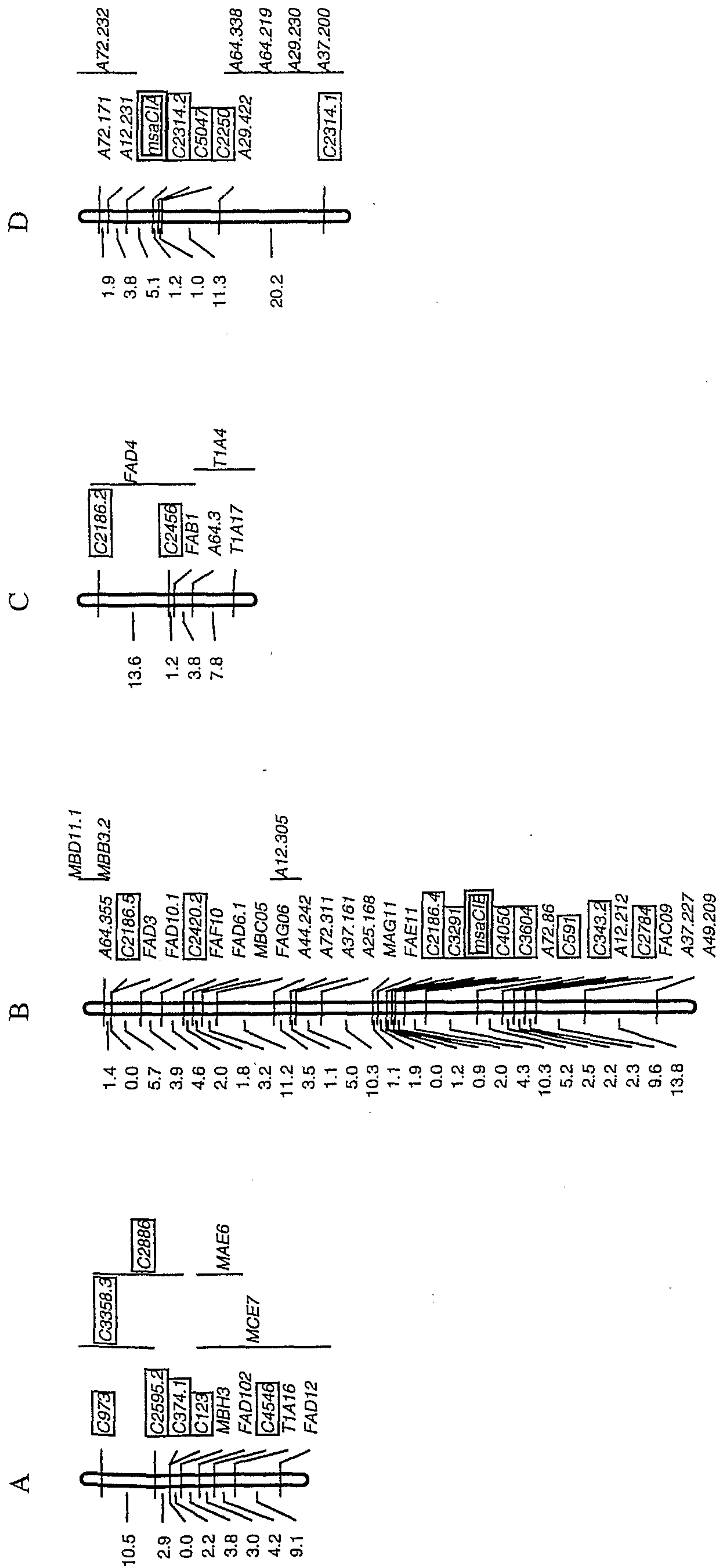


Figure 1

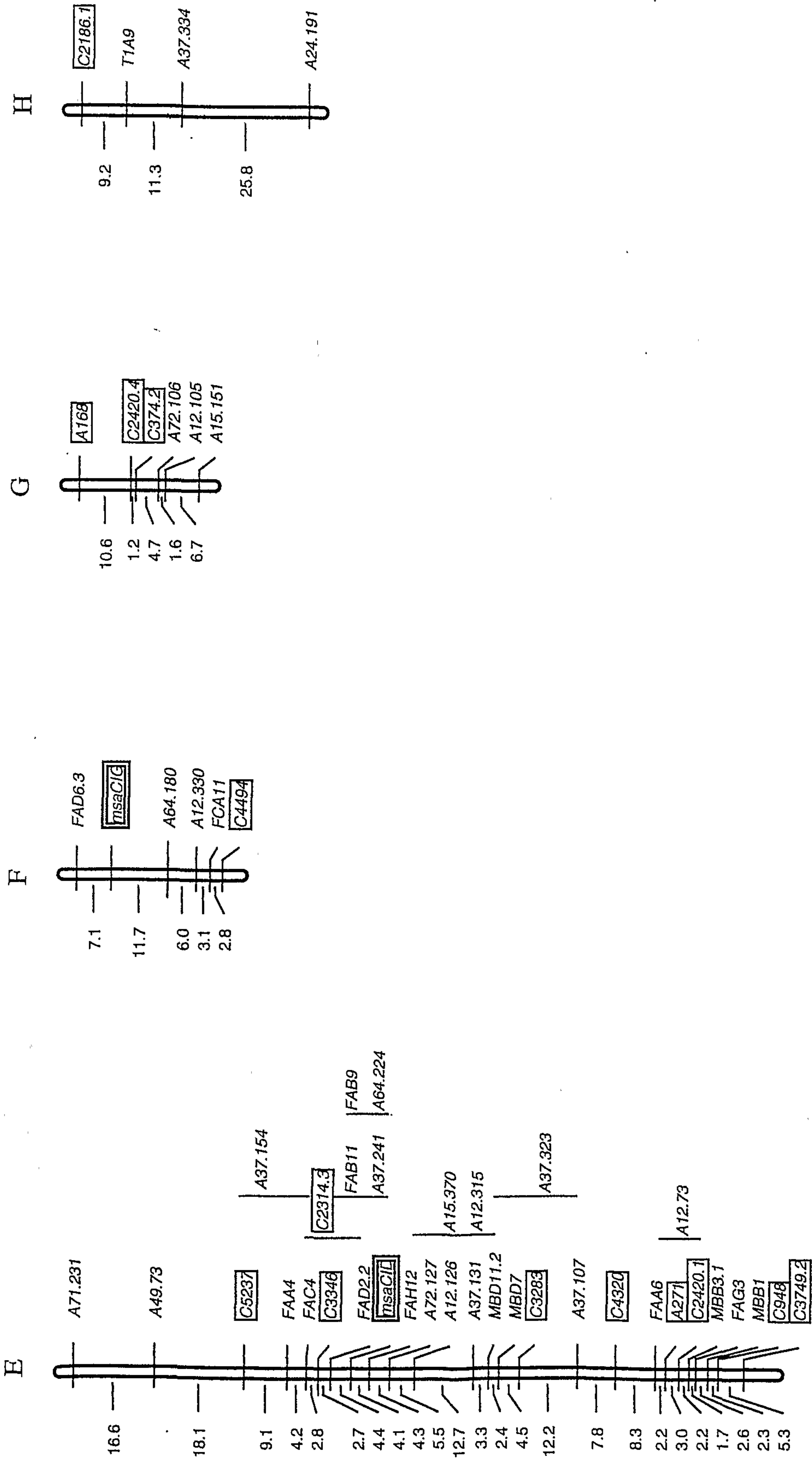


Figure 1 con't

3/13

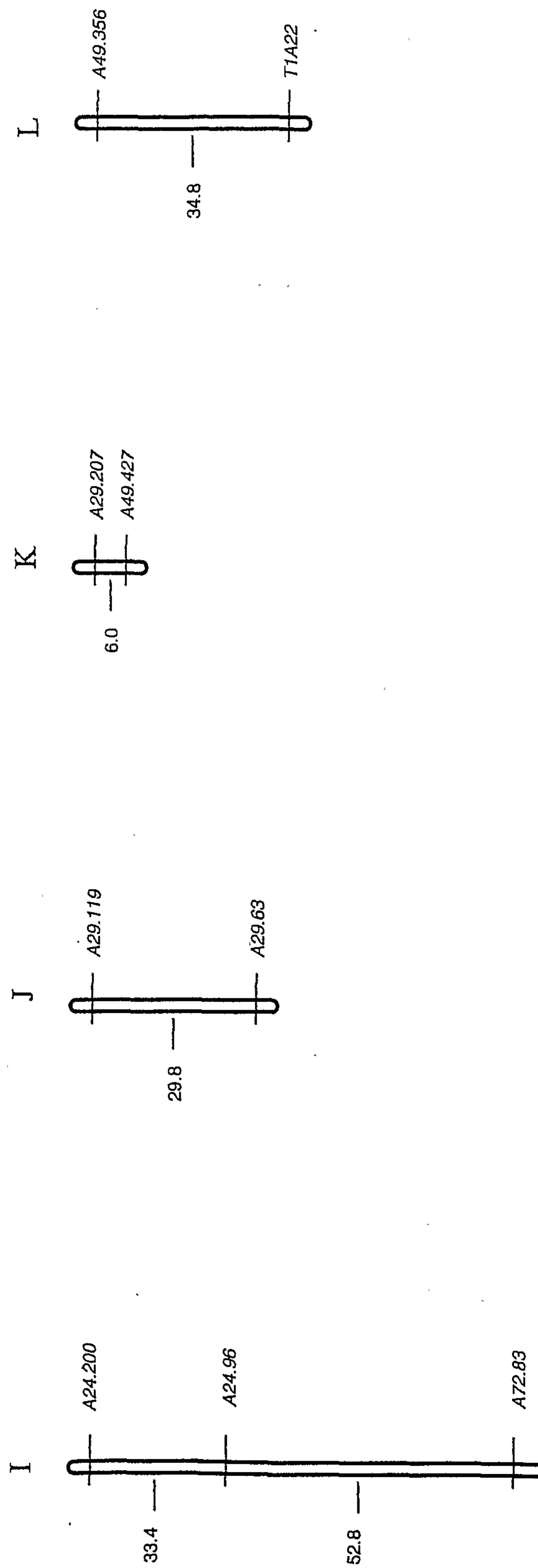


Figure 1 con't

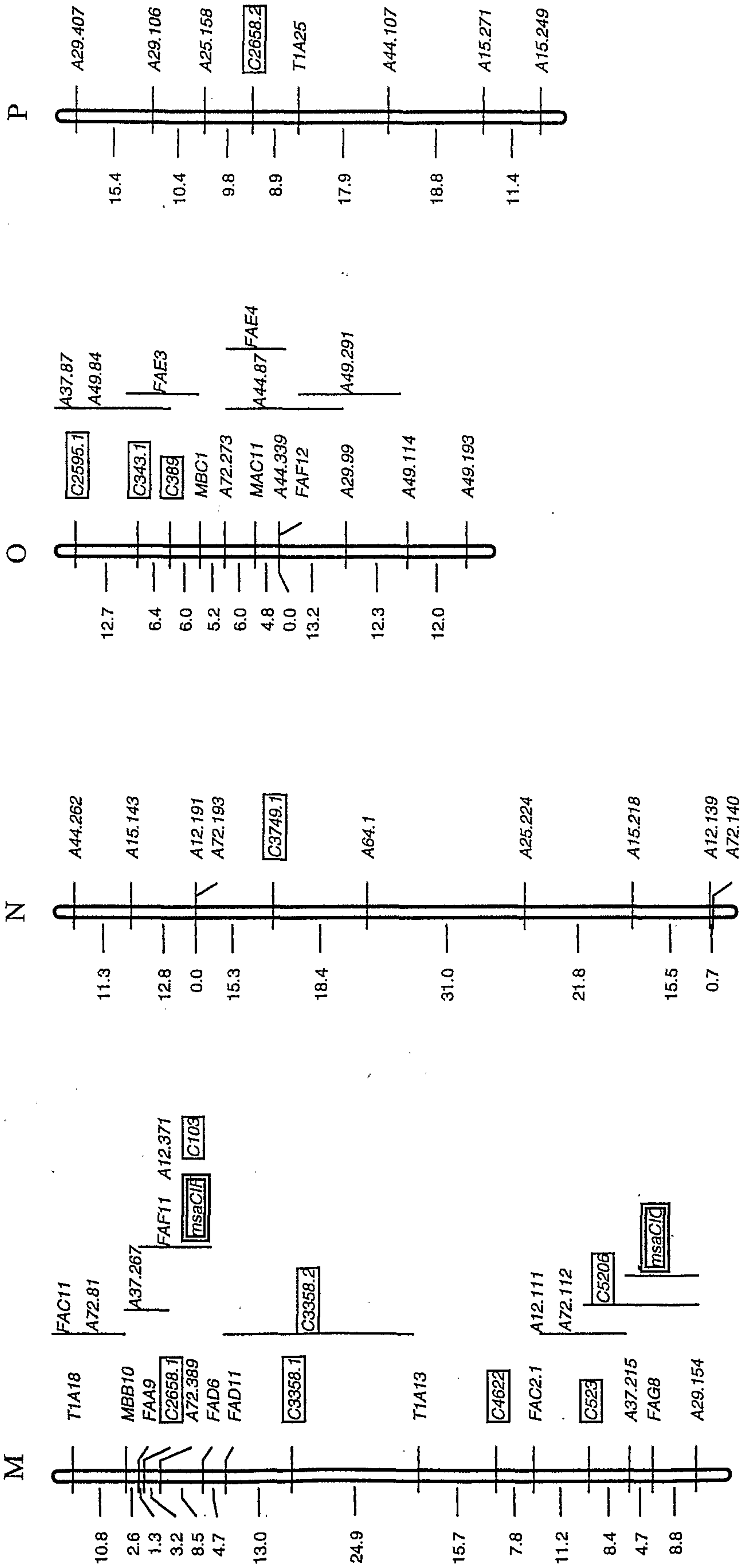


Figure 1 con't

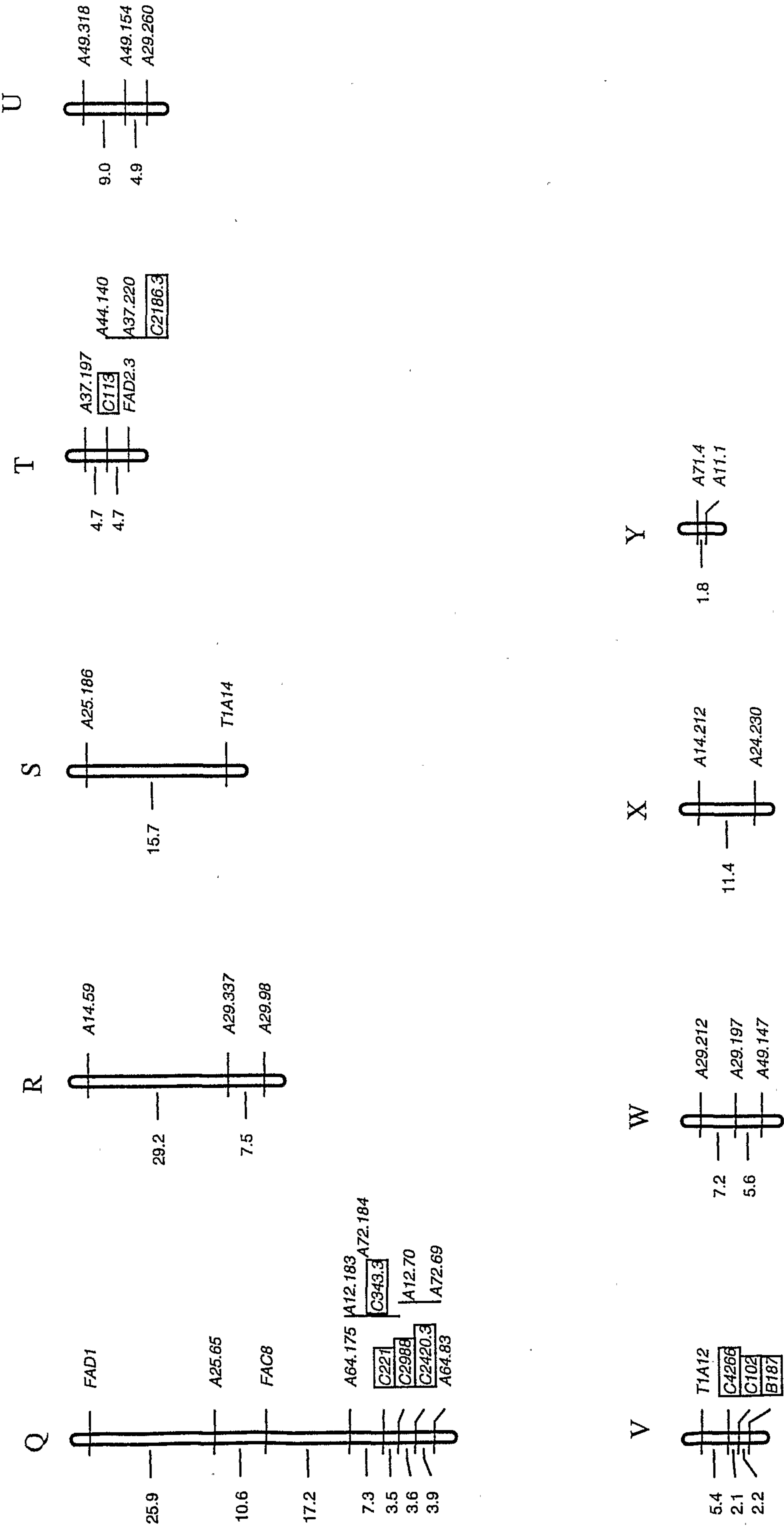
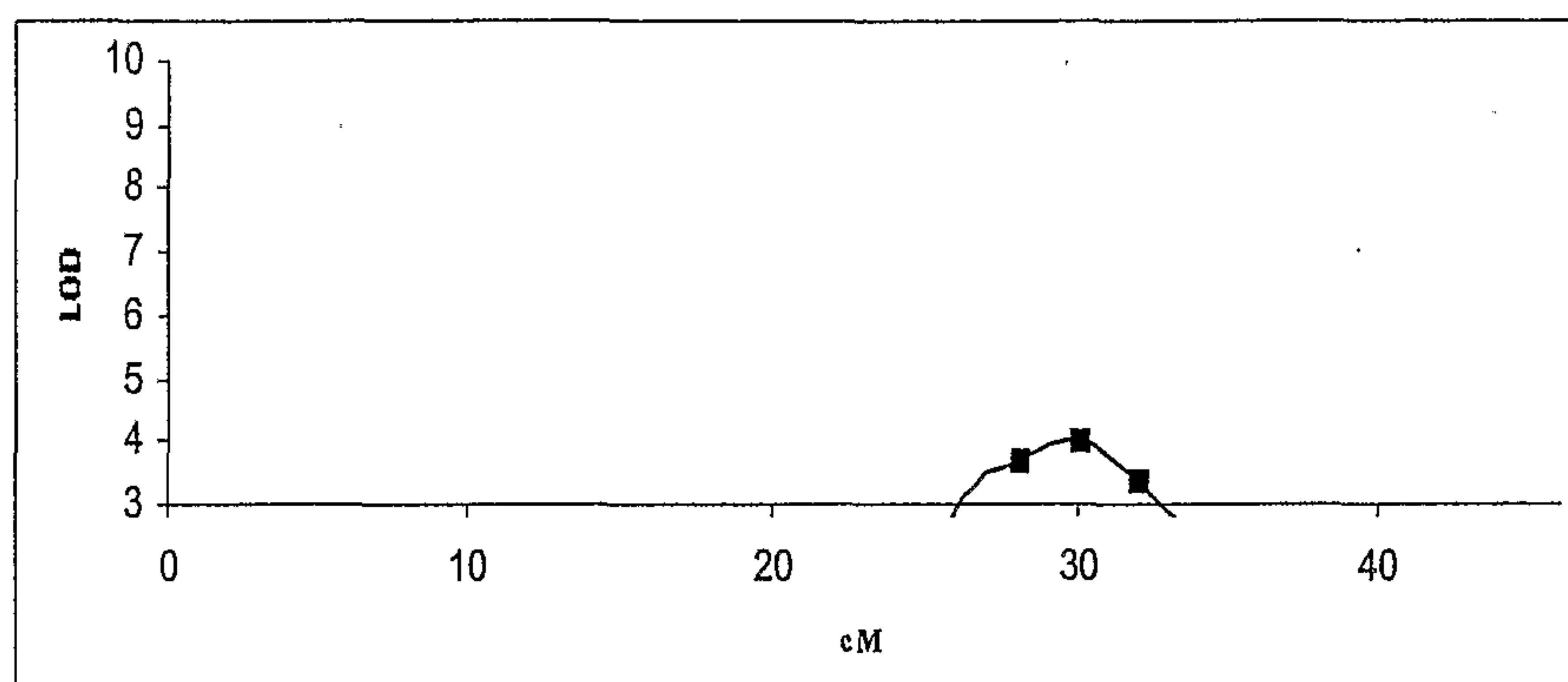


Figure 1 con't

6/13

Linkage group A

Crown

 msaCIC

C973 C2595.2 C374.1 C123 MBH3 FAD10.2 C4546 T1A16

Leaf

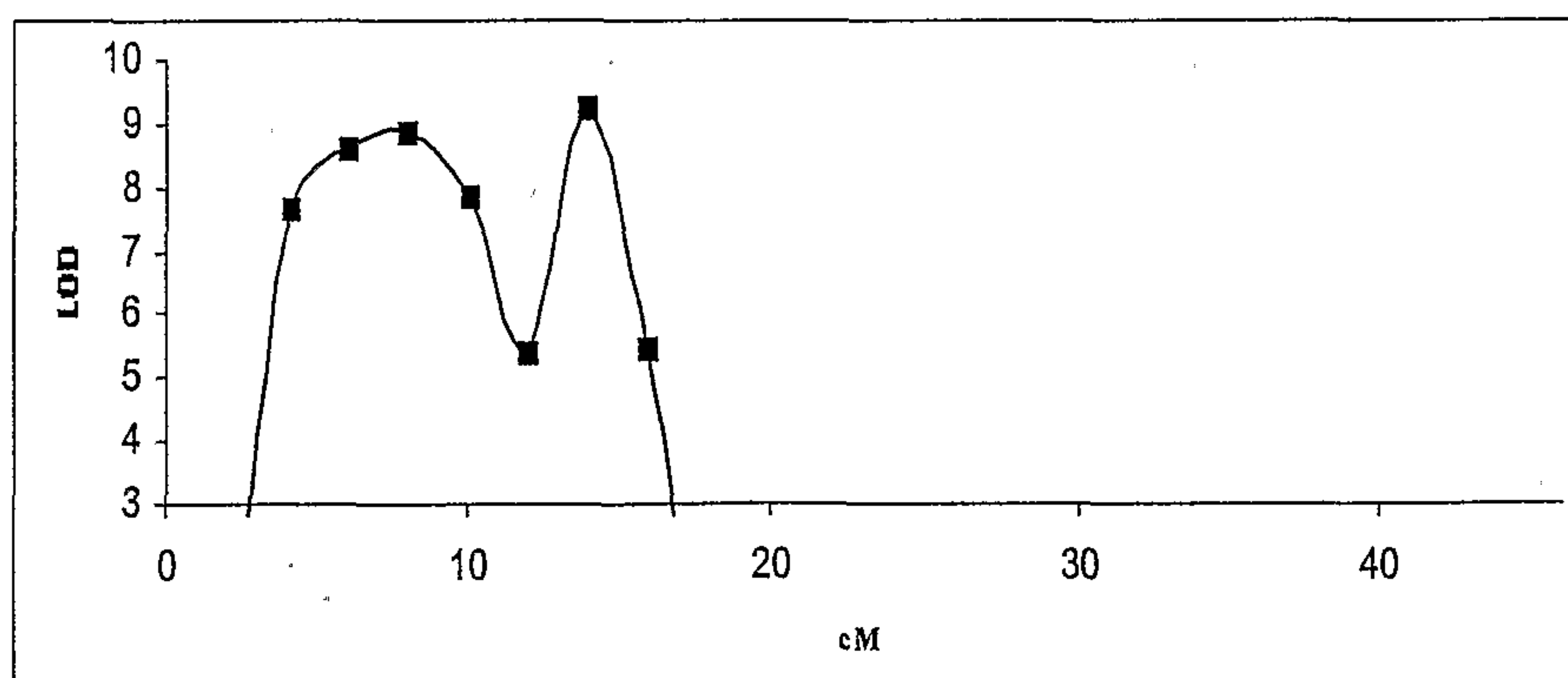


Figure 2

7/13

Linkage group B

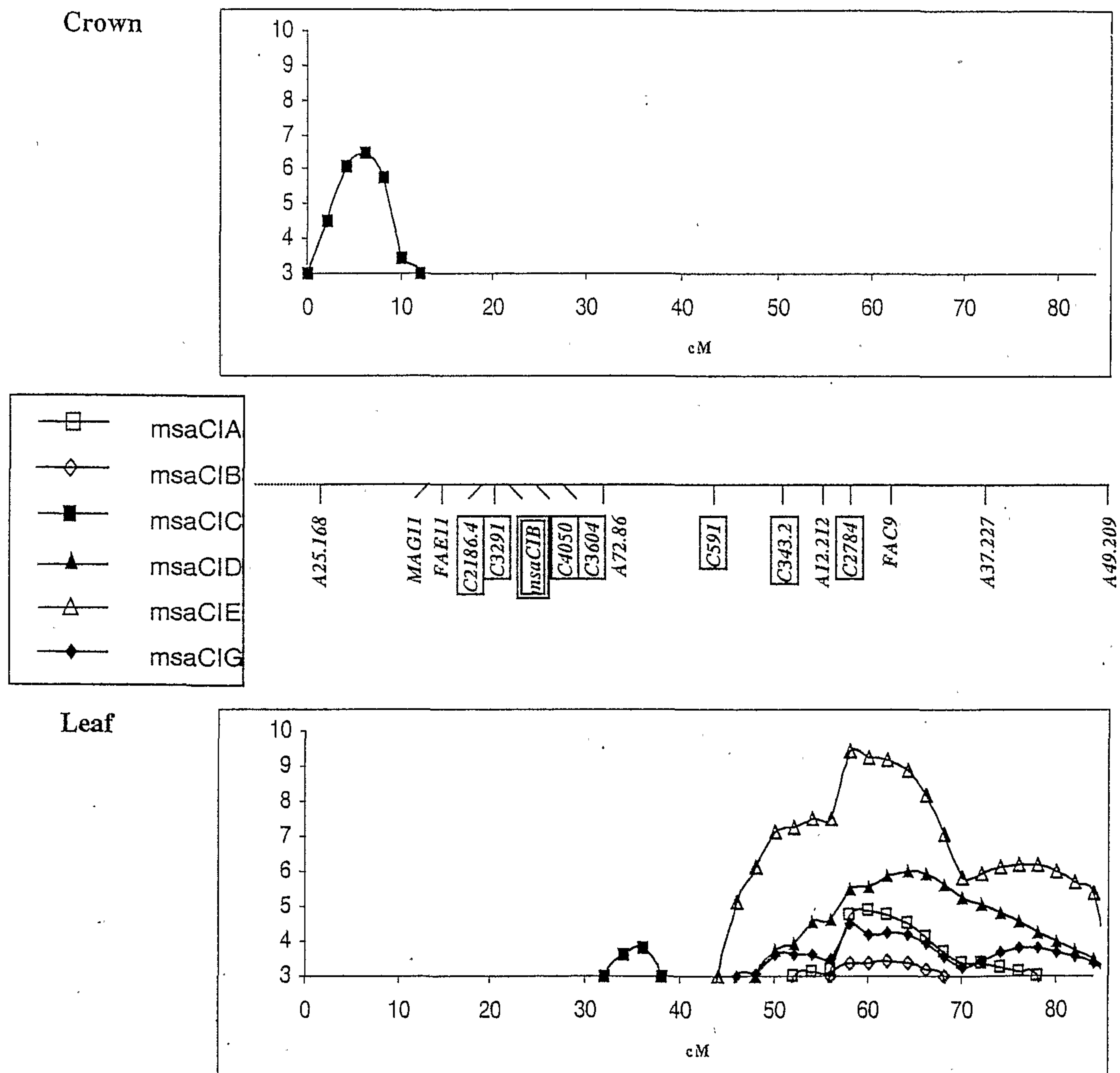


Figure 2 con't

Linkage group C

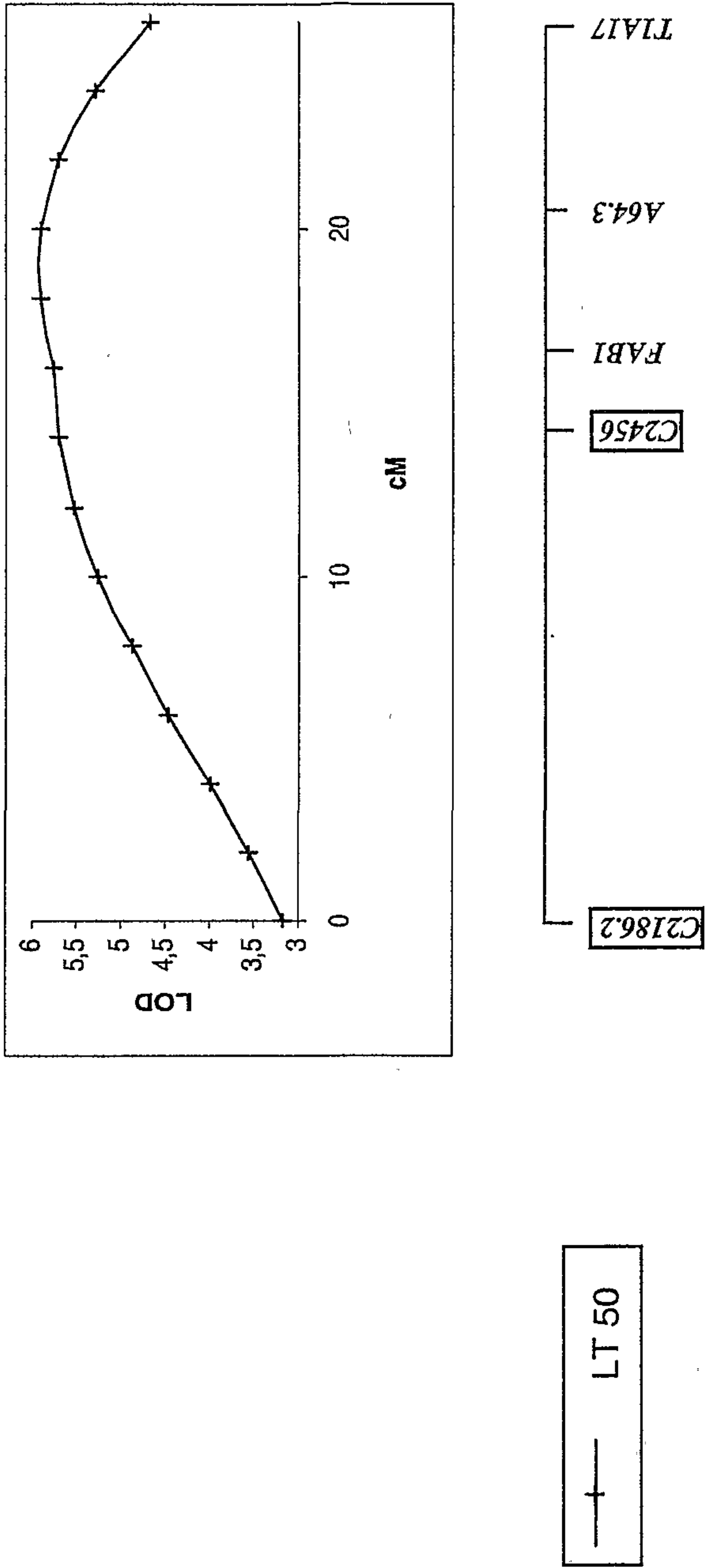
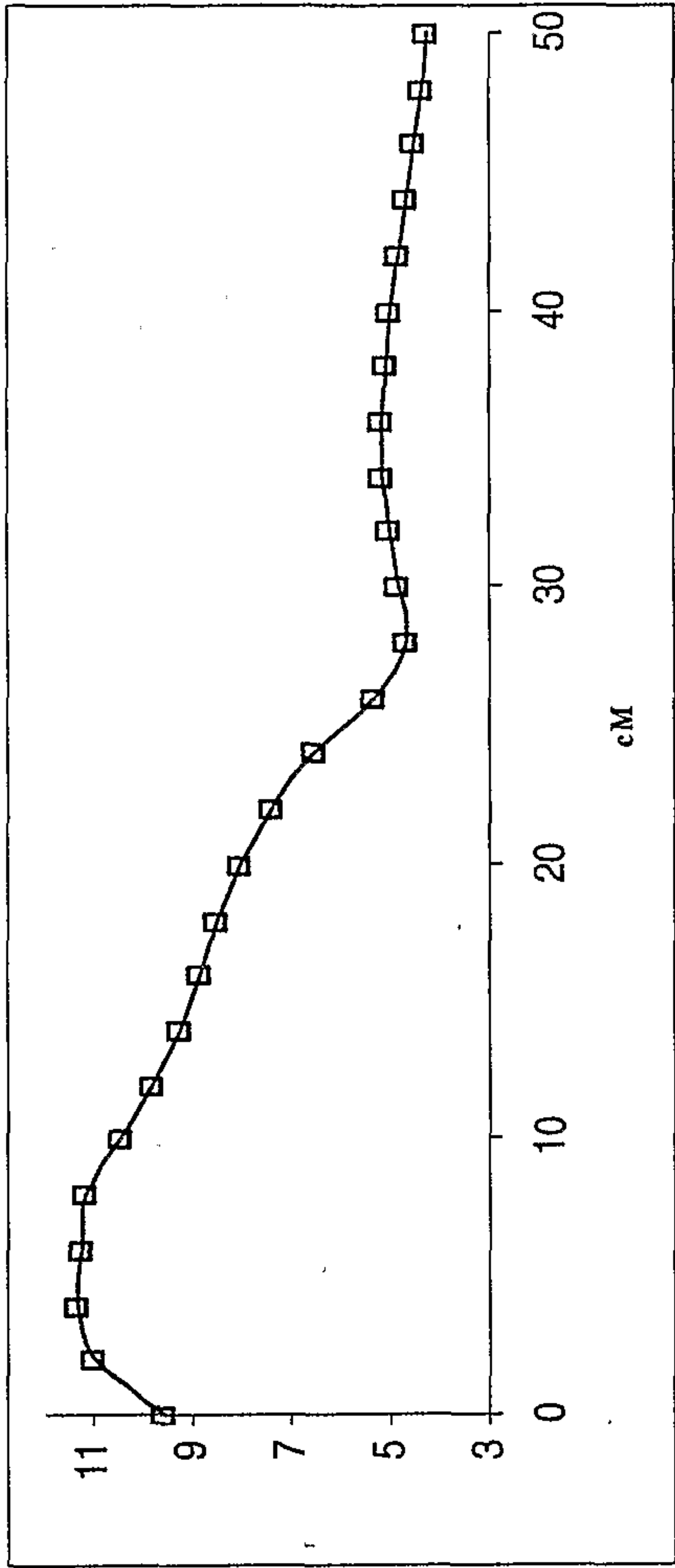


Figure 2 con't

Linkage group D



Crown

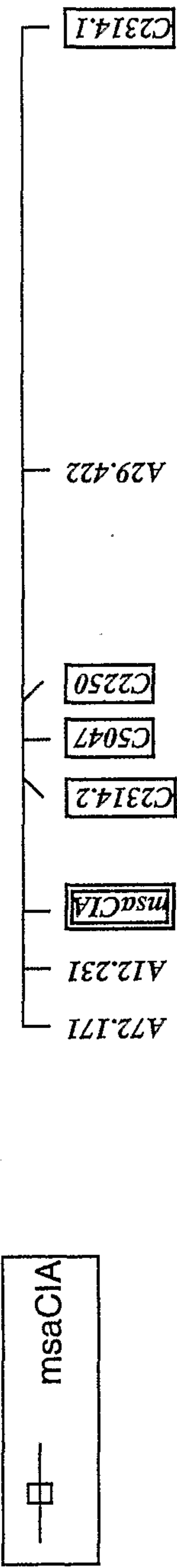
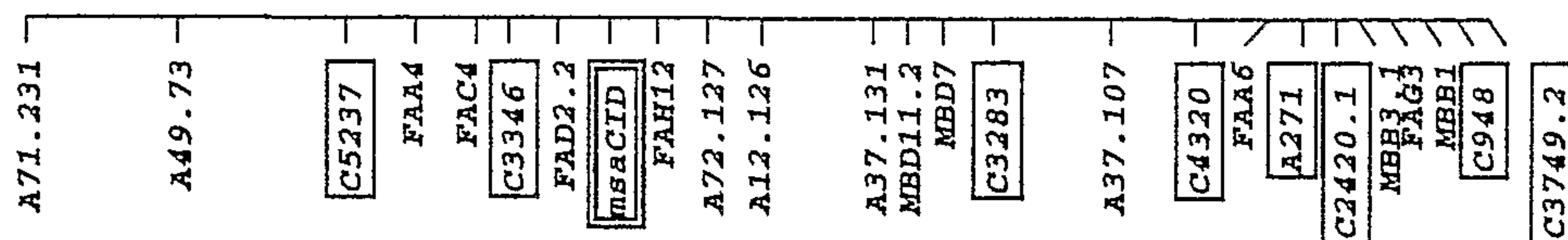
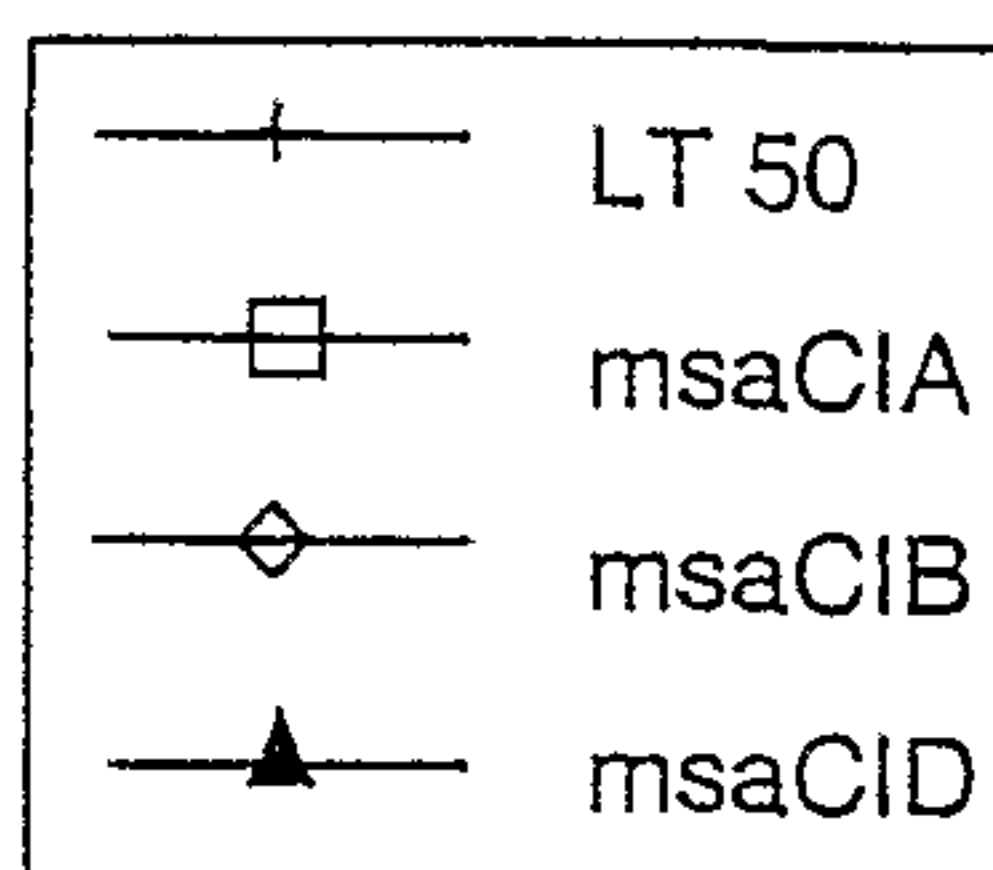
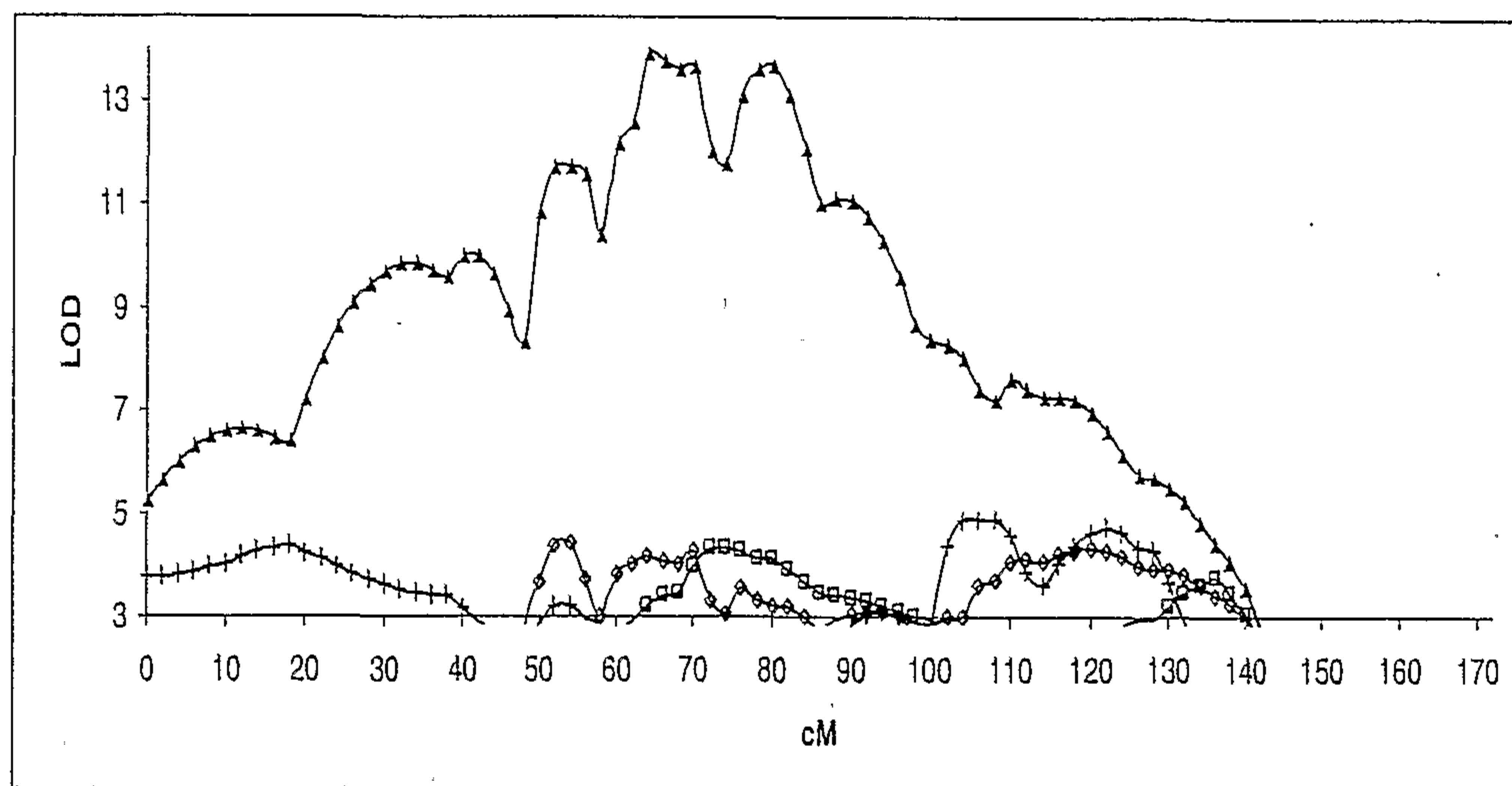


Figure 2 con't

10/13

Linkage group E

Crown



Leaf

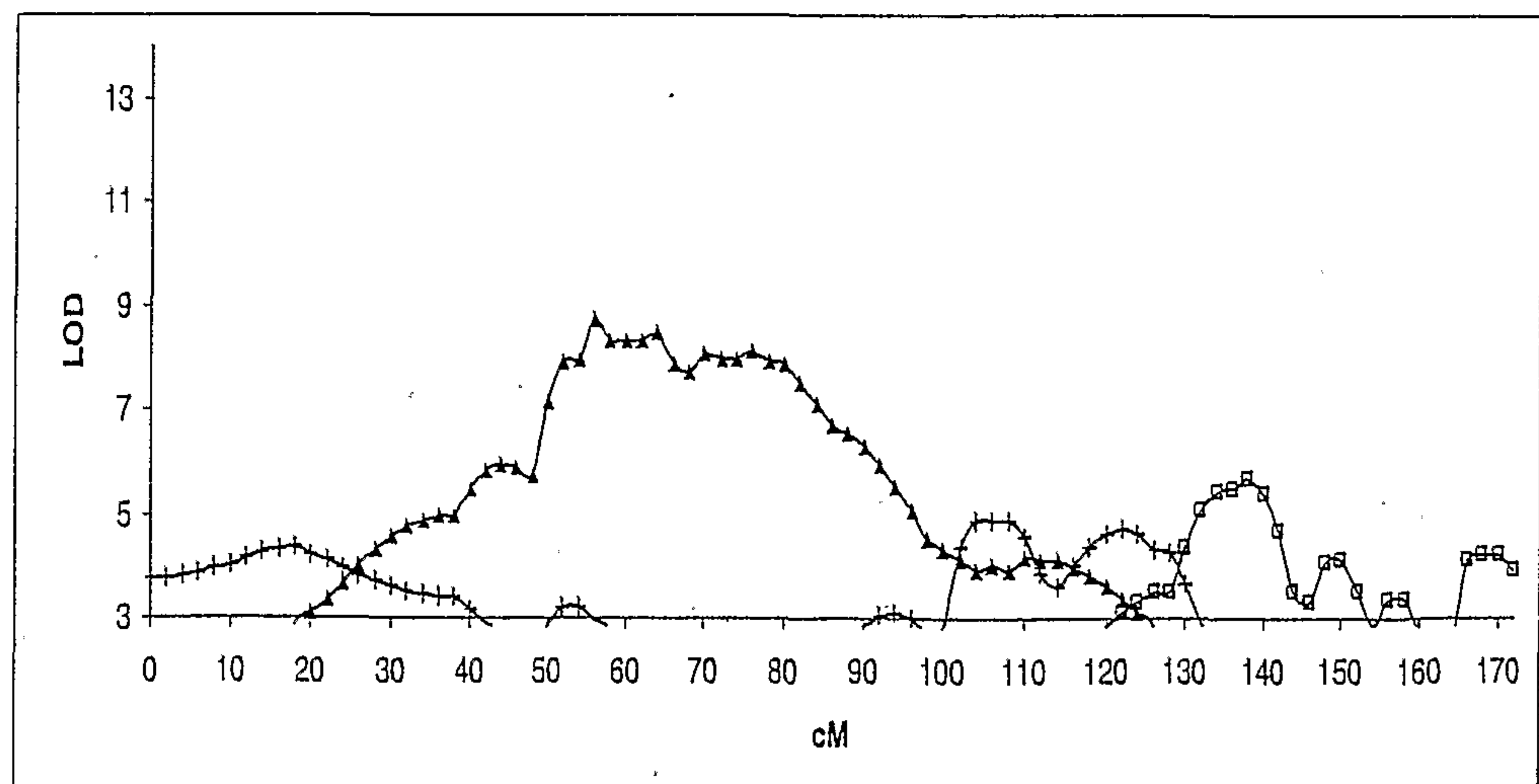
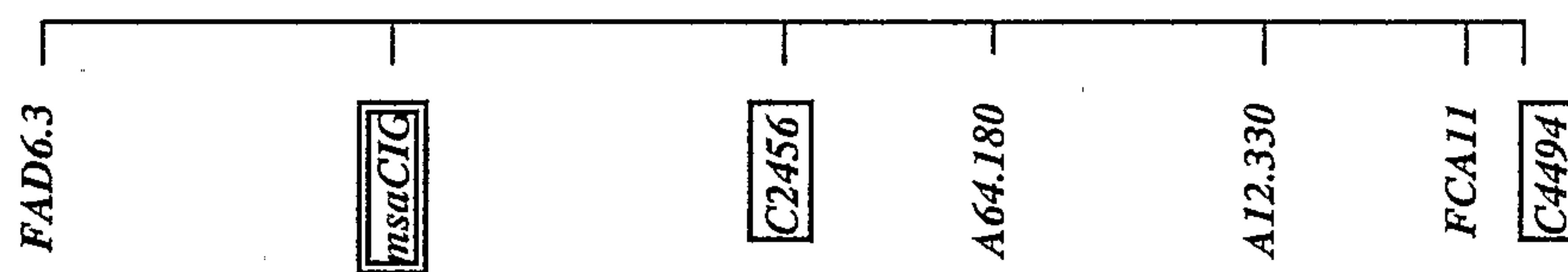
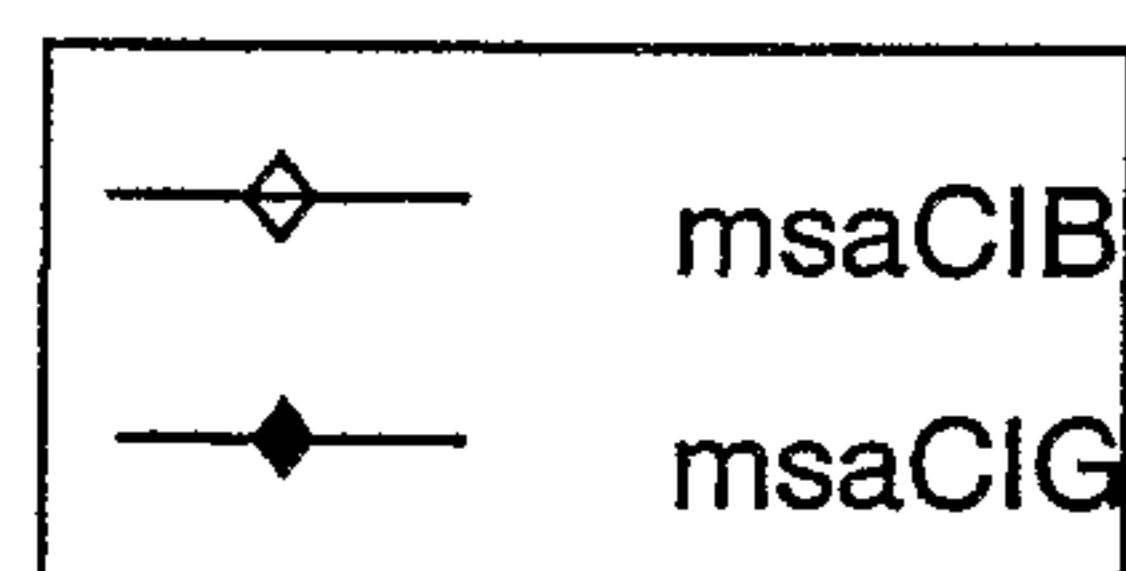
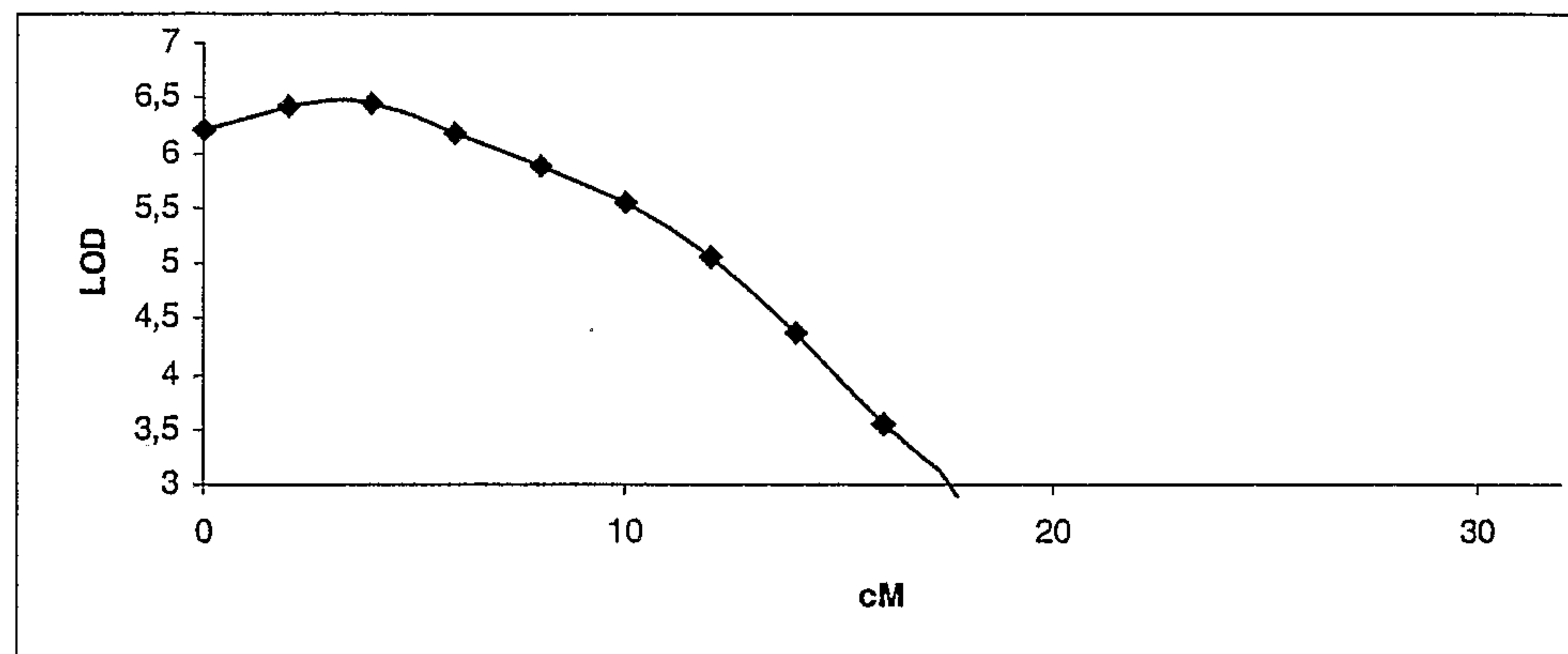


Figure 2 con't

11/13

Linkage group F

Crown



Leaf

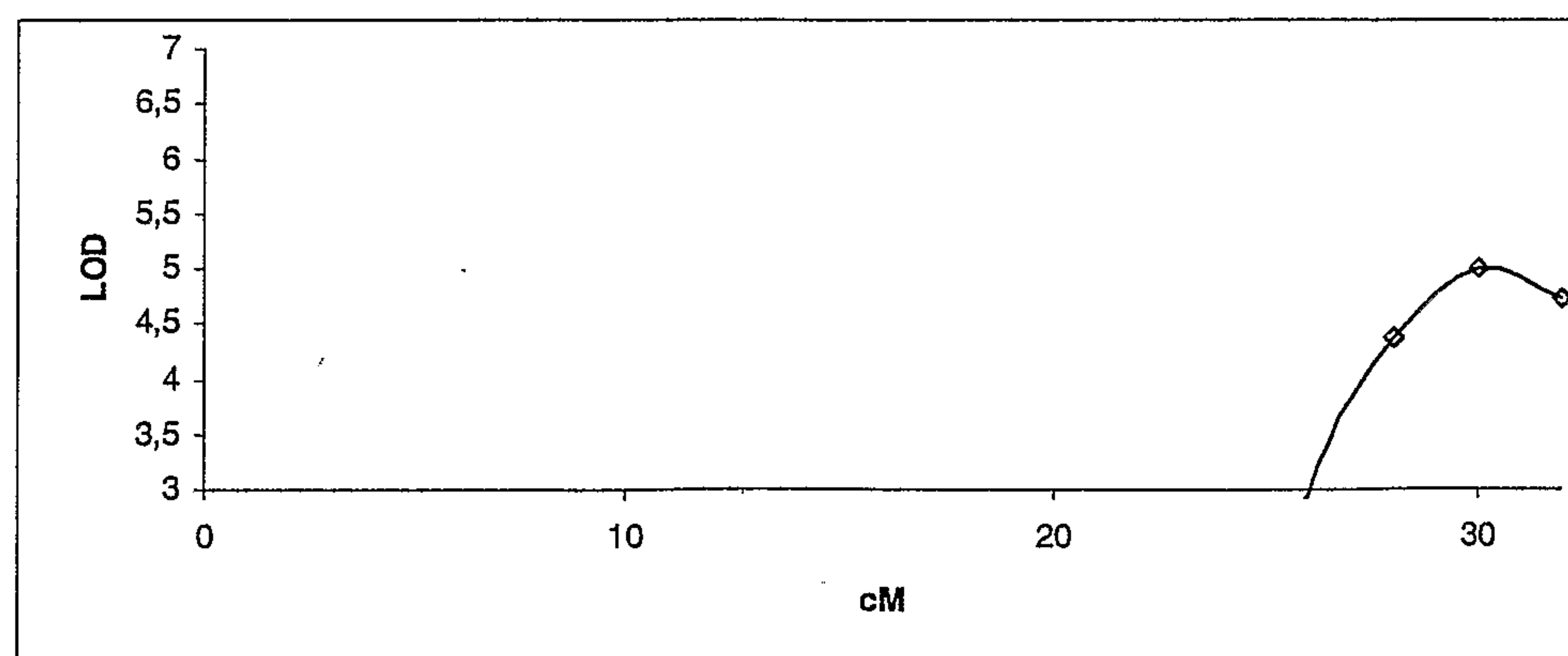


Figure 2 con't

12/13

Linkage group G

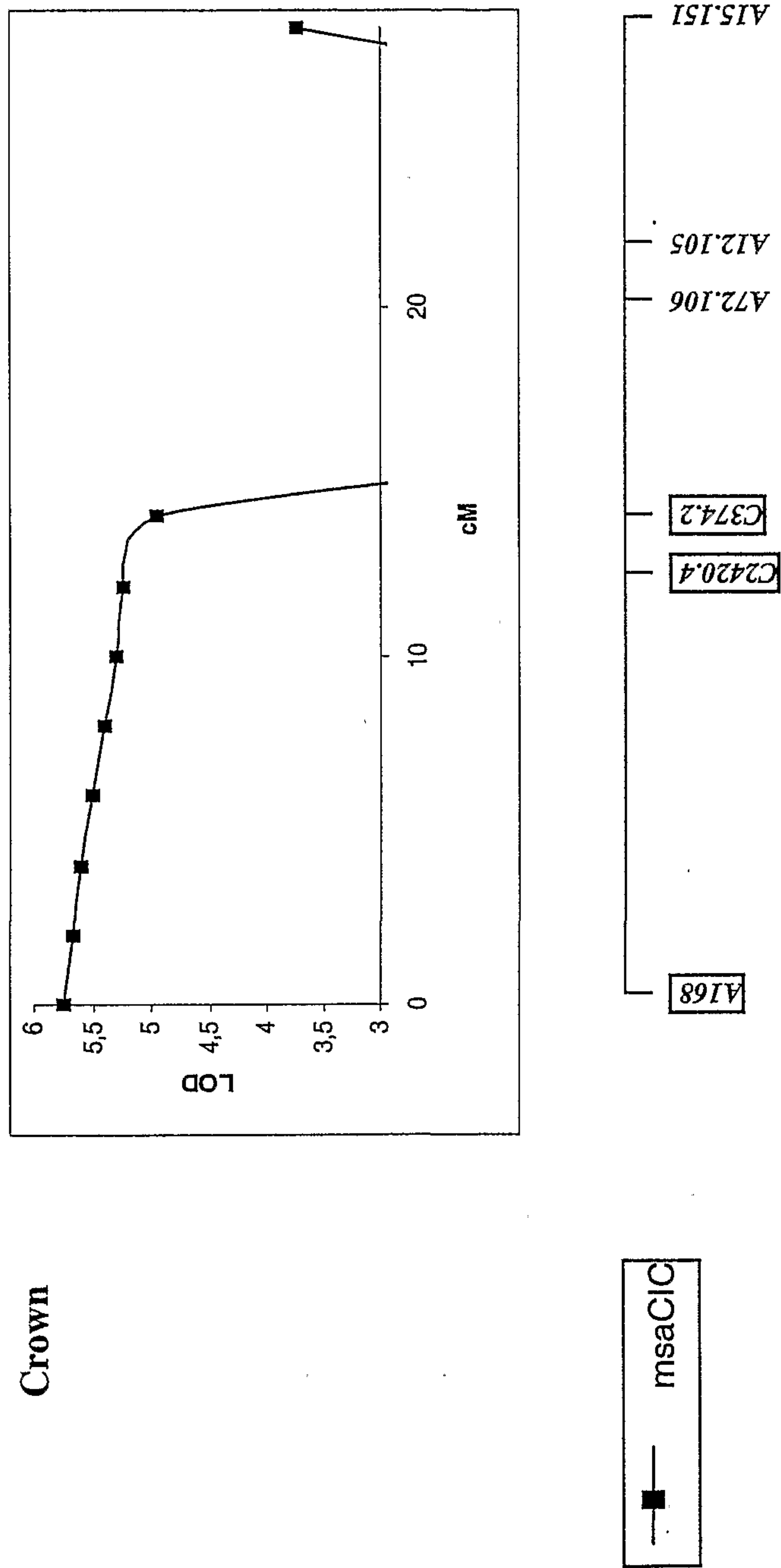


Figure 2 con't

13/13

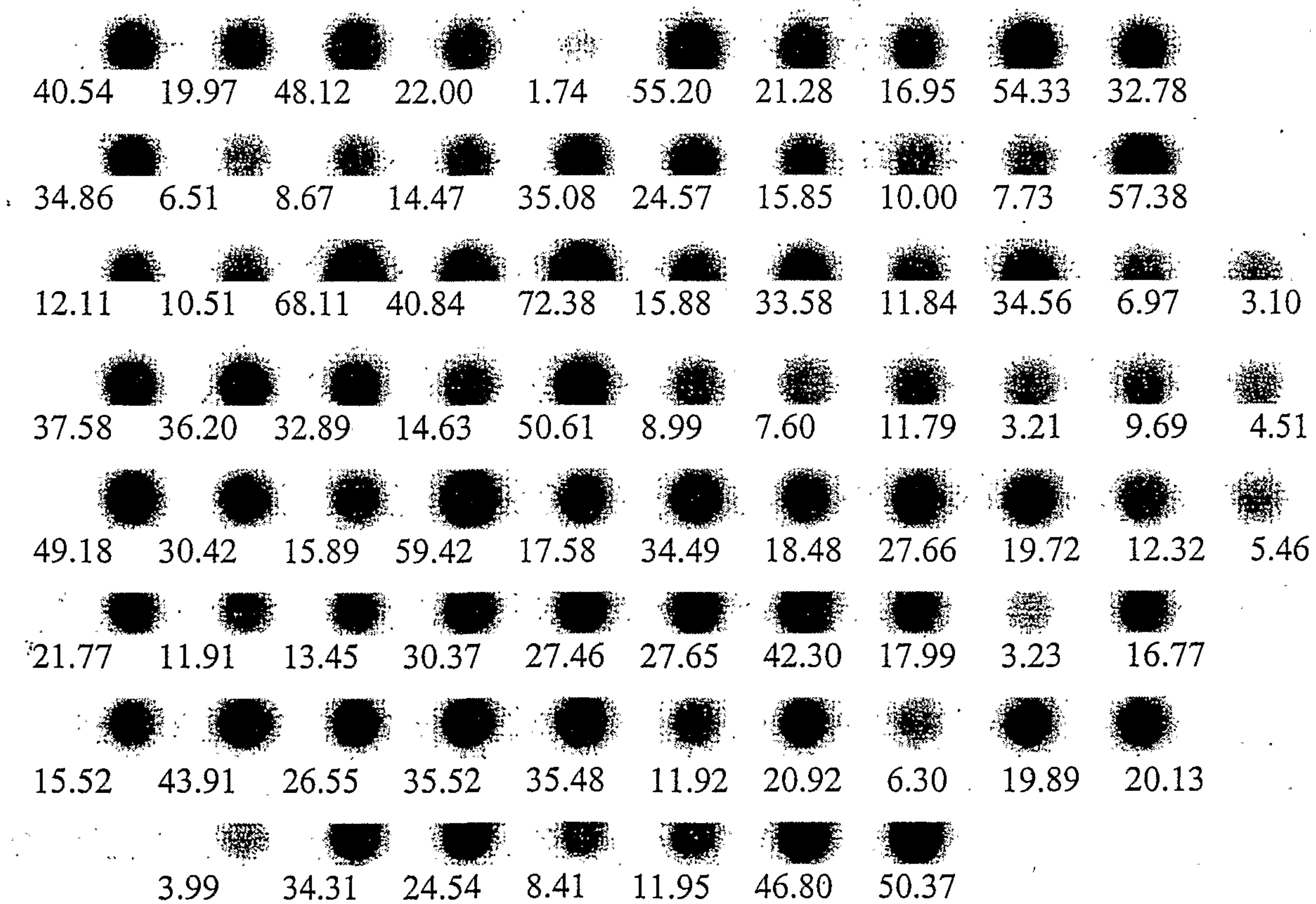


Figure 3

