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(54) Title: POTATOES WITH INCREASED RESISTANCE TO ROT

(57) Abstract: The invention relates to the breeding and selection of potatoes. The invention provides a potato plant or part derived thereof having at least one *amf*-allele said potato plant or part further provided with an increased resistance to rot as characterized by an increased resistance to soft rot caused by *Erwinia spp*. Furthermore, the invention provides a method for breeding and selecting a potato with an increased resistance to rot comprising crossing a first parent potato with at least one *amf*-allele with a second parent potato without an *amf*-allele, and selecting progeny for the presence of at least one *amf*-allele with an increased resistance to soft rot.

Title: Potatoes with increased resistance to rot

The invention relates to the breeding and selection of potatoes. Apart from being an important staple food, potato is classically the raw material for industrial production of starch from potato tubers. Where, chemically, potato starch in potato tubers essentially consists of two components: amylopectin and amylose in a proportion of approximately 80% to 20%, potato proteins in tubers essentially consist of enzyme inhibitors that help protect the tuber against disease such as parasite infestations or fungal or bacterial rot and storage proteins such as patatin in a proportion of approximately 60% to 40%.

For various reasons, starch producers prefer potatoes with different ratios of amylopectin and amylose. An earlier induced gene mutation in potatoes that affects the synthesis of the enzyme granule bound starch synthase (GBSS), and the subsequent molecular cloning of this gene (Hovenkamp-Hermelink et al., 1987, Theor. Appl. Genet. 75:217-221; Visser et al., 1989, Plant Science 64:185-192) has opened possibilities for altering the starch composition of potatoes – either through established breeding methods or through modern techniques of genetic modification.

The GBSS mutation in potato is similar to the so-called waxy (*wx)* mutation in maize and prevents the production of amylose, when expression or specific function of the GBSS protein is absent. Therefore, this mutation has been designated as amylose-free (*amf*) mutant of potato. Herein, the *amf* gene mutation stands for a modification of the GBSS-gene that leads to a complete functional loss of GBSS-activity, notwithstanding that GBSS-like gene products, without the specific activity, may still be expressed from the gene's transcripts in question, whereby the *Amf*-gene stands for a gene from which gene products with GBSS-activity can still be obtained. The *amf*-gene character is determined by a monogenic mendelian recessive gene, the phenotype of which can be detected in various plant parts such as columella cells of root tips, tubers, plastids in the stomatal guard cells and in microspores (Jacobsen et al., 1989, Euphytica 44:43-48). When these parts are stained with a potassium iodine solution (Lugol), starch is stained red in mutants and dark blue in the wild type.

Unlike many other phenotypic genetic markers, the mutated or functionally deleted GBBS- or *amf*-gene offers certain special advantages for genetic analysis as well as for breeding. For example, the progeny can be classified at a very early seedling stage as well as in adult plants, through pollen staining, homo- and heterozygotes can be unambiguously classified: the dosages 2-4 of the mutant allele in a tetraploid can be easily detected through the ratios 5:1, 1:1 and 0:1 in stained pollen samples; different types of 2n-gametes in diploid clones can be detected and their influence on the phenotype and genotype of tetraploid progeny from 4x*2x crosses can be predicted.

Prospects of using the material in conventional as well as in analytic breeding of potato have been opened since the generation of the *amf*-gene potato mutant of Hovenkamp-Hermelink. A disadvantage for breeding is the recessive nature of *amf*, which complicates the combination of this character with other agronomic traits at the tetraploid level.

Therefore, the analytic breeding method advocated by Chase (1963, J. Genet. Cytol. 5:359-364), which involves breeding of potato at the diploid level and returning to the tetraploid condition through the use of 2n-gametes, could be of considerable value for breeding *amf*-varieties. The aim of such investigations are at least two fold: a. to combine *amfamf* and *Amfamf* genotypes with that of 2n-gamete formation, and b. to create fertile, nulliplex clones as basic material for breeding amylose-free potatoes. On the other hand, development of suitable diploid material that produces high frequencies of 2n-pollen and 2n-eggs would also open the way for unilateral and bilateral sexual polyploidization (Mendiburu and Peloquin, 1976, Theor. Appl. Genet.48:137-143). Such diploid breeding material may be homozygous (*amfamf*) or heterozygous (A*mfamf*), because in both cases selection can be carried out based on pollen phenotype.

The invention relates to the breeding and selection of potatoes. Surprisingly, it is found herein that potatoes with at least one *amf*-allele in the genetic background have a distinct phenotypic advantage when compared with potatoes having a similar genetic background lacking the *amf*-allele. The advantage relates to the resistance to rot, in particular to bacterial rot such as caused by *Erwinia spp.*

Erwinia spp., such as *Erwinia carotovora* and *Erwinia chrysantemi* are plant pathogenic bacteria belonging to the *Enterobacteriaceae* family. They are rod-shaped, Gram-negative, non-spore forming, facultative anaerobes and

characterized by their ability to produce large amounts of pectolytic, cellulitic and proteolytic enzymes which enable them to macerate various plant tissues. There is considerable evidence that pectinases play an important role in the pathogenicity of *Erwinia spp.*, while the role of cellulases and proteases is not clear. In contrast to host-specialized phytopathogenic bacteria, *Erwinia spp* do not show a narrow host specificity and therefore are described as unsophisticated opportunistic or general pathogens that simply macerate host tissue if it is suitable as a substrate.

The invention provides a potato plant or part derived thereof (such as a cell, a protoplast, a tuber, an embryo, a seed or an explant) having at least one *amf*-allele said potato plant or part further provided with an increased resistance to rot (herein also identified as a low rot potato). Within a potato homozygous for the *amf*-gene as provided herein, i.e. an amylose-free low rot potato, such increased resistance to rot is most fully developed. The inventors have gathered the surprising insight that depriving a potato of GBSS-activity allows for increasing rot resistance in said potato, provided it has the genetic capacity to increased, or at least sufficient, levels of resistance. Likely, although not yet fully demonstrated, such increased resistance is at least partly related to a genetic link between the *amf* locus and a resistance locus for *Erwinia spp.*, albeit the inventors are not wishing to be bound to theory., considering that genetically improving amylose-free potatoes with an *amf*-allele is still possible by crossing with wild-type potatoes otherwise provided with higher resistance. Potatoes comprising a *amf*-allele have essentially higher rot resistance than potatoes of otherwise similar genetic background having no *amf*-allele. When potato plants in the field or tubers in storage are infected with the phytopathogenic *Erwinia spp.*, various symptoms may develop depending on environmental factors of which temperature and moisture are most important. In the field, non-emergence occurs when the seed potato rots before development of above ground parts. Rot of the seed potato by *Erwinia* after emergence may be followed by colonization of the vascular stem tissue. Especially under wet conditions, the bacteria may cause a basal rot in one or more stems. The symptom is called blackleg. Infection of tubers is storage, under certain conditions, results in decay as well. This is called soft rot. Originally, the *amf*-mutation was induced in a monohaploid which had been selected only for flowering (Hovenkamp-Hermelink et al., 1987, *ibid*) but not for fertility and agronomic characters. Therefore, in order to incorporate this

recessive mutant in other potatoes the inventors crossed this genotype with agronomically more desirable clones which, however, have the wild type of the *Amf* gene. As a first step in this process, fertile diploids that are homozygous for the mutant character (*amfamf*), were produced. When these diploids are somatically doubled through in vitro adventitious shoot regeneration, the resulting tetraploids proved to be less fertile (both male and female). However the 4x plants obtained through meiotic doubling – using 4x x 2x crosses- gave rise to fertile nulliplex tetraploids. Thus, in spite of high levels of sterility and expression of lethal factors in the initial stages, more fertile and vigorous diploid and tetraploid breeding material were created with the desired *amf*-genotypes. Availability of vigorous, fertile and agronomically useful tetraploid genotypes than led to conventional breeding of *amf*-mutants of potato. The inventors, when breeding with potatoes comprising the *amf*-allele, observed by chance that certain lots of potatoes kept in storage in between breeding, notably those at least comprising one copy of the *amf*-allele, had generally higher resistance to soft rot than the wild-type potatoes had. Especially descendants of crosses of amylose-free potatoes with wild-type parents that had increased resistance to rot on their own, i.e. progeny with a background genotype of increased resistance in addition to the *amf*-allele showed this beneficial effect.

It was thus surprisingly found that amf-mutants, resulting from crosses with wild-type potatoes had increased rot resistance, in particular to soft root. The resistance found so far seems partial and most likely polygenic and may be further combined with several other agronomic traits, which is however difficult and time-consuming, especially considering that the cultivated potato is a tetraploid species, wherein the genetics behind breeding goals are usually more complicated when compared with diploid crops, which negatively affects the speed of the selection process.

However, now knowing that the presence of the *amf*-allele allows for a better expression of resistance to soft rot, the invention also provides a method for breeding and selecting a potato with an increased resistance to rot comprising crossing a first parent potato with at least one *amf*-allele with a second parent potato without an *amf*-gene, and selecting progeny for the presence of at least one *amf*-allele and for increased resistance to soft rot..

In a preferred embodiment, the invention provides a method for breeding and selecting a potato with an increased resistance to rot comprising crossing a first

parent potato with at least one *amf*-allele with a second parent potato without an *amf*-gene, and selecting progeny by testing it for the presence of at least one *amf*-allele and testing it for soft rot resistance with a method as described herein and selecting progeny with a higher resistance than detected in either parent..

Furthermore, the invention provides a potato selected with a method according to the invention, use of a potato as provided herein for the industrial production of starch and/or protein and use of a potato as provided herein in breeding and selection programmes of potatoes. In particular, the invention provides use of a potato plant or part derived thereof having at least one *amf*-allele in a breeding and selection programme directed at providing potatoes with an increased genetic resistance to rot.

Figures 1 to 4.

Examples of breeding schemes for breeding and selecting potatoes

Detailed description

Using an amylose-free (amf)mutant of diploid potato (*Solanum tuberosum)*, diploid and tetraploid clones with different genotypes at the *amf*-locus were produced. In order to make use of the diploid material in analytic breeding of *amf*-potatoes, clones were selected that produced a considerable frequencies of 2n-pollen and 2n-eggs. Successful attempts were made to select normal synaptic as well as desynaptic clones producing 2n-gametes. When for example microspores are stained with a potassium iodide solution (Lugol), starch is stained red in mutants (comprising only the *amf*-gene) and dark blue in the wild type (comprising only the A*mf*-allele). Based on the phenotype of starch in the microspores, tetraploid clones with nulliplex, simplex, duplex, triplex and quadriplex genotypes at the *Amf*-locus were selected.

Plant material. Monoploid amylose-free (amf) clone 86.040 and the parent clone AM79.7322 are described in Hovenkamp-Hermelink et al. (1987, *ibid*). Doubled amf-plants were obtained by adventitious shoot regeneration on leaf explants, which were taken from *in vitro* propagated shoots of monoploid 86.040. After root

induction in MS_{30} (Murashige & Skoog, 1962, Physiol. Plant 15:473-497) (MS) medium supplemented with 30 g/l sucrose) a number of these diploid amf-plants were transferred into a glasshouse, at 19°C at day: 17°C at night and 16 h daylength, in sterilized leaf containing soil. For better flowering, part of the doubled plants was grafted onto tomato rootstock. Pollen fertility was estimated, after aceto carmine staining. For the crosses, a variety of wild-type potato pollen was used. The crosses were made on open flowers of diploid (2x) clones of 86.040.

Starch analysis. Starch granules in micropores and tubers were stained with I_2-KI solution according to Hovenkamp-Hermelink et al. (1987), in stomatal guard cells and other leaf cells according to the treatment described for microspores and in root cap cells by treatment of root tips with a mixture of Lugols-solution and choralhydrate (1:1, v/v). Four gram of choralhydrate is dissolved in 2 ml of water. The amylose percentage in starch solutions of tubers was measured according to Hovenkamp-Hermelink et al., 1988, Potato Res. 31:241-246). Roottips were fixed and stained according to Pijnacker and Ferwerda (1985, Can. J. Genet. Cytol. 26:415-419) for chromosome counts and karyotypic investigations. When for example microspores are stained with a potassium iodine solution (Lugol), starch is stained red in mutants (comprising only the *amf*-allele) and dark blue in the wild type (comprising only the A*mf*-allele) (Jacobsen et al., 1989, Euphytica 44:43-48).

Embryo culture. Unripe berries were surface sterilized by treatment for 1 minute with 70% alcohol and for 15 minutes with a saturated solution of Ca-hypochlorite, containing a few drops of 1%SDS (sodium dodecylsulphate) solution per 100 ml. The sterilized berries were cut open aseptically. Ovules were collected and cultured on medium EC2 (MS-medium supplemented with 1.10^{-5} g/l kinetin, 1.10^{-6} g/l LAA, 8g/l agar and 30 g/l sucrose) as defined by Neal & Topoleski (1983, J. Amer. Soc. Hort. Sci. 108:434-438; 1985 J. Amer. Soc. Hort. Sci. 110:869-873) for embryo culture of tomato. During ovule culture, the integument rapidly attained a brow color and was removed; this was followed later by entire excision of the embryo from the endosperm, as described by Haynes (1959). The excised embryos were also cultured on medium EC2, at 23°C and 16 h light. The rescued plantlets were propagated and rooted in MS_{30}.

Test for tuber resistance to soft rot. Tubers free of mechanical damage and disease symptoms were washed and conditioned at room temperature overnight. On the next day, tubers were sprayed with 70% (v/v) ethanol before inoculation. For inoculation, tubers were pricked once by inserting an empty sterile pipette tip in the central part of the tuber. The phytopathogenic *Erwinia carotovora* isolate IPO 161 was obtained from the DLO-Research Institute for Plant Protection (IPO-DLO), Wageningen, the Netherlands.The tubers were then point inoculated by adding 50 microliter bacterial suspension of the phytopathogenic *Erwinia carotovora*) at 6 to 7 x 10^6 colony forming units/millilitre to each tip and pressing the tip 10 mm deep into the tuber. Tubers were incubated in the dark in mist chambers at 27 degrees Celsius and 95% humidity. After 72 hrs incubation, tubers were vertically sliced and the width of deacyed tissue was measured in milimeters. Resistance level of each genotype was calculated as the mean and standard deviation of 5 infected tubers.

Results

Identification of amf-gene mutants

Based on iodine staining of microspores, genotypes corresponding to nulliplex (no wild-type GBSS-allele), simplex, duplex, triplex and quadruplex for the wild-type GBSS allele were selected.

Table 1. Erwinia carotovora subsp. atroseptica IPO 161 resistance of offspring when duplex plants (HZ91-RUG-025 x HZ91-RUG-075) are crossed. These genotypes were distinguished after iodine staining by their segregation of blue and red microspores. Mean diameter of rotted tissue (mm) of slices and small tubers of different genotypes, after inoculation with *Erwinia carotovora subsp. atroseptica* IPO 161 and subsequent incubation.

plant	Tuber slices		Small tubers	
genotype	Exp. 1	Exp.2	Exp. 1	Exp.2
aaaa	11.2	11.9	9.7	12.0
Aaaa	10.9	12.6	10.4	11.0
AAaa	11.1	12.7	9.4	10.3
AAAa	10.8	12.5	9.8	11.5
AAAA	16.2	18.4	14.8	16.9
Mean	12.0	13.6	10.8	12.3
LSD (P<0.01)	2.2	1.6	1.7	1.9

Claims

1. A potato plant or part derived thereof having at least one *amf*-allele said potato plant or part further provided with an increased genetic resistance to rot.

2. A potato plant or part according to claim 1 wherein said rot comprises soft rot.

3. A potato plant or part according to claim 1 or 2 wherein said rot is caused by a phytopathogen selected from *Erwinia spp.*

4. A potato according to anyone of claims 1 to 3 characterized in that it is an amylose-free potato.

5. A method for breeding and selecting a potato comprising crossing a first parent potato with at least one *amf*-allele with a second parent potato without an *amf*-allele and selecting progeny by testing it for the presence of at least one *amf*-gene and testing it for resistance to soft rot.

6. A method according to claim 5 further comprising selecting progeny homozygous for the *amf*-allele

7. A potato plant or part derived thereof selected with a method according to anyone of claims 5 or 6.

8. Use of a potato plant or part derived thereof having at least one *amf*-allele in a breeding and selection programme directed at providing potatoes with an increased genetic resistance to rot.

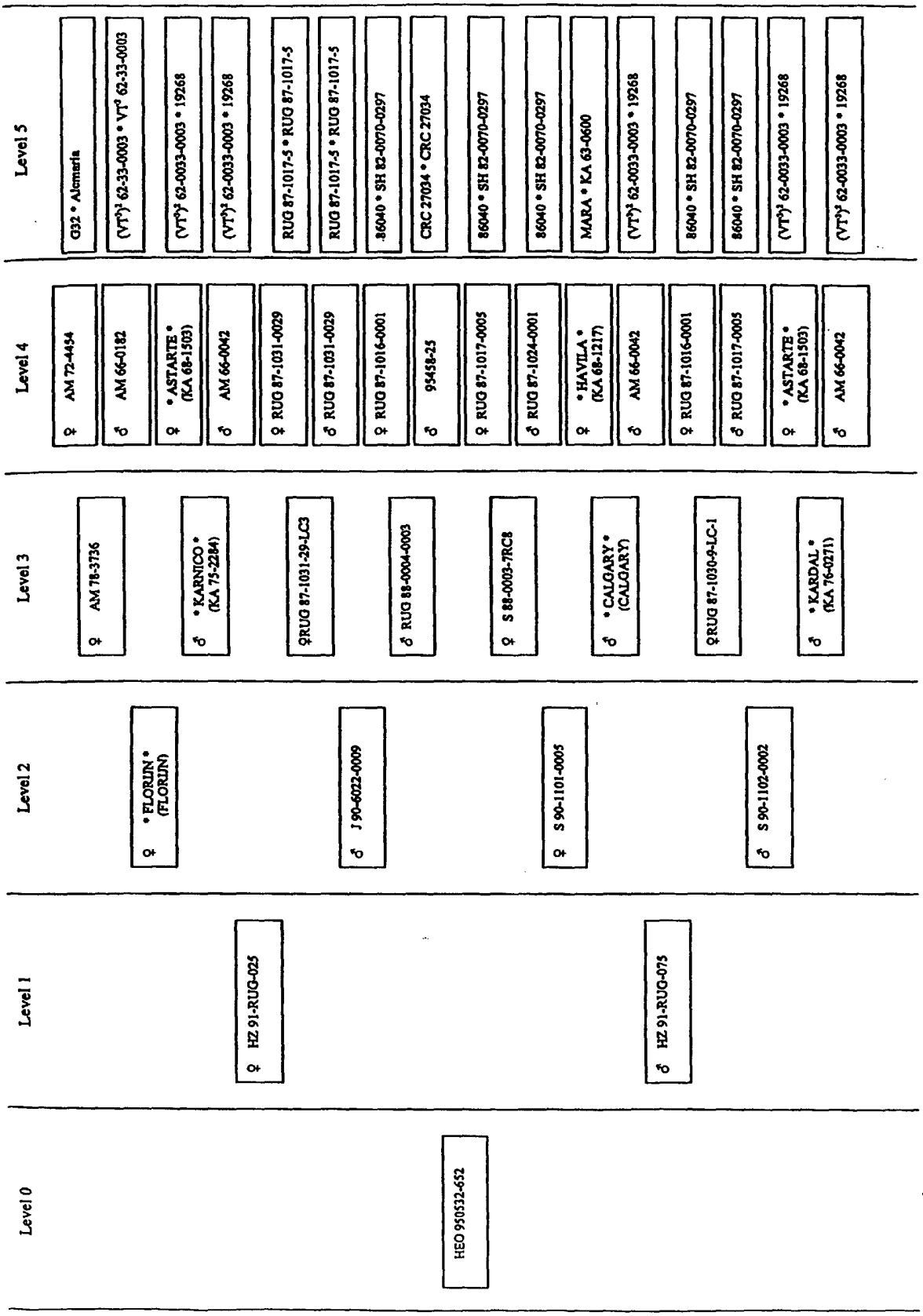
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Fig. 1

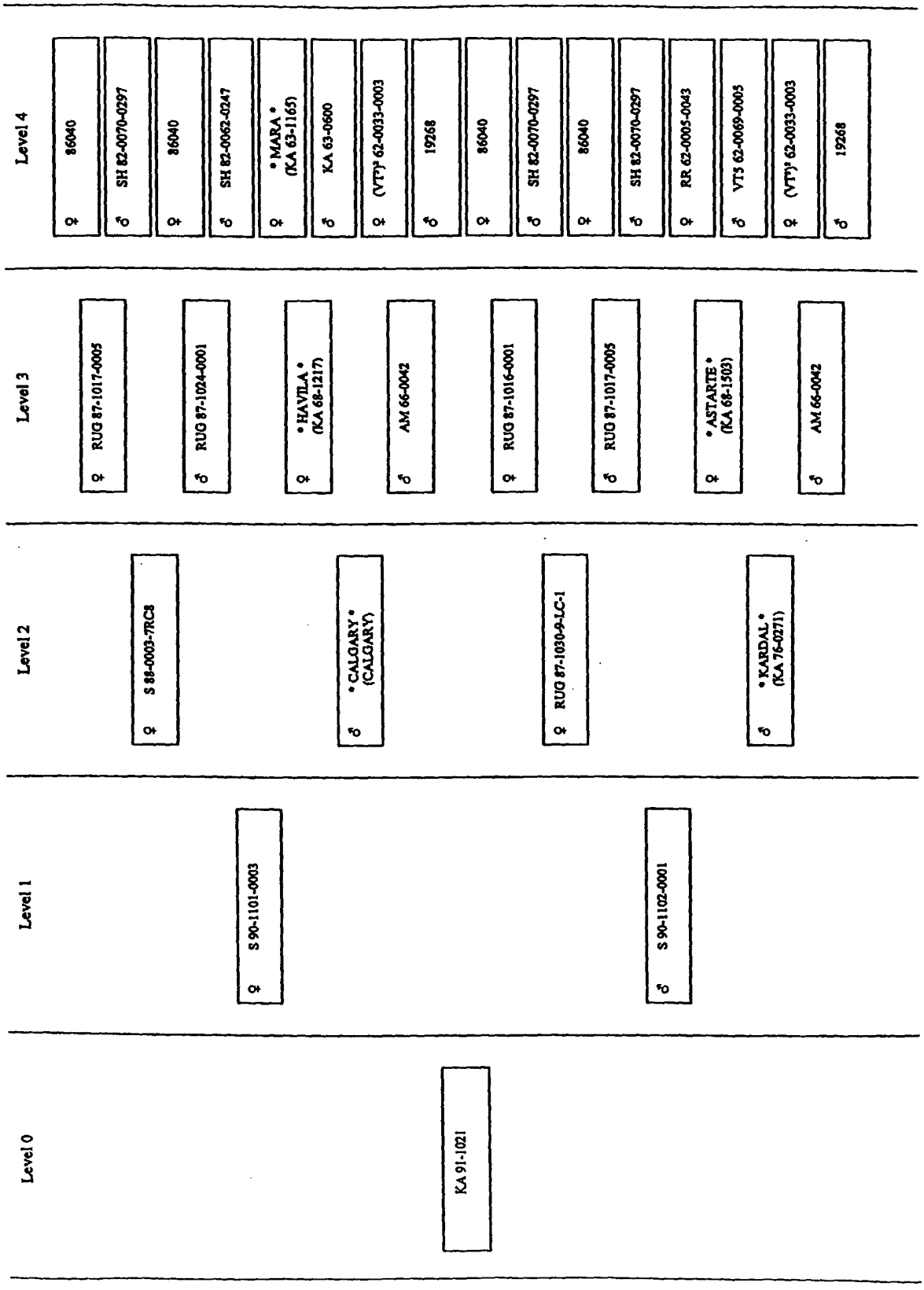
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Fig. 2

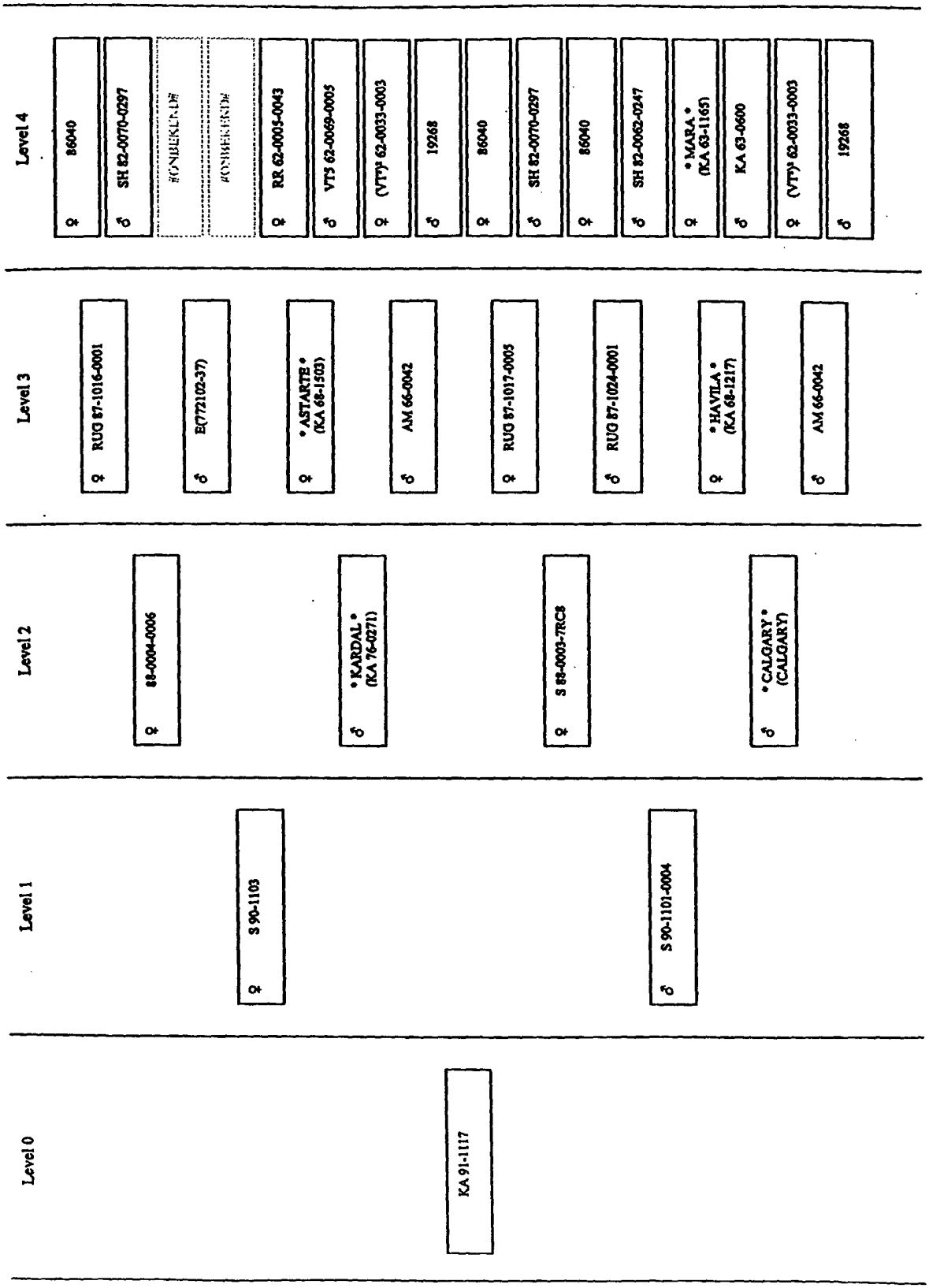
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Fig. 3

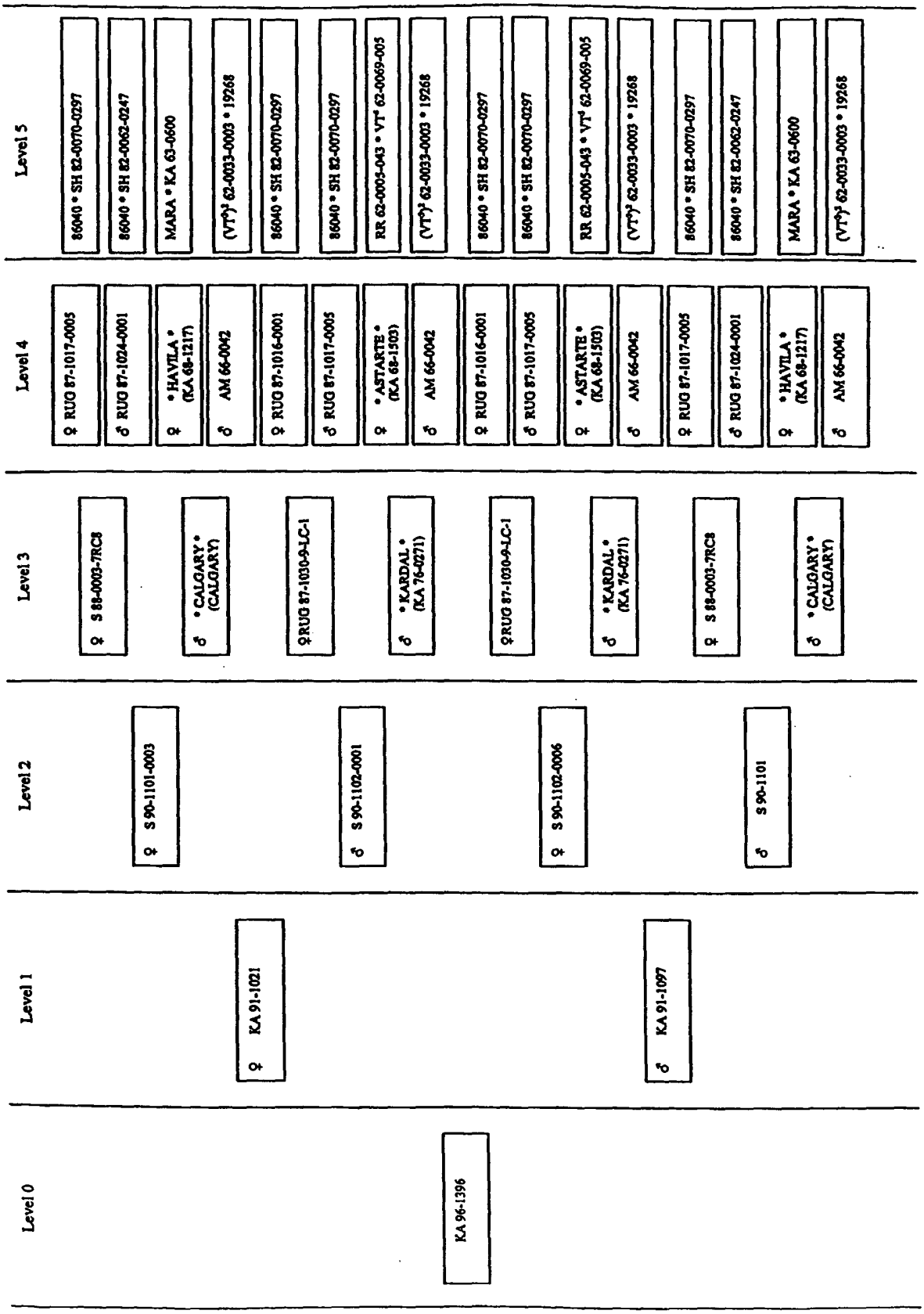
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Fig. 4

INTERNATIONAL SEARCH REPORT

PCT/NL 03/00852

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, BIOSIS, EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BASTIAANSSEN HELEEN J M ET AL: "Postmeiotic restitution in 2n-egg formation of diploid potato." HEREDITY, vol. 81, no. 1, July 1998 (1998-07), pages 20-27, XP009006115 ISSN: 0018-067X tables 1,2 ---	1-4,7
X	JACOBSEN E ET AL: "INTRODUCTION OF AN AMYLOSE-FREE (AMF) MUTANT INTO BREEDING OF CULTIVATED POTATO, SOLANUM RUBEROSUM L" EUPHYTICA, KLUWER ACADEMIC PRESS, AMSTERDAM, NL, vol. 53, 1991, pages 247-253, XP000610759 ISSN: 0014-2336 page 248, left-hand column, paragraph 2 --- -/--	1-4,7

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2004	04/03/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bilang, J

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	VAN DE WAL M H B J ET AL: "Multiple allelism as a control mechanism in metabolic pathways: GBSSI allelic composition affects the activity of granule-bound starch synthase I and starch composition in potato." MGG MOLECULAR GENETICS AND GENOMICS, vol. 265, no. 6, August 2001 (2001-08), pages 1011-1021, XP002231971 ISSN: 1617-4615 table 2 -----	1-4,7