

Fishing Genes from germplasm: Cloning,
testing and use of gene-like fragments
associated with root-knot nematode resistance
in tuber-bearing *Solanum* genetic resources
Patent: WO03/080838 A1

Watanabe, J. A.,^{1,2)} M. Ikegawa^{1,3)}, T. Yamada¹⁾
& K. N. Watanabe^{1,2)}

1. Dept. Biotech. Sci., Kinki University, Uchita, Wakayama, Japan
2. Gene Research Center, Univ. of Tsukuba, Ibaraki, Japan
3. Nara Advanced Inst. for Sci. and Technol., Ikoma, Nara, Japan



Root-knot nematodes
infest over 2000
plant species

*Mi is a known plant
resistance gene to some
Meloidogyne incognita*

Root-knot nematode resistance genes in Potato

RKN is an important pest in warm and tropical zones including Southern part of Japan such as Nagasaki

- 1) Original genetics lines developed (Watanabe et al. 1994)
- 2) Genetics and physiology of the trait analyzed well (Iwanaga et al. 1989, Watanabe et al. 1996, 1999)
 - > Quantitative, additive, temperature insensitive in phenotype.
 - > Broad range of reactions to different taxa of the nematode.
- 3) Diverse sources of resistances in various wild and cultivated species (Watanabe and Watanabe 2000)
- 4) Base work and patent filed in potatoes on markers(PCT/JP02/12392)

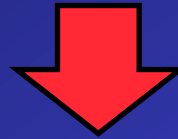
Specific focuses

- Cloning gene-like fragments associated with root-knot nematode (*Meloidogyne* spp) resistance genes in potatoes
- Testing transgenic plants using the fragments
- Use of the gene information for diversity study and molecular breeding

Structure of Plant R genes (Science 292 #5525, Nature 411 #6839)

Common Features in Plant R genes

- LZ:** Leucine Zipper (Coiled coil)
- NBS:** Nucleotide Binding Site
- LRR:** Leucine-Rich Repeat
- TIR:** Drosophila Toll and mammalian interleukin-1 receptor (IL-1R) homologous region
- PK:** Protein Kinase



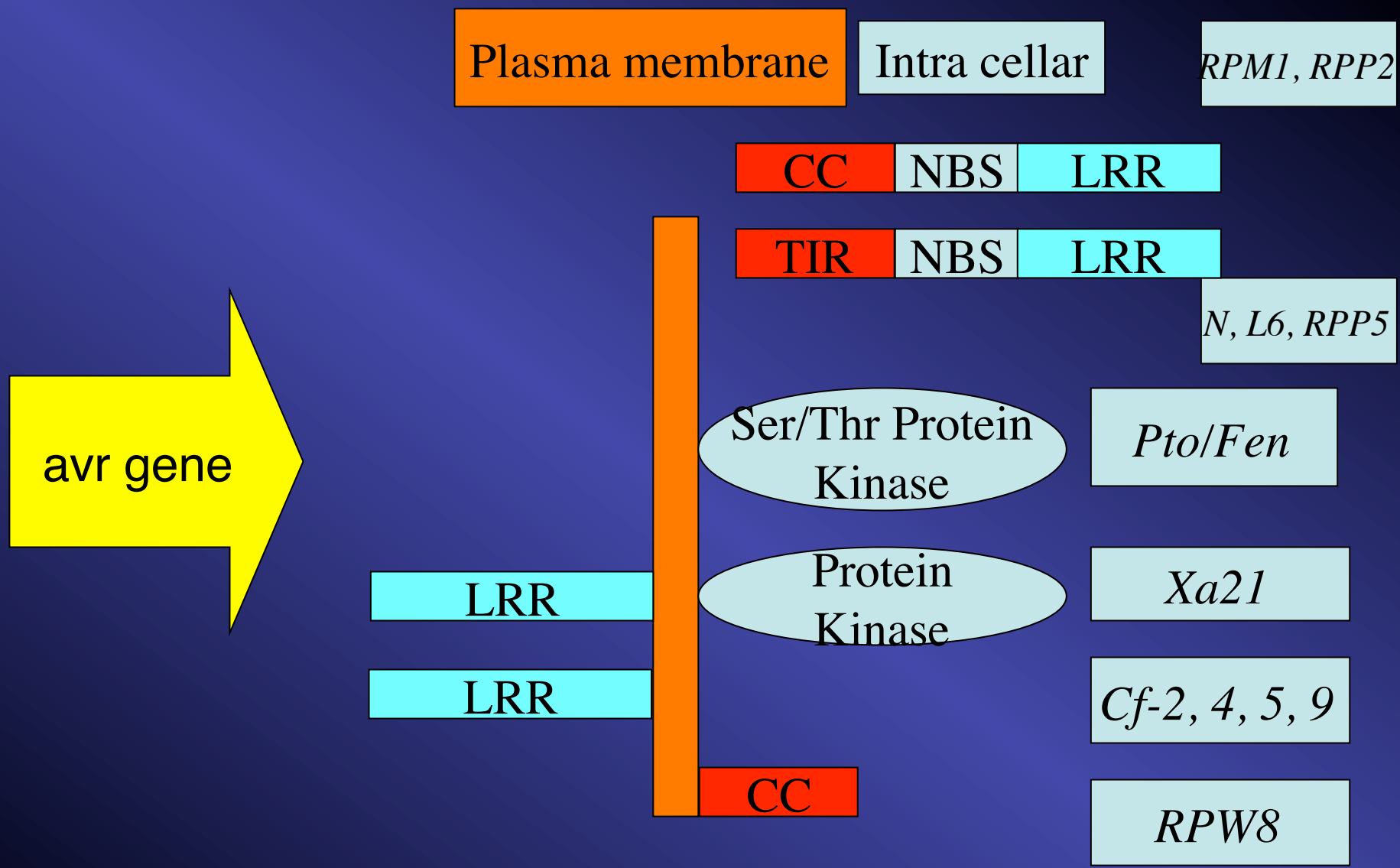
Common Defense Mechanisms among Plants

Signal Transduction Pathway: Nature 415:977-983, 2002

Common features in plant resistance genes



LRR: leucine rich repeats LZ: Leucine zipper/ CC: Coiled coil
NBS: Nucleotide binding site TIR: Homologous region with Toll/IL-1R



Categories of primary products from Plant disease resistance genes
 (Simplified from Dangl & Jones 2001, Science 411:826-827)

RKN resistance gene in Sweetpotato

- Similar structure to known plant R genes but not identical
- Variation occurs in hexasomic hexaploid genome
- Diversity among 6x genotypes
- Possibility of SNPs for identification of R/S
- Weak expression
- Need elaboration of transgenic manipulation I
- Base knowledge filed as patent (JP 2001-223606)

Materials

- Diploid potato breeding lines with high levels of resistance to RKN
(Watanabe et al. 1996, 1999)
- Tetraploid potato cultivars with known S for comparison

Methods

- **PCR:** Primers designed from common motifs in PDR genes especially from *Mi* and sweetpotato information
- **Cloning:** Random cloning of PCR products
- **Sequence comparison:** Difference between fragments from R and S genotypes
- **Amino acid identity:** with known PDR genes
- **Construction of binary vector:** pBI121 or pBE2113 Not
- **Transformation:** *Agrobacterium tumefaciens* LBA4404
- **Resistance evaluation:** regenerants on *N. benthamiana* and Desiree

Deduced amino acid identity with known PDR genes

Solanum acaule NBS-LRR protein, 607/867 (70%)

Solanum tuberosum RGC1 protein, 607/867 (70%)

Solanum tuberosum NBS-LRR protein, 611/867 (70.5%)

Solanum tuberosum disease resistance protein Gpa2 protein, 611/867 (70.5%)

Solanum tuberosum Rx protein protein, 604/867 (70%)

Capsicum chacoense disease resistance protein BS2 protein, 482/870 (55.4%)

Lycopersicon esculentum PRF protein, 403/742 (54%)

Lycopersicon pimpinellifolium PRF protein, 391/726 (54%)

Lycopersicon esculentum root-knot nematode resistance protein, 410/816 (50%)

Arabidopsis thaliana RPP13 protein, 338/682 (50%)

Arabidopsis thaliana rpp8 protein, 399/822 (49%)

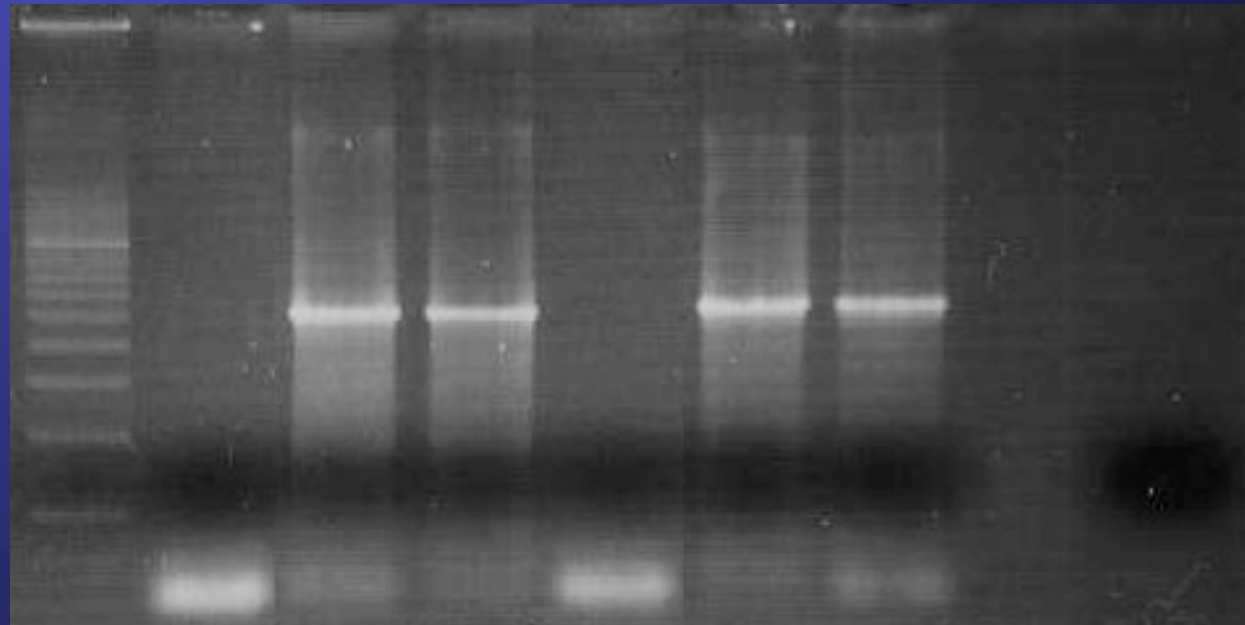
Arabidopsis lyrata NBS/LRR disease resistance protein RPM1, 352/675 (52%)

Summary results of cloning and sequence study

- 2613 bps fragment containing NBS and LRR regions with incomplete CC
- Conserved in NBS and LRR, but not identical to known genes
- Mapped at Chrom VI of potato around *Mi* of tomato
- R & S alleles: probably stop codons at introns in S
- Variation in S alleles

1 2 3 4 5 6 7 8 9

2600bp



RT-PCR using LRR region specific to the potato RKN gene
1. Marker, 2. *N.bensamiana* control, 3. And 4 transgenic plants,
5. *S. tuberosum* Susceptible control, 6 and 7 *S. tuberosum* donor,
8. And 9. Negative control



Resistance evaluation in *N. benthamiana*

- Root-knot nematodes (RKN) were maintained in soil pots with susceptible tomato genotypes.
- Minimum of five cuttings per line were used for the evaluation. Cuttings were made three weeks before transplanting to the RKN infested soil to let rooting enough.
- General infection method Iwanaga et al. (1989) except the incubation temperature of the plants were conducted between 30-35 degree Celsius at day temperature for 16 hours and 25-30 degree Celsius at night temperature for 8 hours at the controlled growth chambers.

Resistance evaluation

- Galls were scored in roots.
- Clones with only no galls were subjected to microscopic observation using erioglaucine (0.05% aqueous solution for 10 min, Yaghoobi et al. 1995) for egg observation.
- Further microscopic observation was conducted on the presence of adults.

Resistance scoring

- HR: No gall and no egg in root
- R: No gall and presence of some egg in root mass
- MR: Some galls @ specific root region and presence of eggs in root mass
- MS: Many galls @ specific root region
- S: Many galls over root system