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Datasheet for the decision of 9 February 2012

Case Number: T 1438/09 - 3.3.08

Application Number: 98915256.6

Publication Number: 1012257

IPC: C12N 15/00

Language of the proceedings: EN

Title of invention:
Methods and materials for making and using transgenic dicamba-degrading organisms

Applicant:
The Board of Regents of the University of Nebraska

Headword:
Dicamba Oxygenase/REGENTS NEBRASKA

Relevant legal provisions:
EPC Art. 56

Keyword:
"Main request and Auxiliary Requests 1 to 4 - inventive step (no)"

Decisions cited:
-

Catchword:
-

Case Number: T 1438/09 - 3.3.08

D E C I S I O N
of the Technical Board of Appeal 3.3.08
of 9 February 2012

Appellant: (Applicant)
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Decision under appeal:
Decision of the Examining Division of the European Patent Office posted on 13 February 2009 refusing European patent application No. 98915256.6 pursuant to Article 97(2) EPC.

Composition of the Board:

Chairman: M. Wieser
Members: P. Julià
J. Geschwind

Summary of Facts and Submissions

I. European patent application No. 98 915 256.6, published as International patent application WO 98/45424 (hereinafter "*the application as filed*"), was refused by the examining division. Basis for the refusal were a Main Request and Auxiliary Requests 1 to 6, all filed on 8 September 2008, which were considered not to fulfil the requirements of Article 56 EPC.

II. The applicant (appellant) lodged an appeal against the decision of the examining division, filed a statement setting out its grounds of appeal and maintained the claim requests filed on 8 September 2008. As a precautionary measure, oral proceedings were requested.

III. The examining division did not rectify its decision and referred the case to the board of appeal (Article 109(2) EPC).

IV. On 17 October 2011, the appellant was summoned to oral proceedings and, in a communication pursuant to Article 15(1) of the Rules of Procedure of the Boards of Appeal (RPBA) annexed thereto, it was informed of the board's preliminary, non-binding opinion on the substantive issues of the appeal.

V. No substantive reply to the board's communication was filed.

VI. On 9 February 2012, oral proceedings were held. At these proceedings, the appellant withdrew all its previous requests and filed a new Main Request and new

Auxiliary Requests 1 to 4 essentially based on its previous requests.

VII. Claim 1 of the **Main Request** and of all of **Auxiliary Requests 1 to 4** reads as follows:

"1. An isolated DNA molecule comprising a DNA sequence encoding a dicamba-degrading oxygenase, wherein said dicamba-degrading oxygenase is selected from the group consisting of:

a) a dicamba-degrading oxygenase having the amino acid sequence of SEQ ID NO:4; and

b) a dicamba-degrading oxygenase having an amino acid sequence which is at least 85% homologous to the amino acid sequence of SEQ ID NO:4, which comprises an iron-sulfur cluster and which catalyzes the oxidation of dicamba to 3,6-dichlorsalicylic acid (DCSA)."

VIII. The following document is cited in the present decision:

D5: Wang, X-Z, Ph Thesis, 1996, University of Nebraska, Lincoln, Nebraska.

IX. The appellant's arguments, insofar as they are relevant to the present decision, may be summarised as follows:

Article 56 EPC

Starting from the closest prior art, document D5, the technical problem to be solved was the provision of a DNA sequence encoding the oxygenase component of the dicamba demethylase from *Pseudomonas maltophilia*,

strain DI-6. Although document D5 gave a skilled person an incentive to look for this DNA sequence, it did not provide the necessary information to obtain this sequence without the exercise of skill and inventive ingenuity. The skilled person was faced with technical problems that required inventive effort. In particular, the following problems were encountered:

i) The N-terminal sequence of the dicamba-degrading oxygenase disclosed in document D5 was not complete. The amino acid residue at position seven was not specified and could be any of the known 21 amino acid residues encoded by any possible codon. The degeneracy of probes based on peptide fragments containing this unknown residue was thus very high and not viable. Although the codons of the neighbouring residues had a low degeneracy and a probe based on a peptide fragment centred on the seven residue could seemingly be the one with the lowest degeneracy, the presence of an unknown residue in the middle of this peptide taught away from using it when designing an oligonucleotide probe. A mismatch in the middle of a probe was known to be disadvantageous, since central positions were less flexible than other positions within a probe sequence. Probes based on other peptide fragments of the N-terminal amino acid sequence disclosed in document D5 were too degenerate to achieve a successful cloning.

Even if document D5 on page 117 referred to the design of a degenerate oligonucleotide sequence based upon the N-terminal amino acid sequence of the dicamba-degrading oxygenase and its use for cloning the oxygenase gene, this reference did not prompt the skilled person to re-sequence the complete N-terminal amino acid sequence

in order to determine the unknown residue at position seven. Such an interpretation implied an *ex-post facto* analysis based on hindsight knowledge of the invention. This reference only prompted the skilled person to use the N-terminal sequence as disclosed in document D5, i.e. with an unknown residue at position seven, for designing a degenerate oligonucleotide probe. Based on the teachings of document D5 and the disclosed N-terminal sequence of the dicamba-degrading oxygenase, it was not possible for a skilled person to identify the unknown residue at position seven as a tryptophan residue. There was not enough information in document D5 for such an identification. Moreover, probes derived from short peptide fragments of the N-terminal sequence of the dicamba-degrading oxygenase (12-mers derived from fragments of 4 residues) were not specific enough for a successful binding, requiring less stringent conditions for hybridization that resulted in a higher background.

ii) Even if a skilled person could have derived from document D5 that the residue at position seven of the N-terminal sequence of the dicamba-degrading oxygenase was a tryptophan, a 17-mer probe based on a peptide fragment (NAWYVA) centred on this tryptophan resulted in a 64-fold degenerate probe set that failed to turn out, in all hybridization conditions used, any positive signal in both a genomic and a plasmid DNA library from *P. maltophilia* strain DI-6. It was only after narrowing the degeneracy of the 17-mer probe to a 32-fold degenerate oligonucleotide probe set by using the codon preference of Pseudomonas bacteria that a single positive hybridization with a single fragment of DI-6 genomic DNA could be identified and isolated. However,

there was no reference in document D5 to codon preference in Pseudomonas, which was also known in the art not to be the same for all Pseudomonas strains. Moreover, there was also discussion in the prior art as to whether or not the *P. maltophilia*, strain DI-6 used in document D5, was actually a Pseudomonas strain.

iii) Even if a skilled person could have derived the information of points i) and ii) above from document D5, other technical difficulties would still have been encountered for which this document did not provide any assistance. In particular, a large amount of work and perseverance was required to find out appropriate <u>hybridization conditions</u>. As stated above, for a 64-fold degenerate oligonucleotide probe set, no positive signals were identified even though several hybridization conditions were tried. A positive clone was obtained only when a size fractioned DI-6 genomic DNA library was constructed with the plasmid vector pBluescript II KS+. Previous genomic and plasmid libraries constructed with other vectors failed to provide any positive clone. Different combinations of restriction enzymes were also tried for digesting the DI-6 genomic DNA and it was only with the *Xho*I/*Hind*III enzymes that a positive fragment could be identified using the 32-fold degenerate oligonucleotide probe set.

iv) In absence of the information discussed in points i) to iii) above, the skilled person was left with <u>no alternative techniques</u> for cloning the oxygenase gene. As shown in document D5, other molecular and genetic approaches (complementation or rescue assays) were not successful and were all bound to fail. Moreover, the isolation and characterization of internal peptides of

the dicamba-degrading oxygenase for designing further oligonucleotide probes was not a real practical option because the information provided in document D5 was highly misleading. The fractions with oxygenase activity eluting from a phenyl Sepharose column (a first step in the oxygenase purification) reported in document D5 (fractions 90-102) were not the same as those identified in the application (fractions 128-145). Furthermore, the method of purification disclosed in document D5 provided only a 90% pure dicamba-degrading oxygenase. The oxygenase preparation contained still 10% contaminants that rendered the isolation of the oxygenase internal peptides, from other peptides of contaminant proteins in the peptide mixture resulting from internal protein cleavage, highly uncertain and extremely difficult.

According to the case law of the Boards of Appeal, a reasonable expectation of success was not to be confused with the hope to succeed and, irrespective of whether or not a reasonable expectation of success existed, it was necessary to assess the real difficulties encountered by the skilled person when following the indicated route. In the present case, the well-known technical difficulties involved in attempting to clone genes using degenerate oligonucleotide probe sets and the actual difficulties encountered when attempting to clone the dicamba-degrading oxygenase, demonstrated that the skilled person would not have had a reasonable expectation of success in attempting to obtain the claimed nucleic acids, based on the minimal teaching of document D5 and within acceptable time limits.

X. The appellant (applicant) requested that the decision under appeal be set aside and that a patent be granted on the basis of the Main Request or one of the first to fourth Auxiliary Requests filed during oral proceedings.

Reasons for the Decision

Main Request

Article 56 EPC

The closest prior art and the technical problem to be solved

1. Document D5, the closest prior art, discloses the purification and characterization of the three components of the dicamba demethylase enzyme system from *Pseudomonas maltophilia*, strain DI-6, namely an oxygenase, a ferredoxin and a reductase (components I, II and III, respectively). The purification and specific activities of the oxygenase component are summarized in Table 2.3 (cf. pages 68 and 69) and described in more detail in sections 2.2.3.3.1 and 2.3.4 (cf. pages 42 and 65, respectively). After four steps of column chromatography, a preparation of 90% pure oxygenase was obtained. This partially purified oxygenase was further purified by isoelectric focusing (IEF) gel and loaded onto SDS-PAGE to check for purity (cf. Figure 2.4, pages 71 and 72). The SDS-PAGE gel slice containing the purified oxygenase was used for sequencing the N-terminal amino acids which is shown in Figure 2.10 (cf. pages 91 and 92) and described in detail in sections 2.2.3.4 and 2.3.7.4 (cf. pages 46 and 86, respectively). Based on these studies and their results, document D5 further reports the molecular and genetic approaches followed for cloning the dicamba

demethylase genes from *P. maltophilia* strain DI-6 as well as the preliminary results obtained from these experiments (cf. page 187, Appendix).

2. Starting from this closest prior art, the technical problem to be solved is the provision of a DNA sequence encoding the oxygenase component of the dicamba demethylase enzyme system from *P. maltophilia*, strain DI-6. As a solution to this problem, the application proposes an isolated DNA molecule encoding the amino acid sequence of SEQ ID NO:4 or at least 85% homologous thereto (cf. Section VII *supra*). The board is convinced that the technical problem is solved.

Obviousness and reasonable expectation of success

3. There is no doubt that the skilled person had a clear incentive from document D5 to clone the gene encoding the dicamba-degrading oxygenase from *P. maltophilia*, strain DI-6. In the board's view, this document not only informs the skilled person of the routes which result in failure (complementation approach) and are thus to be avoided but also of a successful route for cloning the desired gene. It is explicitly stated in document D5 that "*... in experiments which are an extension of the work presented in this thesis, a degenerate oligonucleotide sequence was designed based upon the N-terminal amino acid sequence of the oxygenase component of dicamba demethylase and used to clone a gene with amino acid sequences highly homologous to the oxygenase component of vanillate demethylase ...*" (cf. page 117, lines 5 to 11). This reference, in the board's view, provides the skilled person with a reasonable expectation of success.

Technical problems alleged by the appellant
The N-terminal sequence and the design of a degenerate oligonucleotide probe set

4. According to document D5, a partially (90%) purified oxygenase was further purified by IEF and SDS-PAGE electrophoresis. The SDS-PAGE gel slice was used for determining the N-terminal amino acid sequence of the dicamba-degrading oxygenase shown in Figure 2.10 (cf. pages 91 and 92, point 1 *supra*). Certainly, the obtained oxygenase was highly purified and of a very good quality. This is also clearly reflected in the length of this N-terminal sequence, namely 28 amino acid residues. It is known in the field that, typically, the background significantly increases at about 15-20 cycles rendering the amino acid determination more and more difficult.

5. This good quality is further confirmed by the presence of difficult residues - known to be partly destroyed by the harsh conditions of the Edman chemistry - at high positions of the N-terminal amino acid sequence of the dicamba-degrading oxygenase. In particular, Ser and Thr (high dehydration) at positions 17 and 24, Lys (easily oxidized) and Arg (poorly extracted) at positions 19 and 23, and aspartic acid (labile peptide bond) at positions 27 and 28. In principle, there is no reason why they should not also have been detected when present at a low position, such as at position seven, when they have been easily detected at a higher position. Thus, the determination of these residues at a high position rules out their presence at position

seven, where the background and the difficulties for their determination are essentially much lower.

6. The board does not agree with the appellant that a skilled person would have expected any of the 21 known amino acids at position seven of the N-terminal amino acid sequence of the oxygenase disclosed in document D5 (cf. Section IX *supra*). On the contrary, the term "X" depicted at position seven is technically meaningful to, and immediately identified by, a skilled person as standing for a difficult amino acid residue. As for the reasons given in point 5 above, several of these difficult to identify amino acids can be ruled out, the skilled person is aware that the term "X" can only specify other difficult residues, such as cysteine (not detectable if previously not derivatized) or tryptophan (completely destroyed depending on the neighbouring residues). The more so in view of the good quality of the peptide used for the sequencing of this N-terminal amino acid sequence, as explained above.

7. Moreover, in view of the incentive and the explicit information given on page 117 of document D5 (cf. point 3 *supra*), the board is convinced that the incomplete N-terminal amino acid sequence of the dicamba-degrading oxygenase disclosed in document D5 would be the starting point for a skilled person to design a degenerate oligonucleotide probe. Following well-known criteria in the field, a skilled person would first look for fragments or regions of low degree of degeneracy within this N-terminal sequence and easily identify the region centred around the unknown residue (NAXYVAA) which is by far the longest region with a significant low degeneracy.

8. Contrary to the appellant (cf. Section IX *supra*), the board does not consider the presence of the unknown residue to teach a skilled person away from this region. As argued in points 5 and 6 *supra*, only a very limited number of residues would come into question to fill the gap designated by the term "X", such as cysteine or tryptophan. These two amino acids are encoded, respectively, by one and two codons that have the same first and second bases and differ only in the third base. Thus, they do not significantly increase the degeneracy of this region. Moreover, alternatives were available to a skilled person for reducing the degeneracy of the probe, such as the use of "neutral" bases (inosine) at positions of ambiguity, codon preference (*infra*), etc. as cited in standard literature and laboratory manuals of the field (cf. *inter alia* Sambrook, J. et al., (1989) "Molecular Cloning: A Laboratory Manual", 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, cited in the "List of References" on page 182 of document D5).

Codon preference and hybridization conditions

9. Indeed, the selection of codons that most commonly code for a particular amino acid in the specific species under study, i.e. the codon preference, is a well-known strategy for reducing the degeneracy of oligonucleotide probe sets. Comprehensive lists of relative codon frequencies in several species were available in the art and referred to in standard literature and laboratory manuals of the field (cf. *inter alia* Sambrook, *supra*, and page 10, lines 23 to 29 of the application as filed). Thus, the selection of the

Pseudomonas codon preference for designing a degenerate oligonucleotide probe set is not considered to include any inventive merit.

10. Although, as argued by the appellant (cf. Section IX *supra*), not all Pseudomonas strains share the same codon preference, this is rather the exception than the rule. There was no reason for a skilled person to consider the *P. maltophilia* strain DI-6 to be such an exception and not to use the known codon preference in Pseudomonas species as a first step for reducing the degeneracy of the oligonucleotide probe set. Moreover, even though, as also argued by the appellant, the assignment of strain DI-6 to Pseudomonas species was the subject of certain controversy, there is nothing in document D5 to support, let alone to confirm, these alleged doubts and to lead the skilled person away from the codon preference in Pseudomonas species.

11. Likewise, the board is also convinced that the selection of appropriate hybridization conditions, even if laborious, lengthy and difficult as alleged by the appellant (cf. Section IX *supra*), cannot confer any inventive merit upon the claimed subject-matter. Protocols and instructions to guide the skilled person to find suitable hybridization conditions were available in the art (cf. *inter alia* Sambrook, *supra*), and none of the conditions referred to in the appellant's grounds of appeal are so far removed from usual, normal hybridization conditions to justify an inventive contribution. In this regard, it is noted that, in Example 2 of the application as filed concerning the "*Identification And Sequencing Of A Clone Coding For The Oxygenase Of Dicamba O-Demethylase*

Of Pseudomonas maltophilia DI-6", explicit reference is made to commercial protocols for the hybridization and detection protocols only. No further detailed information or suggestion is provided that these conditions were critical for attaining the desired result (cf. page 49, lines 31 to 33 of the application as filed).

Cloning of the dicamba-degrading oxygenase gene

12. As stated in point 3 *supra*, relevant information is disclosed in document D5 to guide the skilled person when attempting to clone the dicamba-degrading oxygenase gene. The skilled person does not start from scratch but in the knowledge of the whole disclosure and information disclosed in that document. In particular, there is a clear indication of successful cloning when using a degenerate oligonucleotide sequence based upon the N-terminal amino acid sequence of the oxygenase (cf. page 117, lines 5 to 11 of document D5).

13. Even though no conclusive results were achieved by curing plasmid DNA of DI-6 strain, there is also a clear indication in document D5 that the oxygenase gene is located on a plasmid (cf. page 241, first four lines from the bottom in document D5). The presence of multiple large plasmids in *P. maltophilia*, strain DI-6 was known in the art and it was also well established in the art that genes of catabolic enzymes used to degrade halogenated organic compounds were often located on large plasmids (cf. *inter alia* Chaudray, G.R. and Chapalamandugu, S. (1991) "Biodegradation of halogenated organic compounds", (1991) Microbiol. Rev.,

Vol. 55, pages 59 to 79, cited in the "List of References" on page 172 of document D5).

14. The board does not see any inventive merit in the selection of the specific vector used to construct the size-fractioned genomic library. The pBluescript II KS+ vector, cited in Example 2 of the application as filed, is a multipurpose, versatile, high-copy-number pUC-based plasmid commercially provided by Stratagene (La Jolla, CA). It is a well-known, advantageous plasmid vector widely used in the construction of genomic and cDNA libraries since the early 1990s. The selection and use of this plasmid vector for the construction of a size-fractioned genomic library, wherein genomic DNA is digested with different restriction enzymes, does not go beyond the normal competence and the ordinary capacity of a skilled person.

Alternative methods

15. Document D5 describes a detailed protocol for the purification of the dicamba-degrading oxygenase which provides a preparation of this enzyme of 90% purity or, after additional IEF and SDS-PAGE electrophoresis, of complete homogeneity (cf. pages 71 and 72, Figure 2.4 of document D5; point 1 *supra*). Moreover, the dicamba-degrading oxygenase is identified as having characteristics that are similar to those of other known oxygenase proteins which contain a Rieske iron-sulfur cluster. Purification protocols for Rieske-type oxygenases similar to that described in document D5 and based essentially on anion-exchange, hydrophobic-interaction and gel-filtration

chromatographies, were known and well established in the art (cf. page 86, lines 6 to 8; page 116, line 3 from the bottom to page 117, line 2; page 178, last paragraph to page 179, first paragraph of document D5).

16. Chromatographic fractions containing the three components of the dicamba demethylase enzyme system of *P. maltophilia*, strain DI-6, including the oxygenase, are identified in document D5 by their activity (cf. page 42, lines 2 to 8 of document D5). Thus, although the specific numbering of the chromatographic elution fractions containing the desired enzyme may vary or differ depending on the particular characteristics of the chromatographic column, elution gradient, flow rate, etc., there is no doubt that a skilled person can easily and immediately identify them.

17. The description of a purification protocol, the availability of homogeneous oxygenase preparations, the (partial) characterization of the structural and kinetic properties of this enzyme and further detailed information disclosed in document D5 and referred to above, would, in the board's view, certainly motivate the skilled person to simply follow the straightforward and clear indication of the route for cloning the dicamba-degrading oxygenase of *P. maltophilia,* strain DI-6, suggested in that document (cf. *inter alia* page 243, last sentence of document D5). In the light thereof and in the absence of evidence to the contrary, the board is also convinced that the preparation, isolation and characterization of internal peptides of the dicamba-degrading oxygenase that could allow the skilled person to design further (alternative, longer,

etc.) degenerate oligonucleotide probe sets, does not require any inventive contribution.

Conclusion

18. According to the established case law of the Boards of Appeal, inventive step cannot be acknowledged if a skilled person can expect to perform the cloning and expression of a gene in a fairly straightforward manner, and the cloning, although requiring much work, does not pose such problems as to prove an expectation of success to be ill-founded. Such problems can only be taken in consideration when convincing evidence is provided that real technical difficulties were encountered when carrying out the actual cloning (cf. "Case Law of the Boards of Appeal of the EPO", 6th edition 2010, I.D.6, page 177). In the present case, the board considers that the appellant has failed to provide this evidence. Therefore, in the light of all the above considerations, the subject-matter of the Main Request does not fulfil the requirements of Article 56 EPC.

Auxiliary Requests 1 to 4

19. Since claim 1 of Auxiliary Requests 1 to 4 is identical to claim 1 of the Main Request (cf. Section VII *supra*), the same arguments as for the Main Request apply to all these Auxiliary Requests. None of them is considered to fulfil the requirements of Article 56 EC.

Order

For these reasons it is decided that:

The appeal is dismissed.

The Registrar: The Chairman:

A. Wolinski M. Wieser