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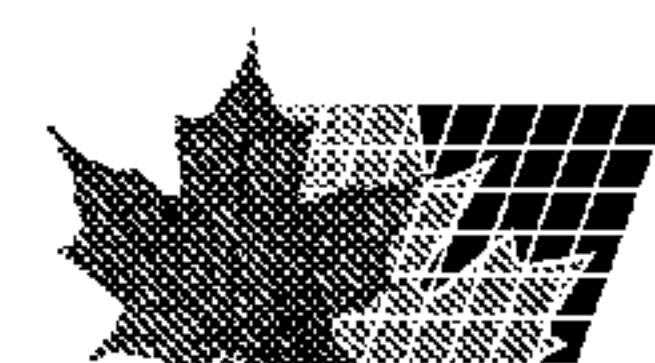
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(54) Titre : GENE SPL7 INHIBANT LA FORMATION DE LSIONS CHEZ LES VEGETAUX ET SON APPLICATION  
(54) Title: PLANT LESION FORMATION SUPPRESSING GENE, SPL7 AND USE THEREOF

(57) **Abrégé/Abstract:**

The present inventors successfully isolated a lesion formation suppressing gene (Sp17) from rice via a linkage analysis. It was revealed that introduction of the gene into plants enhances their heat stress resistance and that lesion formation can be suppressed.



**ABSTRACT**

The present inventors successfully isolated a lesion formation suppressing gene (*Sp/7*) from rice via a linkage analysis. It was revealed that  
5 introduction of the gene into plants enhances their heat stress resistance and that lesion formation can be suppressed.

**DESCRIPTION****PLANT LESION FORMATION SUPPRESSING GENE, *Sp17* AND USE  
5 THEREOF**Technical Field

The present invention relates to a gene involved in lesion formation on plants and heat stress resistance in plants, as well as methods of modifying  
10 plants via the gene. The present inventions have utility in agriculture fields, including plant-breeding technology.

Background Art

Various mutant strains of rice have been produced in the past. These  
15 include mutant strains where lesion-like necrotic spots are formed on the leaf in connection with the growth of the rice (Figure 1) (Rice Genetics Newsletter 12: 9-153 (1995)). This lesion formation occurs in these mutant strains under high temperature and strong light, thereby suggesting a relationship between the mutant genes and avoidance of damage by light or heat (Fuse T. et al.,  
20 Physiol.Plant 89: 799-804 (1993)). On the other hand, these genes are also postulated to be related to hypersensitivity reaction in plant cells and resulting cell death, because some plants that form lesions become resistant to infections from pathogenic microorganisms (Kawasaki T. and Shimamoto K., Cell Engineering Supplement "Plant Cell Engineering Series 8: Disease  
25 Resistance of Plants at Molecular Level" pp.124-130 (1997)).

Therefore, identification of this mutant gene in these mutant strains along with the isolation of the corresponding wild type gene may enable suppression of lesion formation and an increased resistance to stress, such as light and heat, in rice plant by introducing the gene to arbitrary cultivars via  
30 transformation methods.

Hence, there was a need in the art to identify the mutant gene in these mutant strains and also to isolate the corresponding wild type gene.

### Disclosure of the Invention

This need in the art led to the present invention, the object of which is to isolate a novel gene related to suppression of lesion formation on plants. Another object of the present invention is to modify plants by utilizing this  
5 novel gene.

In order to accomplish the object to identify the mutant gene and also isolate the corresponding wild type gene, the present inventors conducted extensive research by focusing on *Sp17*, in which a mutant gene exists on chromosome 5, among mutant strains forming lesion-like necrotic spots on  
10 the leaf in connection with the growth of rice.

First, the present inventors conducted linkage analysis and aligned *Sp17* gene region of yeast artificial chromosome (YAC) clones. Specifically, linkage analysis of large segregating population, essential for the isolation of the *Sp17* gene, was conducted. Furthermore, YAC clones existing adjacent to  
15 the *Sp17* locus were identified by utilizing the YAC genomic clone library constructed in the Rice Genome Research Program. Then, the end fragments of the identified YAC clones were isolated and aligned to demonstrate that the YAC clones include an *Sp17* gene region (Figure 2C).

The present inventors selected 9 PAC clones from a Nipponbare  
20 genomic PAC clone library, constructed in the Rice Genome Research Program, using STS primer sets prepared from the RFLP marker C11368, a gene on the same locus as the *Sp17* locus. These PAC clones were aligned to indicate the PAC clone containing an *Sp17* locus (Figure 2D).

According to a fine scale genetic map, constructed using end  
25 fragments of YAC and PAC clones aligned in the *Sp17* region as novel RFLP markers or CAPS markers, the *Sp17* locus was demonstrated to exist in a genomic region of about 16 kb between RFLP markers P461H4T and P693G10S (Figure 2).

The nucleotide sequence of the candidate genomic region was  
30 analyzed. Based on the sequence information, a novel CAPS marker was constructed to further delimit the candidate region, and it was concluded that the *Sp17* gene existed on a genomic region of about 3 kb between CAPS markers S12C6-6d and HsfC3-3' (Figure 3).

Gene prediction and similarity search was conducted against the nucleotide sequence of the candidate genomic region. It revealed that there was only one predicted ORF, that has similar sequence with the *Hsf* gene isolated from plants such as tomato and *Arabidopsis*. Thus, the gene was  
5 deemed to be a candidate for the *Sp17* gene, and the nucleotide sequence of the corresponding region in a mutant strain KL210 was determined. The result revealed a nucleotide substitution in the nucleotide sequence of the mutant gene when compared with the wild type gene (Figure 4). An amino acid substitution correlated with the nucleotide substitution was considered to be  
10 the cause of the loss or decrease in function of the *Sp17* protein.

Furthermore, the present inventors introduced the genomic region (specified as the candidate for the *Sp17* gene) into the *Sp17* mutant strain by inserting this region into a vector, which can be transformed using *Agrobacterium*. The transformed plants were grown in an isolated growth  
15 chamber under natural photoperiod conditions to monitor the formation of lesions. Lesions were observed on both control plants (transformed only with the vector) and mutant strains at the late phase of growth, whereas none of the plants transformed with the candidate gene showed any lesion formation (Figure 5). Hence it can be concluded that, the candidate gene region  
20 suppressed the lesion formation in the mutant strain KL210. Furthermore, in selfed progeny of transgenic plants segregation ratio between plants forming lesions and those that did not form any, was fitted to the expected ratio. Therefore, it was concluded that the candidate gene was the *Sp17* gene.

Moreover, the present inventors tested necessary conditions for lesion  
25 formation. It was discovered that both high temperature (Figure 6) and ultraviolet irradiation are conditions that enhance lesion formation (Figure 7). These facts indicate that the *Sp17* gene plays an important role in preventing high-temperature stress in plants. Thus, by breeding transgenic plants expressing the *Sp17* gene, it will be possible to enhance the inherent ability of  
30 plants to avoid heat stress and to suppress lesion formation on plants.

In other words, the present inventors have succeeded in isolating a gene involved in suppressing lesion formation on plants. The present inventors also found that this gene can suppress lesion formation on plants

and that heat stress resistance of plants can be enhanced via this gene as well, thereby completing the present invention.

More specifically, this invention provides the following:

(1) a DNA encoding a protein derived from plants that suppresses the lesion formation on plants, which DNA is selected from the group consisting of:

(a) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2;

(b) a DNA including the coding region of the nucleotide sequence of SEQ ID NO: 1;

(c) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2 in which one or more amino acids are substituted, deleted, added and/or inserted; and

(d) a DNA capable of hybridizing under stringent conditions with the DNA consisting of the nucleotide sequence of SEQ ID NO: 1;

(2) a DNA encoding a protein derived from plants that enhances the heat stress resistance of plants, wherein said DNA is selected from the group consisting of:

(a) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2;

(b) a DNA including the coding region of the nucleotide sequence of SEQ ID NO: 1;

(c) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2 in which one or more amino acids are substituted, deleted, added and/or inserted; and

(d) a DNA capable of hybridizing under stringent conditions with the DNA consisting of the nucleotide sequence of SEQ ID NO: 1;

(3) a vector comprising the DNA of (1) or (2) ;

(4) a transformed cell into which the DNA of (1) or (2), or the vector of (3) has been introduced;

(5) the transformed cell of (4), wherein said transformed cell is a plant cell;

(6) a protein encoded by the DNA of (1) or (2) ;

(7) a method for producing the protein of (6), wherein said method comprises:

cultivating the transformed cell of (4); and

5 collecting an expressed protein from the transformed cell or culture supernatant thereof;

(8) a plant transformant comprising the transformed cell of (5);

(9) a plant transformant which is a progeny or a clone of the plant transformant of (8);

(10) a breeding material of the plant transformant of (8) or (9);

10 (11) a method for producing the plant transformant of (8), wherein said method comprises:

(a) introducing the DNA of (1) or (2) into a plant cell; and

(b) regenerating a plant from said plant cell;

15 (12) a method for suppressing lesion formation on plants, wherein said method comprises the step of expressing the DNA of (1) in cells of said plant body;

(13) a method for enhancing the heat stress resistance of plants, wherein said method comprises the step of expressing the DNA of (2) in cells of the plant body;

20 (14) an antibody that binds to the protein of (6); and

(15) a polynucleotide comprising at least 15 nucleotides that are complementary to a DNA consisting of the nucleotide sequence of SEQ ID NO: 1 or a complementary strand thereof.

The present invention provides a DNA encoding a *Spl7* protein.  
25 Nucleotide sequence of the *Spl7* genomic DNA of rice Nipponbare is set forth in SEQ ID NO: 1, and the amino acid sequence of a protein encoded by said DNA is set forth in SEQ ID NO: 2. The *Spl7* gene has been known to be involved in suppressing the lesion formation in rice. It exists somewhere within the vast region of chromosome 5. However, the *Spl7* gene had not been  
30 previously identified or isolated. After performing extensive research, the present inventors finally identified the region where the gene exists, and were the first to isolate this gene as a single gene.

*Spl7* protein has a function to suppress lesion formation on rice caused

by heat stress. Furthermore, the relation of Spl7 protein to the transcriptional regulation of heat shock protein genes is suggested from the structure of the Spl7 protein. Recently, one of heat shock proteins was reported to be closely related to the avoidance of heat stress in plants (Queitsch C. et al., Plant Cell 12: 479-492 (2000)). Therefore, it is concluded that heat stress resistance of plants can be enhanced and lesion formation thereby suppressed, by transforming plants with DNA encoding the Spl7 protein.

DNA encoding the Spl7 protein of the present invention includes genomic DNA, cDNA, and chemically synthesized DNA. A genomic DNA and cDNA can be prepared according to conventional methods known to those skilled in the art. More specifically, genomic DNA can be prepared as follows: (1) extract genomic DNA from rice cultivars having *Spl7* gene (e.g., Nipponbare); (2) construct a genomic library (utilizing a vector, such as, plasmid, phage, cosmid, BAC, PAC, etc.); (3) spread the library; and (4) conduct colony hybridization or plaque hybridization using a probe prepared based on the DNA encoding a protein of the present invention (e.g., SEQ ID NO: 1). Alternatively, a genomic DNA can be prepared via PCR using primers specific for a DNA encoding the protein of the present invention (e.g., SEQ ID NO: 1). On the other hand, cDNA can be prepared as follows: (1) synthesize cDNAs based on mRNAs extracted from rice cultivars having the *Spl7* gene (e.g., Nipponbare); (2) prepare a cDNA library by inserting the synthesized cDNA into vectors, such as  $\lambda$ ZAP; (3) spread the cDNA library; and (4) conduct colony hybridization or plaque hybridization as described above. Alternatively, cDNA can be also prepared by PCR.

The present invention includes DNAs encoding proteins (Nipponbare) that are functionally equivalent to the Spl7 protein of SEQ ID NO: 2. Although there is no limitation regarding the plant species from which the DNA of the present invention is derived, they are preferably Gramineae, and most preferably rice. Herein, the phrase "functionally equivalent to the Spl7 protein," as used herein, indicates that the object protein has the function of suppressing lesion formation on plants and/or the function of enhancing heat stress resistance of plants.

Examples of such DNAs include those encoding mutants, derivatives,

alleles, variants, and homologues comprising the amino acid sequence of SEQ ID NO: 2 wherein one or more amino acids are substituted, deleted, added and/or inserted.

5 Examples of methods for preparing a DNA encoding a protein comprising altered amino acids well known to those skilled in the art include the site-directed mutagenesis (Kramer, W. and Fritz, H. -J., "Oligonucleotide-directed construction of mutagenesis via gapped duplex DNA." Methods in Enzymology, 154: 350-367 (1987)). The amino acid sequence of a protein may also be mutated in nature due to a mutation of its corresponding  
10 nucleotide sequence. A DNA encoding protein having the amino acid sequence of the natural Spl7 protein where one or more amino acids are substituted, deleted, and/or added are also included within the scope of the DNA of the present invention, provided they encode a protein functionally equivalent to the natural Spl7 protein (SEQ ID NO: 2). Additionally, nucleotide  
15 sequence mutants that do not give rise to amino acid sequence changes in the protein (degeneracy mutants) are also included within the scope of the DNA of the present invention.

The present inventors were able to access whether a DNA encoded a protein that suppressed lesion formation on plants by inserting the subject  
20 DNA into an appropriate vector, transforming a Spl7 mutant strain therewith, and observing whether the lesion formation on the mutant strain was suppressed or not (described in Example 6). On the other hand, whether a DNA encodes a protein having a function that enhances heat stress resistance of plants or not can be assessed by the following steps: inserting  
25 the subject DNA into an appropriate vector; transforming the wild type strain with said vector; observing the growth of the strain under both high temperature conditions (approximately 40°C) and low temperature conditions (approximately 25°C); and then comparing the decrease in growth rate of this transformed wild type strain under high temperature with that of the wild type  
30 strain. The subject DNA is considered to have a function in conferring heat stress resistance to plants when the decrease in growth rate under high temperature growth conditions is smaller as compared to that of the wild type strain.

A DNA encoding a protein functionally equivalent to the Spl7 protein described in SEQ ID NO: 2 can be produced, for example, via methods well known to those skilled in the art. These methods include: methods using hybridization techniques (Southern, E.M. Journal of Molecular Biology, 98: 503, (1975)); and polymerase chain reaction (PCR) techniques (Saiki, R. K. et al. Science, 230: 1350-1354, (1985); Saiki, R. K. et al. Science, 239: 487-491, (1988)). It is routine for a person skilled in the art to isolate DNA with a high homology to the *Spl7* gene from rice and other plants using the nucleotide sequence of the *Spl7* gene (SEQ ID NO: 1) or parts thereof as a probe, and oligonucleotides hybridizing specifically to the nucleotide sequence of *Spl7* gene as a primer. Such DNA encoding proteins functionally equivalent to the Spl7 protein, obtainable by hybridization techniques or PCR techniques, are included within the scope of the DNA of this invention.

Hybridization reactions to isolate such DNAs are preferably conducted under stringent conditions. Stringent hybridization conditions according to the present invention include conditions such as: 6 M urea, 0.4% SDS, and 0.5x SSC. DNAs with higher homology are expected when hybridization is performed under conditions with greater stringency, such as: 6 M urea, 0.4% SDS, and 0.1x SSC. The DNAs isolated under such conditions are expected to encode a protein having a high degree of amino acid homology with the Spl7 protein (SEQ ID NO: 2). As used herein, the phrase "high homology" through the entire amino acid sequence means an identity of at least 50% or more, preferably 70% or more, and most preferably 90% or more (e.g., 95% or more). The degree of sequence homology can be determined via programs of BLASTn (nucleotide level) and BLASTx (amino acid level) (Altschul et al. J. Mol. Biol. 215: 403-410, (1990)). These programs are based on the BLAST algorithm by Karlin and Altschul (Proc. Natl. Acad. Sci. USA, 87: 2264-2268, (1990) and Proc. Natl. Acad. Sci. USA, 90: 5873-5877, (1993)). To analyze a nucleotide sequences according to BLASTN, the parameters are set, for example, as score= 100 and word length= 12. On the other hand, the parameters used for the analysis of amino acid sequences by the BLASTX are set, for example, as score= 50 and word length= 3. Default parameters of each program are used when using BLAST and Gapped BLAST programs.

Specific techniques for such analysis are known in the art.

The DNA of the present invention can be used, for example, to prepare recombinant proteins, to produce plant transformants with suppressed lesion formation or enhanced heat stress resistance, etc.

5        According to the present invention, a recombinant protein is generally prepared by inserting a DNA encoding a protein of the present invention into an appropriate expression vector, introducing the vector into an appropriate cell, culturing the transformed cells, allowing the cells to express the recombinant protein, and purifying the expressed protein. A recombinant  
10       protein can be expressed as a fusion protein with other proteins so as to be easily purified, for example, as a fusion protein with maltose binding protein in *Escherichia coli* (New England Biolabs, USA, vector pMAL series), as a fusion protein with glutathione-S-transferase (GST) (Amersham Pharmacia Biotech, vector pGEX series), or tagged with histidine (Novagen, pET series). The host  
15       cell is not limited to any particular type as long as the cell is suitable for expressing the recombinant protein. It is possible to utilize yeast, various animal, plant, or insect cells besides the above-described *E. coli*. A vector can be introduced into a host cell by a variety of methods known to one skilled in the art. For example, a transformation method using calcium ions (Mandel, M.  
20       and Higa, A. Journal of Molecular Biology, 53: 158-162 (1970), Hanahan, D. Journal of Molecular Biology, 166: 557-580 (1983)) can be used to introduce a vector into *E. coli*. A recombinant protein expressed in host cells can be purified and recovered from the host cells or the culture supernatant thereof via standard methods. When a recombinant protein is expressed as a fusion  
25       protein with maltose binding protein or other partners, the recombinant protein can be easily purified by affinity chromatography.

      The resulting protein can be used to prepare an antibody that binds to the protein. For example, a polyclonal antibody can be prepared by immunizing animals (such as rabbits) with a purified protein or part thereof, of  
30       the present invention, followed by collecting blood from the animal after a certain period and removing the clots. A monoclonal antibody can be prepared by fusing myeloma cells with antibody-forming cells of animals immunized with the above protein or part thereof, isolating a monoclonal cell

expressing a desired antibody (hybridoma), and recovering the antibody from said cell. The antibody produced by this method can be used to purify or detect a protein of the present invention. Accordingly, another aspect of the present invention includes antibodies that bind to proteins of the invention.

5           A plant transformant with suppressed lesion formation or enhanced heat stress resistance can be created using DNAs of the present invention. More specifically, a DNA encoding a protein of the present invention is inserted into an appropriate vector; the vector is introduced into a plant cell; and the resulting transformed plant cell is regenerated.

10           Vectors used for the transformation of plant cells are not limited to any particular type as long as the vector can express inserted genes into plant cells. For example, vectors comprising promoters for constitutive gene expression in plant cells (e.g., cauliflower mosaic virus 35S promoter); and promoters inducible by exogenous stimuli can be used. The term "plant cell"  
15           used herein includes various forms of plant cells, such as cultured cell suspensions, protoplasts, leaf sections, and callus.

          A vector can be introduced into plant cells by known methods, such as the polyethylene glycol method, electroporation; *Agrobacterium*-mediated transfer, and particle bombardment. Plants can be regenerated from  
20           transformed plant cells by known methods depending on the type of the plant cell (Toki et al., Plant Physiol. 100:1503-1507 (1995)). Some of the transformation and regeneration methods for rice plants include: (1) introducing genes into protoplasts using polyethylene glycol, and regenerating the plant body (suitable for *indica* rice cultivars) (Datta, S.K., in "Gene  
25           Transfer To Plants", Potrykus I and Spangenberg Eds., pp66-74 (1995)); (2) introducing genes into protoplasts using electric pulse, and regenerating the plant body (suitable for *japonica* rice cultivars) (Toki et al., Plant Physiol. 100: 1503-1507 (1992)); (3) introducing genes directly into cells by particle bombardment and regenerating the plant body (Christou et al.,  
30           Bio/Technology, 9: 957-962 (1991)); (4) introducing genes using *Agrobacterium*, and regenerating the plant body (Hiei et al., Plant J. 6: 271-282 (1994)). These methods are well established in the art and are widely used in the technical field of the present invention. These methods can be

suitably used in the present invention.

Once a transformed plant, with the DNA of the present invention is introduced into the genome, is obtained, it is possible to obtain descendants from that plant body by sexual or vegetative propagation. Alternatively, plants  
5 can be mass-produced from breeding materials (for example, seeds, fruits, ears, tubers, tubercles, tubs, callus, protoplast, etc.) obtained from the plant, as well as descendants or clones thereof. Plant cells transformed with the DNA of the present invention, plant bodies including these cells, descendants and clones of the plant, as well as breeding material obtained from the plant,  
10 its descendant and clones, are all included within the scope of the present invention.

Plants produced above have enhanced heat stress resistance and suppressed lesion formation compared to wild type plants. Employing the method of the present invention, productivity of valuable crops like rice can be  
15 increased which is greatly beneficial.

Furthermore, the present invention provides polynucleotides comprising at least 15 nucleotides that are complementary to a DNA of the present invention consisting of the nucleotide sequence of SEQ ID NO: 1 or to the complementary strand thereof. The term "complementary strand," as used  
20 herein, refers to one strand of a double stranded DNA composed of A:T and G:C base pairs. In addition, "complementary," as used herein, refers to not only those completely matching within a region of at least 15 continuous nucleotides, but also those having a homology of at least 70%, preferably at least 80%, more preferably 90%, and most preferably 95% or higher within  
25 that region. Such DNAs are useful as probes to detect or isolate a DNA of the present invention, or as primers to amplify a DNA of the present invention.

#### Brief Description of the Drawings

Figure 1 depicts photographs of a leaf of the *Spl7* mutant KL210 (left  
30 panel) and a leaf of the Nipponbare (right panel).

Figure 2 shows a fine scale linkage map of the *Spl7* gene region and an alignment map of genomic clones. A and B represent genetic maps constructed from a segregating population of 298 individuals and 2944

individuals, respectively. C and D represent alignment maps constructed from YAC and PAC clones of Nipponbare, respectively.

Figure 3 shows a fine scale genetic map of the *Spl7* gene region, and a map indicating candidate genomic region and the predicted gene thereon.

5        Figure 4 illustrates the structure of the *Spl7* candidate gene and compares the genomic nucleotide sequence of Nipponbare with that of KL210.

Figure 5 is photographs of a leaf of the *Spl7* mutant KL210 (left panel) and a leaf of the transformant (4 panels on the right).

10        Figure 6 represents the relationship between days required for lesion formation on *Spl7* mutants and mean temperature under natural growth conditions.

Figure 7 is photographs of a leaf of *Spl7* mutant KL210 grown by blocking ultraviolet rays (left panel) and a leaf grown without blocking  
15        ultraviolet rays (right panel).

#### Best Mode for Carrying out the Invention

The present invention is specifically illustrated below with reference to Examples, but it is not to be construed as being limited thereto.

20

#### [EXAMPLE 1] Construction of a genetic map

Fine scale linkage analysis of *Spl7* region essential for map base cloning was conducted using a large scale segregating population. 298 individuals of the F2 population obtained by crossing *Spl7* mutant strain with  
25        SL18 were used as the population for linkage analysis. SL18 has a genetic background of and Nipponbare, and its chromosome 5 is substituted with that of Kasalath. According to a linkage analysis using RFLP markers, the *Spl7* locus was found to exist between RFLP marker S869 and R2781, and to be cosegregated with C11368, S2581, S1762, S1831, and B344 (Figure 2A).

30        Furthermore, 2646 individuals of the F2 population were used for linkage analysis to construct a fine scale genetic map of the *Spl7* region. CAPS (Cleaved Amplified Polymorphic Sequence) markers were used for efficient analysis procedures. More specifically, plants with chromosome

recombination adjacent to *Sp17* were screened in the F2 population using CAPS markers S869 and R2781, each marker adjacent to *Sp17*. Accordingly, 65 recombinants could be selected (Figure 2B). Using these recombinants, a fine scale linkage map was constructed with RFLP markers constructed as described below.

[Example 2] Alignment of the *Sp17* gene region using yeast artificial chromosome (YAC) clones and P1 derived artificial chromosome (PAC) clones

YAC clones comprising the nucleotide sequence of DNA markers, S869, C11368, S2581, and R2781, existing adjacent to the *Sp17* locus were specified using the alignment map of Nipponbare YAC clones constructed in the Rice Genome Research Program (Figure 2C). Additionally, end fragments of the specified YAC clones, Y4666, Y2205, Y3824c, Y6089, and Y2288, were isolated by the cassette method to align the specified YAC clones. The results revealed that the YAC clone Y4666, Y2205, and Y3824c comprised the *Sp17* gene region (Figure 2C).

Furthermore, to delimit the candidate region for the *Sp17* gene, STS primer set (primers 5'-GACCTGTGCTCTGCCTTTCT-3'/SEQ ID NO: 3 and 5'-GTATGCCAACTGCTCAACTT-3'/SEQ ID NO: 4; amplified genomic fragment of 0.4kb), prepared from the RFLP marker C11368 existing on the same position as the *Sp17* locus, was used for screening the Nipponbare PAC clone library (mean insert length of 112kb; 18432 clones; about 4.5 times of the rice genome) constructed in the Rice Genome Research Program (Baba et al., Bull. Natl. Inst. Agrobiol. Resour. 14: 24-36 (2000)). As a result, 9 PAC clones were selected. By aligning these PAC clones, 8 PAC clones, represented by P0029H1, were shown to contain the *Sp17* locus (Figure 2D).

[Example 3] Delimitation of the *Sp17* gene region

The end fragments of YAC and PAC clones aligned within the *Sp17* region were cloned and used as new RFLP or CAPS markers for constructing a fine scale genetic map. The result revealed that the *Sp17* locus is located on a genomic region between RFLP markers P461H4T and P693G10S and is

cosegregated with RFLP marker C11368B. Accordingly, the *Sp/7* locus was demonstrated to exist on a genomic region of about 16 kb between the 2 markers (Figure 2).

5 [Example 4] Identification of the candidate genomic region by nucleotide sequence analysis

The genomic candidate region of 16kb in the PAC clone P0029H1, which is expected to contain the *Sp/7* gene, was subjected to nucleotide sequence analysis. The nucleotide sequence was analyzed by subcloning the  
 10 candidate region first with restriction enzymes *Not* I and *Sal* I, then with various other restriction enzymes, followed by the dye-primer method. CAPS markers were newly constructed using the nucleotide sequence information of the candidate gene region, which was determined by the linkage analysis, to further delimit the candidate region. The *Sp/7* gene cosegregated with the  
 15 CAPS marker HsfC2-1 (primers 5'-TCTCTCTCGTTCGTTCCCCG-3'/SEQ ID NO: 5 and 5'-TGGATAAATGGAGATGGGCA-3'/SEQ ID NO: 6; restriction enzyme *Apa* I), and 3 and 7 recombinants, respectively, at S12C6-6d (primers 5'-TCGGCATCGGCTATTATCGG-3'/SEQ ID NO: 7 and 5'-GATTTCGGGATACTGTGCGT/SEQ ID NO: 8; restriction enzyme *Nla* III) and  
 20 HsfC3-3' (primers 5'-ACGATGTGTTTTGGGAGCGG-3'/SEQ ID NO: 9 and 5'-GACCTGTGCTCTGCCTTTCT-3'/SEQ ID NO: 10; restriction enzyme *Nla* III) were identified.

Thus, the *Sp/7* gene was shown to exist on a genomic region of about 3 kb between the CAPS markers S12C6-6d and HsfC3-3' (Figure 3).  
 25 Furthermore, gene prediction and similarity search was conducted against the nucleotide sequence of the candidate genomic region, predicting only a gene, that has similar nucleotide sequence with the *Hsf* gene that is isolated from plants such as tomato and *Arabidopsis* (Figure 3). The *Hsf* gene is shown to function as a transcription-regulating factor of a heat shock induced protein.

30

[Example 5] Nucleotide sequence analysis of the *Sp/7* candidate gene

Using the *Hsf* like gene as the candidate for the *Sp/7* gene, the nucleotide sequence of corresponding region in mutant strain KL210 was

determined. Specifically, the nucleotide sequence analysis was conducted by cloning products of genomic PCR and RT-PCR using primers designed to amplify the corresponding nucleotides based on the already obtained nucleotide sequence information of Nipponbare. A substitution of a nucleotide  
5 could be detected by comparing the nucleotide sequence of the wild type and those of mutant genes. As predicted, the nucleotide substitution results in a substitution of tryptophan to cystein (Figure 4). The substituted amino acid is a part of amino acids highly conserved in the *Hsf* gene. Thus, the amino acid substitution is considered to be the cause of the loss or decrease in the Spl7  
10 protein function in mutant strains.

#### [Example 6] Verification of the candidate gene function by transformation

The 5.6 kb *Nsp* V-Bgl III, Nipponbare genomic region fragment, comprising the predicted 5' upstream promoter region specified as the  
15 candidate for *Spl7* gene, was inserted into vector pPZP2H-lac so that it could be used for transformation via *Agrobacterium*. Transformation was performed according to the method of Toki (Plant Mol. Biol. Rep. 15: 16-21 (1997)) using either the vector #178, inserted with the fragment, or the vector alone. Spl7 mutant strain KL210 was used as the strain for transformation. 150 and 50 of  
20 hygromycine resistant individuals, respectively, were obtained by the transformation experiment using the 5.6 kb *Nsp* V-Bgl II fragment and the vector alone. The integration of the candidate gene was confirmed by PCR method using candidate gene specific primers (sense strand 5'-GTCTCCGTGGCCGTGGCTGA-3'/SEQ ID NO: 11 and antisense strand 5'-  
25 AACGAGGAATCTTAGAAGGG-3'/SEQ ID NO: 12).

The result revealed that all 150 transformants contained the candidate gene. The transformants were grown in an isolated green house under natural day length condition (transferred into an isolated green house (Tsukuba) from a growth chamber in March) to investigate the formation of lesions. As a  
30 result, the plant transformed with the vector alone formed lesions at the late stage of growth similar to the mutant strain KL210, whereas no lesion formation was observed on any plant where the candidate gene was introduced (Figure 5). Furthermore, lesions were formed on 6 plants and none

were formed on the 18 plants out of the 24 self-pollinated progenies, which were expected to be transformed only with one copy of the transgene. The resulting segregation ratio fitted to an expected rate of 1:3. These results demonstrate that the function of the candidate gene region (*Nsp* V-*Bgl* II of 5.6 kb) is to suppress lesion formation on the mutant strain, KL210, and also that the candidate gene is the *Spl7* gene.

[Example 7] Conditions necessary for lesion formation

The days required for lesion formation on *Spl7* mutant strain KL210 were investigated by seeding KL210 and Nipponbare having the wild type gene at an interval of 14 days starting May 17, 1999. The days required for lesion formation decreased for plants seeded till August 23, and increased for those seeded on September 6 (Figure 6). On the other hand, the mean temperature from seeding to lesion formation increased until August 9 and decreased thereafter (Figure 6). The shortest time till lesion formation was 11 days for those seeded on August 23, when even the mean temperature had declined. However, the highest temperature of 36°C in 1999 was recorded shortly before lesion formation. More days (more than 60 days after seeding) are needed for lesion formation on plants grown in growth chambers in winter. Furthermore, the degree of lesion formation was also suppressed in winter. Moreover, when plants were grown in a growth chamber where the temperature was 26°C or less, lesions did not appear during any growth period, whereas lesions appeared after a month in a growth chamber where the temperature was 37°C. According to these results, it was suggested that high temperature is essential for lesion formation to some extent, and additionally, that high temperature enhances lesion formation.

Next, KL210 and Nipponbare were grown by covering them with sheets that blocked ultraviolet rays or with plastic sheets which do not block UVs. Whatever sheet KL210 was grown covered with, lesion formation was observed on the 25th day from seeding. However, the degree of lesion formation was suppressed on plants that were blocked from UV irradiation as compared to those where UV was allowed through (Figure 7). These results demonstrate that UV irradiation is one of the conditions required for

enhancing lesion formation.

According to the results obtained above, it was concluded that the *Hsf* like gene is the *Sp17* gene candidate. Then by the map base cloning method it was established to be the *Sp17* gene, that the gene which suppresses lesion  
5 formation on rice. Hence, the biological function of *Hsf* like genes in plants was demonstrated for the first time in the present invention.

#### Industrial Applicability

Tropical plants, for example, rice, are adapted to a certain degree of  
10 high temperature compared to barley, and hence acquire resistance against heat stress. However, the growth temperature of plants is climbing higher due to global warming. Under such conditions, methods to efficiently confer heat stress resistance to plants have been desired for stabilizing crop production in the future. Many points in the mechanism of heat stress resistance remain  
15 unexplained and no efficient method for positively enhancing resistance by modification has been known. By effectively conferring heat stress resistance to plants, not only stable food production at today's level's will be maintained in spite of global warming, but cultivation of crops in regions where it was unable to grow plants will also be possible. The *Sp17* gene of the present  
20 invention enhances heat stress resistance in plants and has a function in suppressing lesion formation on plants. Hence, the *Sp17* gene of the present invention greatly contributes to the above-mentioned objectives.

## SEQUENCE LISTING

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<120> PLANT LESION FORMATION SUPPRESSING GENE, Sp17 AND USE THEREOF

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We claim:

1. A DNA encoding a protein isolated from a plant that suppresses lesion formation in plants, said DNA is selected from the group consisting of:
  - 5 (a) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2;
  - (b) a DNA comprising the coding region of the nucleotide sequence of SEQ ID NO: 1; and
  - (c) a DNA encoding a protein having at least 90% identity to a  
10 protein comprising the amino acid sequence of SEQ ID NO: 2.
2. A vector comprising the DNA of claim 1.
3. A transformed cell into which the DNA of claim 1 or the vector of  
15 claim 2 has been transformed.
4. The transformed cell of claim 3, wherein said transformed cell is a plant cell.
- 20 5. A protein encoded by the DNA of claim 1.
6. A method for producing the protein of claim 5, wherein said method comprises:
  - cultivating the transformed cell of claim 3; and
  - 25 collecting an expressed protein from said cultivated transformed cell or culture supernatant thereof.
7. A method for producing a plant transformant, wherein said method comprises:
  - 30 (a) transforming the DNA of claim 1 into a plant cell; and
  - (b) regenerating a plant from said plant cell.
8. An antibody that binds to the protein of claim 5.

9. The DNA of claim 1, wherein the DNA encodes a protein having at least 95% identity to a protein comprising the amino acid sequence of SEQ ID NO:2.

Application number / numéro de demande: JP01/09153

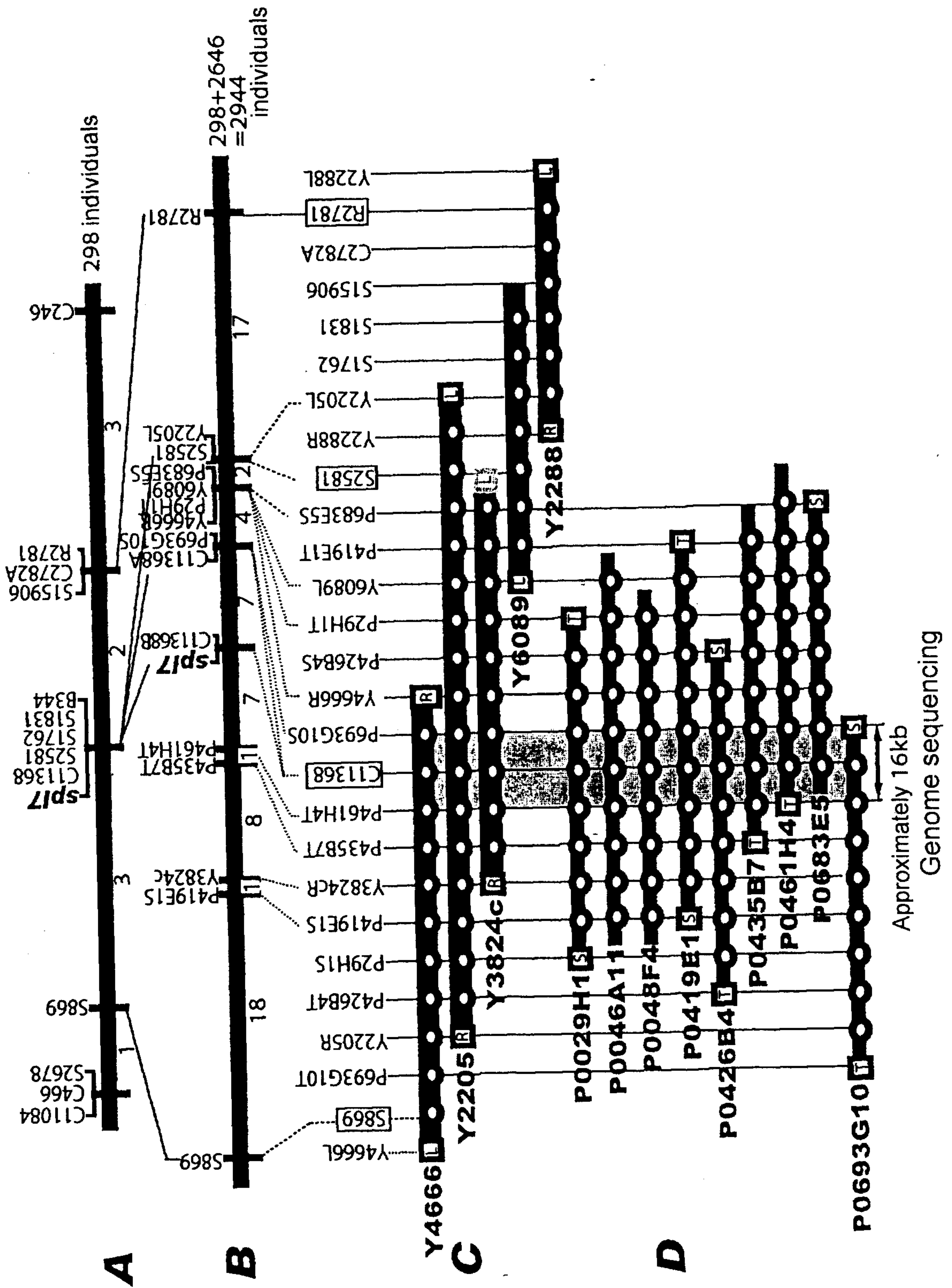
Figures: 1-5-7

Pages: \_\_\_\_\_

Unscannable items  
received with this application  
(Request original documents in File Prep. Section on the 10<sup>th</sup> floor)

Documents reçu avec cette demande ne pouvant être balayés  
(Commander les documents originaux dans la section de préparation des dossiers au  
10ème étage)

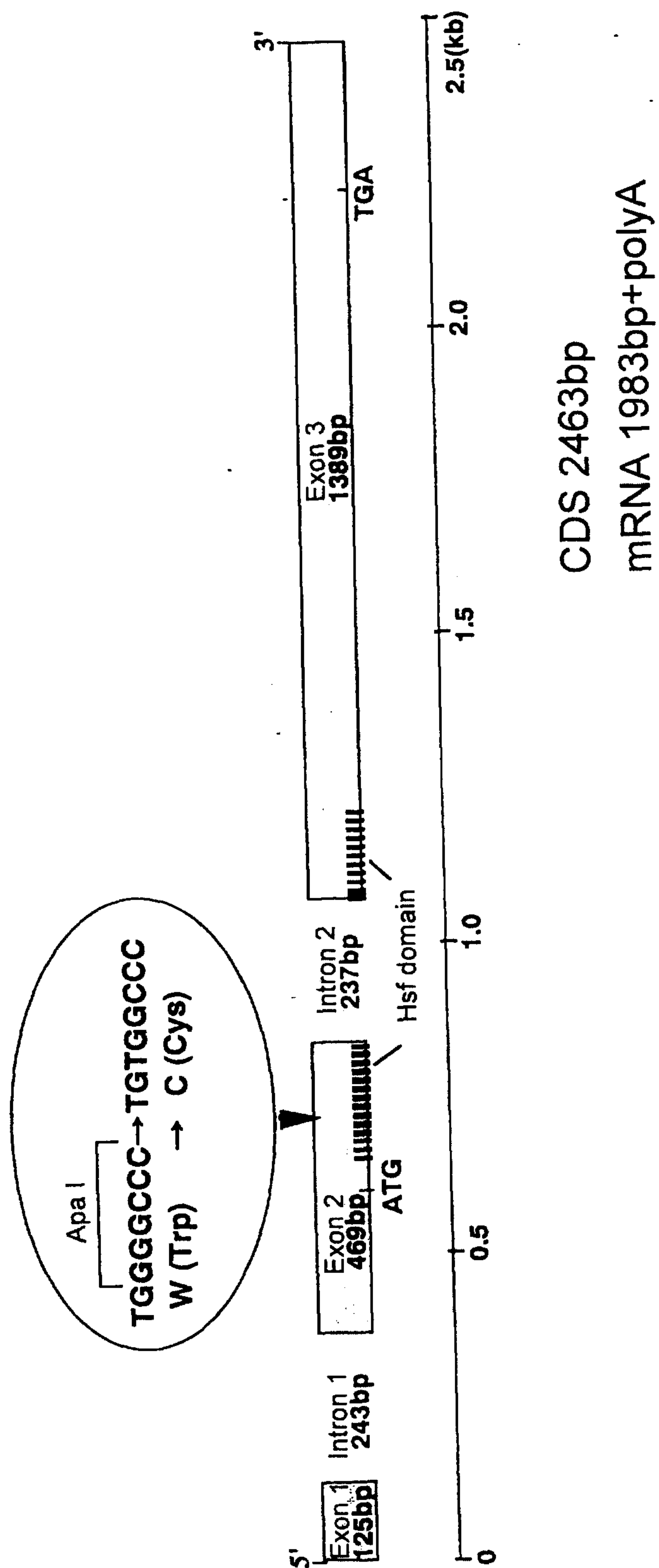
Figure 2



Genomic map of the Nipponbare genome showing the location of the *Spl7* gene region and various genes. The map includes a scale bar from 0 to 15 kb. The *Spl7* gene region is indicated by a double-headed arrow between approximately 10 kb and 12 kb. Genes shown include P461H4T, S12 C1-1, S12 C3-3, S12 C4-4a, S12 C4-4b, S12 C6-6a, S12 C6-6b, S12 C6-6d, Hsf C2-1, Hsf C3-3', C11368 C4-2, P693G10S, Hsf C3-3', C11368 C4-2, C11368A, and P693G10S. A legend indicates that black arrows represent Hsf (Heat shock transcription factor) and black squares represent Predicted ORF. Arabidopsis and Maize orthologs are also shown.

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Figure 4



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Figure 6

