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D E C I S I O N
of 9 December 2004

Case Number: T 1006/02 - 3.3.8

Application Number: 92919292.0

Publication Number: 0601092

IPC: C12N 15/65

Language of the proceedings: EN

Title of invention:

Method for the selection of genetically transformed cells and compounds for use in the method

Patentee:

Syngenta Participations AG

Opponents:

Unilever PLC
BASF Aktiengesellschaft
CAMBIA Center for the Application of Molecular Biology to
International Agriculture

Headword:

Selection of transformed cells/SYNGENTA

Relevant legal provisions:

EPC Art. 54, 56, 83, 84, 123(2)

Keyword:

"Main request: added matter (no)"
"Novelty (yes)"
"Inventive step (no)"
"First auxiliary request: Inventive step claim 1 (yes)"
"Sufficiency of disclosure (no)"
"Second auxiliary request: Sufficiency of disclosure (yes)"
"Inventive step (yes)"

Decisions cited:

-

Catchword:

-



Case Number: T 1006/02 - 3.3.8

D E C I S I O N
of the Technical Board of Appeal 3.3.8
of 9 December 2004

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Decision under appeal: Interlocutory decision of the Opposition
Division of the European Patent Office posted
28 August 2002 concerning maintenance of
European patent No. 0601092 in amended form.

Composition of the Board:

Chairman: L. Galligani
Members: T. J. H. Mennessier
C. Rennie-Smith

Summary of Facts and Submissions

- I. The patent proprietor (appellant I), opponent 02 (appellant II) and opponent 03 (appellant III) each lodged an appeal against the interlocutory decision of the opposition division given at oral proceedings on 27 June 2002 with written reasons posted on 28 August 2002, whereby the European patent No. 0 601 092 was maintained on the basis of the second auxiliary request (claims 1 to 5) filed on 27 June 2002. The patent had been granted on European application No. 92 919 292.0 which originated from an international application published as WO 93/05163 (to be referred to in the present decision as the application as filed).
- II. The patent had been opposed also by a further party (opponent 01) which did not appeal against the decision of the opposition division. Opponent 01 was respondent to appellant I's appeal.
- III. The patent had been opposed on the grounds that (i) the invention was not new and did not involve an inventive step (see Article 100(a) EPC), (ii) the invention was not sufficiently disclosed (see Article 100(b) EPC), and (iii) the subject-matter of the patent extended beyond the content of the application as filed (see Article 100(c) EPC).
- IV. The granted claims had been found by the opposition division to comply with the requirements of Articles 54 and 123(2) EPC but were refused for the reason that they did not involve an inventive step (Article 56 EPC) over document D2 (see section X, *infra*).

V. The three statements of grounds of appeal were filed. Appellant I filed a response to the appeals filed by appellants II and III. Appellant III filed observations in reply to the appellant I's statement of grounds. The Board issued a communication pursuant to Article 11 of the Rules of Procedure of the Boards of Appeal in which provisional and non-binding opinions were expressed. In reply to that communication both appellants I and III filed further observations. Appellant I filed new first to third auxiliary requests, the main request being the claims as granted and the fourth and fifth auxiliary requests being, respectively, the first and second auxiliary requests filed during oral proceedings at first instance.

VI. Oral proceedings took place on 9 December 2004 at which appellant I filed a new first and a new second auxiliary requests in replacement of all previous auxiliary requests. The oral proceedings were not attended by appellant III as announced in its letter of 25 November 2004.

VII. The **main request** (claims as granted) consisted of 7 claims which read:

"1. A method of selecting genetically transformed plant cells from a population of cells which comprises supplying the said population with a compound which can be metabolized by the expression product of a nucleotide sequence which has been introduced into the transformed cells, so as to provide the transformed cells with a physiological advantage when compared to the non-transformed cells, wherein the compound is not an

antibiotic or herbicide and has no direct adverse effect on the non-transformed cells."

- "2. A method according to claim 1, in which the compound is selected from the group consisting of cytokinins, auxins, gibberellins, vitamins, carbohydrates, opines, proteins, sterols and saponins."
- "3. A method according to either of claims 1 or 2, wherein the said nucleotide sequence encodes
- (i) β -glucuronidase when the compound is a cytokinin glucuronide;
 - (ii) mannose-6-phosphate isomerase when the compound is mannose;
 - (iii) UDP-galactose-4-epimerase when the compound is galactose or galactose-containing compound
 - (iv) a permease, or is
 - (v) an opine metabolism or transport gene"
- "4. A method according to any preceding claim, wherein the transformed cells contain a further introduced sequence."
- "5. A method according to the preceding claim, wherein native β -glucuronidase activity is reduced by supplying to the culture medium a pH regulating compound which provides the culture medium, the

cells or compartments of the cells with a pH of between 5.5 and 8.5."

- "6. A method according to claim 2, wherein the opine does not function or only insufficiently functions as a nitrogen source for the non-transformed cells and [t]he introduced nucleotide sequence comprises an opine metabolism or transport gene which upon expression allows the opine to function as a nitrogen source in transformed cells."
- "7. A method according to claim 1, wherein expression or transcription of the introduced nucleotide sequence leads to an increase in the activity of an enzyme found endogenously in the population of cells, such that the activity of the enzyme in transformed cells is greater than the activity of the enzyme in non-transformed cells, or wherein expression or transcription of the introduced nucleotide sequence results in blockage of the metabolism of the compound which is supplied to the population of cells or blockage of the synthesis of a compound in the transformed cells."

VIII. The **first auxiliary request** consisted of 6 claims and differed from the main request in that claim 1 had been amended as shown below, claim 3 had been amended by adding the words "**co-introduced expressible**" between the words "said" and "nucleotide", claim 4 had been deleted and claims 5 to 7 had been renumbered.

Claim 1 of the first auxiliary request read:

"1. A method of selecting genetically transformed plant cells from a population of cells, **wherein the genetically transformed plant cells are transformed with a desired expressible nucleotide sequence containing a regulatory sequence enabling its expression in the transformed cells and a co-introduced expressible nucleotide sequence also containing a regulatory sequence enabling its expression in the transformed cells**, which **method** comprises supplying the said population with a compound which can be metabolized by the expression product of **the co-introduced expressible nucleotide sequence** which has been introduced **with the desired expressible nucleotide sequence** into the transformed cells, so as to provide the transformed cells with a physiological advantage when compared to the non-transformed cells, wherein the compound is not an antibiotic or herbicide and has no direct adverse effect on the non-transformed cells."
(emphasis added by the Board to show the differences with claim 1 of the main request)

IX. The **second auxiliary request** consisted of 4 claims and differed from the first auxiliary request in that claims 5 and 6 had been deleted and claim 3 had been amended to read:

"3. A method according to either of claims 1 or 2, wherein the said co-introduced expressible nucleotide sequence encodes β -glucuronidase when the compound is a cytokinin glucuronide."

X. The following documents are referred to in the present decision:

(D2) Richard A. Jefferson, "New Approaches for Agricultural Molecular Biology: From Single Cells to Field Analysis", in "Gene Manipulation in Plant Improvement II", Gustafson, Ed., Plenum Press, New York (USA), 1990, Pages 365 to 400

(D6) WO-A-89/03880 (published on 5 May 1989)

(D7) Raju S.S. Datla et al., Gene, Vol. 101, 30 May 1991, Pages 239 to 246

(D9) George A. Karlin-Neumann et al., The Plant Cell, Vol. 3, June 1991, Pages 573 to 582

XI. The submissions made by appellant I (patent proprietor), insofar as they are relevant to the present decision, may be summarised as follows:

Main request (granted claims)

- Article 123(2) EPC

There was clear support on page 10, lines 13 to 16, in the application as filed for the feature found in claim 1 that "the compound is not an antibiotic or herbicide".

- Article 54 EPC

The cells according to claim 1 were transformed upon introduction therein of only one nucleotide sequence,

namely a sequence the expression product of which could metabolise a compound supplied to the cells so as to provide the transformed cells with a physiological advantage. The method of claim 1 comprised supplying the afore-mentioned compound to a **unique population of cells** consisting of transformed cells and of untransformed cells. This was in clear contrast to the experiment reported on page 396 of document D2 where **two distinct populations of cells**, one consisting of transformed cells and the other of untransformed cells, were described. Anyway the said experiment lacked enablement as the preparation of the tryptophol-glucuronide conjugate had not been disclosed. Document D6 essentially described a glucuronide permease gene and did not relate to a method of selection.

- Article 56 EPC

Document D2 was not dealing with selection of transformed plant cells but rather with the general problem posed by the development of methods for real time, non-destructive analysis of the regulation of a gene introduced into plants in order to localise gene activity to individual cells within tissues of the transgenic plants.

The relevant part of document D2 for the assessment of inventive step was not the afore-mentioned passage on page 396, which described an experiment only accidentally close to the invention, but rather the passage at the bottom of page 392 starting with the terms "An interesting complement" and ending with the term "be compromised". In said passage, it was proposed to spray or coat onto the surface of the plant tissue

the GUS substrate in order to localise GUS (β -glucuronidase) activity. The proposal was based on the secretion of the enzyme by the transgenic cells transformed with a GUS system generated between a promoter of the gene of interest and the GUS gene, as illustrated in detail on pages 374 and 375 of the document where constructs consisting of a promoter from a patatin gene and the E. coli GUS gene were introduced in tomato.

Therefore, the skilled person facing the technical problem posed by the provision of an alternative to the routine selection systems based on differential growth characteristics, such as typified by antibiotic selection, would not have derived the method of claim 1 from document D2.

First auxiliary request

- Article 123(2) EPC

The remarks made with respect to the main request applied similarly to the first auxiliary request.

- Article 84 EPC

The term "co-introduced", as used in claims 1 and 3, had been unambiguously defined on page 5, lines 16 to 21, in the patent specification. As to the term "expressible", the skilled person would have understood without difficulty that it had been meant that the nucleotide sequences referred to in the claim were capable of expressing a protein.

- Article 83 EPC

The GUS system was clearly indicated, and extensively exemplified, in the patent as a way of carrying out the invention. Other matched compound/expression product pairs were known in the art at the priority and filing dates, and some were mentioned in paragraph 0030 on pages 5 and 6 in the patent specification. It could therefore be seen that there were suitable variants, known to the skilled person through the disclosure of the patent, which provided the same effect of the invention. Other systems would be known from common general knowledge. It was not necessary for these other variants to be exemplified. Thus, the requirements of Article 83 EPC were satisfied.

- Article 56 EPC

The method of claim 1 related to the selection of the cells actually transformed within a population of cells which had been submitted to transformation with two distinct nucleotide sequences, one being a gene of interest and the other a selection gene. This was in clear contrast to the teaching of document D2 which dealt with transgenic plants or, as accidentally disclosed on page 396, with two distinct populations of cells, one consisting of transformed cells and the other of untransformed cells. The said document described systems which did not involve co-introduction into the plant cells of two distinct sequences.

Account being taken of the differences between the method of claim 1 and the disclosure of document D2, for the skilled person faced with the technical problem

of providing an alternative to the routine selection systems based on differential growth characteristics, such as typified by antibiotic selection, there was no incentive to proceed so as to arrive at the invention of claim 1.

As for the other documents: the data presented in document D9 demonstrated the feasibility of using the Agrobacterium tms2 gene as a selectable reporter gene for negative selection in Arabidopsis; document D6 did not relate at all to a method of selection; in document D7, GUS was not used as a positive selection marker.

Thus, the skilled person, faced with the aforementioned technical problem, would not have arrived at the method of claim 1 based on the teaching of any of documents D6, D7 and D9 or any combinations thereof.

Second auxiliary request

- Articles 123(2), 84, 83 and 56 EPC

The remarks made with respect to the first auxiliary request applied similarly to this request.

XII. The submissions made by appellants II and III (opponents 02 and 03) and opponent 01, insofar as they are relevant to the present decision, may be summarised as follows:

Main request (granted claim 1)

- Article 123(2) EPC

The passage to on page 10, lines 13 to 16 in the application as filed referred to by appellant I was concerned with the identification and isolation of transformed cells without the use of selection genes coding for antibiotic or herbicide resistance. Moreover, in that passage there was no explicit mention of any compound which was not an antibiotic or herbicide. Therefore, the feature that "the compound is not an antibiotic or herbicide" as contained in claim 1 had to be regarded as added matter.

- Article 54 EPC

The experiment on page 396 of document D2 undoubtedly described a positive selection method using, as the compound capable of being metabolised by GUS, tryptophol which was an auxin, in the presence of which the transformed tobacco leaf discs remained green and healthy, ie were provided with a physiological advantage compared to the untransformed leaf discs. Thus, D2 disclosed the method of claim 1.

Also document D6, which described β -glucuronidase as an appropriate positive selection marker in plant cells (see pages 2 and 3), disclosed the method of claim 1.

- Article 56 EPC

It was trivially obvious that the results obtained in the experiment on page 396 of document D2 could be used to select, ie identify and isolate, transformed tissue from untransformed tissue. Even if the experiment used two previously prepared, distinct and uniform populations, it was immediately apparent that if the

two populations had been mixed, the transformed tissues would have been identified and isolated, ie selected, by virtue of the fact the transformed tissues survived and were healthy, while the untransformed tissues died.

Various statements in document D2 indicated and repeated the possibility of using GUS-fusion systems to perform positive selection: see on page 388: "These directions include the development of methods for [...] positive and negative selections"; on page 392: "This would obviate genetic selections based on differential growth characteristics, such as typified by antibiotic selection. Transformants could then be screened, obtained and cultured directly without recourse to laborious, lengthy and often impractical antibiotic selections."; on page 394: "Development of general methods for conditional positive and negative selections based on gene fusion action must be an important goal .."; and on page 395: "This could therefore provide a positive selection for the activity of the gene fusion.". Thus, the skilled person would have found in document D2 the incentive to develop the method of claim 1.

First auxiliary request

- Article 123(2) EPC

The objection made with respect to claim 1 of the main request also applied to claim 1 of the first auxiliary request.

- Article 84 EPC

Both the terms "co-introduced" as used in claims 1 and 3 and "expressible" as used in claim 1 rendered the claimed subject-matter unclear.

- Article 83 EPC

In the examples the transformed shoots were selected (identified) subsequently by conventional X-Gluc assay, ie using a substrate which upon metabolisation by β -glucuronidase stained the transformed cells rather than providing them with a physiological advantage. It was on that basis - not as a result of the use of cytokinin-glucuronide - that the transformed shoots could be identified, counted and (if desired) separated. Therefore, the examples did not enable what was claimed.

Given the evident difficulties in performing what was claimed in respect of a GUS-based system in connection with the supposed pH-dependency of native GUS expression, it could not be concluded that the description was enabling for other systems, without anticipation of undue difficulty. It should be presumed that other systems would have their own problems and specific requirements; so that the patent could not be presumed to disclose sufficiently other systems.

- Article 56 EPC (claim 1)

For the reasons given with respect to the main request, claim 1 of the first auxiliary request was similarly not inventive in view of document D2 alone.

Document D2 could also be considered in association with document D6 or document D7 which described the assembly in vivo of a gene encoding a bifunctional fusion peptide between E. coli β -glucuronidase and neomycin transferase II as well as a potential application of that peptide as a marker for facilitating the direct selection and characterisation of plant regulatory sequences.

Moreover, claim 1 was also not inventive in view of document D9 taken on its own which showed that the *tms2* gene can serve as a regulatable selectable marker in Arabidopsis and, thereby, described a strategy for selecting mutants in the signal transduction pathway.

Second auxiliary request

- Articles 123(2), 84, 83 and 56 EPC

The remarks made with respect to the first auxiliary request applied similarly to this request.

XIII. Appellant I requested that the decision under appeal be set aside and that the patent be maintained as granted, or, in the alternative, on the basis of the first or second auxiliary requests filed during the oral proceedings; and that the appeals of appellants II and III be dismissed.

XIV. Appellants II and III requested that the decision under appeal be set aside and the patent be revoked.

XV. Opponent 01 requested that the appeal of appellant I be dismissed.

Reasons for the Decision

Main request

Article 123(2) EPC

1. It is argued by appellants II and III that there is no support in the application as filed for the feature found in claim 1: "the compound is not an antibiotic or herbicide".
2. According to page 10, lines 12 to 16, in the application as filed, "the method [of the invention] is particularly suitable for the selection of genetically transformed plant cells, thereby allowing identification and isolation of such cells without the use of selection genes coding for antibiotic or herbicide". This excludes the possibility that the compound used in the claimed method in relation to the selection gene be an antibiotic or an herbicide. Therefore, the objected feature does not result in an extension of the subject-matter beyond the content of the application as filed. Thus, the requirements of Article 123(2) EPC are met.

Article 54 EPC

3. Documents D2 and D6 have been cited against the novelty of the subject-matter of claim 1.

4. Document D2 is a review article investigating the possibility of using in agricultural molecular biology DNA constructs (called by the author "gene fusions") in which "DNA sequences from two (or more) genes are combined such that the coding sequences of one gene (the responder) are transcribed and/or translated under the direction of another gene(s) (the controller)" (see page 368, in particular Figure 1). The only detailed gene fusions contain the coding sequence of the E. coli gene β -glucuronidase gene (GUS).

5. A large part of the document is dedicated to gene fusions between a promoter from a patatin gene and the E. coli GUS gene, and their use **in investigating the transcriptional regulation** of the patatin gene in potatoes transgenically transformed with said gene fusions (see page 373 to the top of page 388). Experiments designed and carried out to address this issue are reported and discussed.

6. Apart from these experiments, further experimental results are reported on page 396. Leaf discs were prepared from tobacco plants transformed or untransformed with a construct consisting of a CaMV 35S promoter operatively linked to a GUS gene (the coding sequence of the E. coli GUS gene). The leaf discs were cultured in a medium supplemented with a conjugate of the tryptophol auxin with glucuronic acid. It was observed that only the leaf discs derived from GUS-transformed plants, which thus expressed β -glucuronidase, remained green and healthy, due to the effect of the auxin released from the conjugate.

7. It can thus be said that document D2 describes a process which allows to distinguish between transformed and untransformed plant cell populations (leaf discs), by using a compound (tryptophyl glucuronide conjugate) which is metabolised by the expression product (β -glucuronidase) of the nucleotide sequence (GUS gene) that has been introduced upon transformation in the plant cells. In the experiment on page 396, in contrast to the method of claim 1, two distinct populations, one consisting of transformed cells and the other of untransformed cells, **not** a single cell population consisting of both transformed and untransformed cells were tested. This marks a distinctive difference between the process of document D2 and the method of claim 1 and, strictly viewed, is enough to make the latter novel over the former.

8. Document D6 essentially describes the E. coli glucuronide permease gene as well as the permease per se and its use for selectively altering the permeability of cells. As part of the background relevant thereto, Section 2.3 on pages 2 and 3 briefly reminds the reader of the possibility of using β -glucuronidase from E. coli as a gene fusion marker. In particular, it is mentioned that gene fusions between the E. coli β -glucuronidase gene and the CaMV 35S promoter had been used to transform tobacco plants. In Section 5.2.1., on pages 12 and 13, the possibility is mentioned of using glucuronide permease together with the GUS gene fusion marker in order to allow incorporation of β -glucuronidase substrates into undisrupted cells and, thus, detection of β -glucuronidase reporter gene activity in vivo. However, there is no description of the use of such constructs

in association with a compound which, as required in claim 1 at issue, is metabolised by the enzyme so as to provide, within a population of transformed and untransformed cells, the transformed cells with a physiological advantage, when compared with the untransformed cells. Thus, the method of claim 1 is also novel over document D6.

9. Thus, as neither document D2 or D6 anticipates the subject-matter of claim 1, the requirements of Article 54 EPC are met by the main request as a whole, the remaining claims being all dependent on claim 1.

Article 56 EPC

10. It is argued by the opponents-appellants that the skilled person would have found in document D2 an incentive to develop the method of claim 1.
11. As already explained (see points 6 and 7, supra), an experiment is disclosed on page 396 of document D2 wherein the distinction was made between plant cells having incorporated the E. coli GUS gene (transformed cells) and untransformed plant cells, when cultured as two distinct populations in the presence of tryptophyl conjugate. It is shown that the conjugate is metabolised by the expression product (β -glucuronidase) of the nucleotide sequence (GUS gene) introduced in the cells. The transformed cell population, **but not** the untransformed one, remained green and healthy as a result of its ability to cleave active auxin from the supplemented tryptophyl glucuronide and use it to its advantage.

12. Having regard to document D2, the underlying technical problem in the present case can be defined as finding a method for discriminating between transformed and untransformed cells **within a population of plant cells genetically transformed** with a nucleotide sequence that expresses a protein.
13. The solution proposed in claim 1 is a method of selection which is based on the physiological advantage conferred on the transformed cells by the introduction of a nucleotide sequence capable of expressing a product which metabolises a compound, which is not an antibiotic or herbicide and has no adverse effect on the non-transformed cells.
14. As repeatedly stated above (see points 6, 7 and 11), document D2 describes a process which allows to discriminate (thus, select) **between two distinct plant cell populations**, namely a population of genetically transformed plant cells which express a fusion construct comprising the GUS gene (CaMV 35S promoter - E. coli GUS gene) and an untransformed plant cell population. The process is based on the expression of the GUS gene product which metabolises a compound, which is not an antibiotic or herbicide and has no adverse effect on the non-transformed cells.
15. The relevant question here is whether the skilled person would have used the same approach for discriminating between transformed and untransformed cells **within a population of genetically transformed cells**.

16. In the view of appellant I, document D2 is only accidentally close to the claimed method and, in evaluating inventive step, one should consider that the purpose of document D2 was not to provide a selection method of transformed plant cells but rather the development - via gene fusions - of methods of analysis and monitoring of a gene introduced in plants. The gene fusions described were in particular used for detecting and localising gene activity in subsets of cells, tissues or stages of plant development. There were no suggestions or expectations of using such an approach in the selection of transformed cells within a cell population. Moreover, the experiment on page 396 was not particularly inviting as use was made therein of a very weak auxin (chosen for its ease of synthesis) and there were no instructions how to synthesise glucuronide compounds which would be suitable for a selection method such as the one claimed. In appellant I's view, this renders it non-enabled.
17. In judging the inventive step involved in claim 1, it is important to note that the claim covers in its generality the simple embodiment wherein the genetic transformation of the plant cells is carried out only with the GUS gene, no additional gene being introduced. Such transformation is precisely that described in document D2, where the construct CaMV 35S promoter - E. coli GUS is introduced into plant cells. This is also the preferred construct in the patent in suit. The only difference between the said embodiment and the disclosure in D2 lies in the fact that, while in the latter discrimination based on expression of GUS is carried out on two separated cell populations (transformed and untransformed), in the claimed

embodiment discrimination is made **within the same population**. However, the principle is the same. In the board's judgement, the skilled person would have had no hesitation in proposing the approach of document D2 in order to distinguish GUS-transformed cells from GUS-untransformed cells within the same population, thereby monitoring the successful transformation. Document D2 on page 395 refers to the approach used as a way to achieve **positive selection** for the activity of the gene fusion, ie a selection of only those subsets of cells or tissues in which the GUS gene was successfully introduced and expressed. This is in the document explicitly contrasted to the situation of a **negative selection** in which the aglycone is a toxin such a cell-lethal antibiotic or a herbicide. The positive selection is based on some advantage which is conferred on the transformed cells by way of introduction of the construct in comparison to the untransformed cells where the construct was not introduced. Nothing would prevent the skilled person from readily thinking that this approach could also be used to carry out the discrimination between successfully and unsuccessfully GUS-transformed cells within the same population of cells. The use in document D2 of a "very weak" auxin which is easy to synthesise has no influence on the expectation of the skilled person that such an approach would work. Document D2 on page 396 refers to "thousands of compounds, with diverse biological and chemical properties [which] can be conjugated as glucuronides" and to "more that three thousand $\tilde{A}[\beta]$ -glucuronides known in the literature, and almost limitless possibilities for synthesis or biological preparation of more". So lack of enablement is out of the question.

18. For these reasons, the method of claim 1 does not involve an inventive step and, thus, the requirements of Article 56 EPC are not met by the main request as a whole.

First auxiliary request

Article 123(2) EPC

19. The objection raised against the feature: "the compound is not an antibiotic or herbicide" has been dealt with above (favourably to the patentee-appellant) in respect of the main request (see point 1). The opponents-appellants had no further objections under Article 123(2) and (3) EPC against the amendments introduced in this request in comparison with the claims as granted. Nor does the Board have any objections in this respect as the amendments are of a restrictive nature and are fully supported by the application as filed.

Article 84 EPC

20. The opponents-appellants argued that the terms "co-introduced" as used in claim 1 and "expressible" as used in claims 1 and 3 render the claimed subject-matter unclear.
21. On page 5, lines 16 to 21 in the patent, it is stated that "[T]he fact that a nucleotide sequence is "co-introduced with" the desired nucleotide sequence refers to the fact that the two nucleotide sequences are coupled to each other or otherwise introduced

together in such a manner that the presence of the co-introduced nucleotide sequence in a cell indicates that the desired nucleotide sequence has been introduced into the cell". This is an unambiguous definition in view of which the objection of lack of clarity raised against the term "co-introduced" becomes irrelevant.

22. The term "expressible" is not unusual for a skilled person in the field of genetics, where it is common to refer to the expression of a nucleotide sequence as the process which upon the transcription of the coding DNA sequence thereof into a messenger RNA and the then translation of that RNA into an "expression product" that gives rise to an "expression product". It is evident that if an expression product can be derived from a nucleotide sequence, this in turn can be regarded as an expressible sequence. Therefore, the Board sees no clarity problem in respect of the use of term "expressible".

23. Thus, the requirements of Article 84 EPC are met.

Article 56 EPC

24. Claim 1 of this request requires that the plant cells be genetically transformed with **two co-introduced nucleotide sequences**, one encoding a desired expressible gene, the other encoding the product that metabolises the compound that provides the transformed cells with a physiological advantage, thereby making a positive selection possible. The simple embodiment referred to in point 17 above is no longer covered by this claim.

25. The opponents-appellants argued that the skilled person would have found in document D2 an incentive to develop a method of positive selection as defined in claim 1, in particular in combination with document D6 or D7. They also referred to document D9.
26. As the closest prior art and the underlying technical problem can be defined essentially as in the case of the main request (see points 11 and 12 above), the question to be answered is whether the solution now proposed (see point 24 above) would have readily occurred to a person skilled in the art, having regard to document D2 alone or in combination with any of the other documents referred to.
27. As already stated, the focus of the disclosure in document D2 is the development of methods based on the use of special genetic constructs to investigate the regulation of the expression of a heterologous gene in a plant. For that purpose, use is made of constructs where the coding sequence of a gene encoding a known polypeptide (optionally with the signal sequence) the presence of which can be easily detected (responder gene usually encoding an enzyme) is operatively linked to the coding sequence of another gene (eg a promoter) which directs transcription and/or translation of the first sequence (controller gene) (see pages 368 and 369). In this framework, on pages 395 and 396 it is explained how fusion genetics can serve as a tool for directing the effects of a compound (eg a plant regulator or an herbicide) to subsets of cells, tissues or stages of plant development where the said gene fusion is active. The experiment on page 396 shows that, upon transformation of plant cells with the construct

CaMV 35S promoter - E. coli GUS gene, a positive visual selection for the activity of the gene fusion is feasible, the discs being green and healthy. The gene fusion of document D2 is one where the controller portion (eg the promoter) directs expression of the reporter portion (eg GUS). The said reporter gene is the desired expressible gene and, at the same time, the gene encoding the product that metabolises the compound which provides the transformed cells with a physiological advantage, thereby making a positive selection possible. Document D2, however, does not suggest the possibility of de-coupling the two functions, ie to co-introduce (eg by gene fusion) one gene encoding a desired expressible gene and a second gene encoding the product that metabolises a compound which, by providing the transformed cells with a physiological advantage, allows selection of the transformed cells.

28. The question arises whether any of the other documents referred to by the opponents-appellants would have given the skilled person such a suggestion.
29. Document D6, which has been already discussed (see point 8, supra) does not deal with a method for selecting transformed cells in a cell population also containing untransformed cells. Document D6 cannot therefore complement document D2.
30. As for document D7, it actually shows the utility of a β -glucuronidase (GUS) and neomycin phosphotransferase II (NPT-II) gene fusion as a versatile marker in plants by its introduction into tobacco under the control of the CaMV 35S promoter, by first selecting directly

kanamycin resistance and subsequently assaying enzymatically and histochemically for GUS activity. The authors of document D7, being persuaded that "GUS does not offer a positive selection" (see the last sentence of the first paragraph in the left-hand column on page 240) have performed a **negative selection**. Also the concept of selecting transformed plant cells in a cell population containing also untransformed cells is absent from document D7. Therefore, the skilled person would have found no incentive to combine the teaching of document D2 with that of either document D6 or document D7 and arrive thereby at a selection method such as claimed.

31. The further argument that document D9 is relevant for the assessment of inventive step is also not convincing, at least for the reason that, as explicitly acknowledged on page 577 (see the Section entitled "Discussion"), it reports experiments which demonstrate the feasibility of using the Agrobacterium tms2 as a selectable reporter gene for **negative selection** in Arabidopsis, whereas claim 1 is directed to a method of positive selection.

32. Therefore, in the board's judgment, the skilled person facing the aforementioned technical problem would have found no incentive in document D2 to test any of the gene fusion systems and glucuronide conjugates actually described therein, in particular the CaMV 35S promoter E. coli β -glucuronidase gene construct in association with a tryptophol or cytokinin glucuronide as described on page 396, with a view to developing a method of positive selection of plants having incorporated a further desired nucleotide sequence. Thus, the

subject-matter of claim 1 involves an inventive step over document D2 and the first auxiliary request as a whole meets the requirements of Article 56 EPC.

Article 83 EPC

33. Under this heading, it is objected by the opponents-appellants that in the examples the selection of the transformed shoots was achieved by using not a cytokinin-glucuronide but X-Gluc, ie a substrate which upon metabolism by β -glucuronidase stained the transformed cells rather than providing them with a physiological advantage.
34. This view is contradicted by an analysis of the content of Example 13 (see pages 41 and 42 in the patent specification) which describes a preferred embodiment of the method of claim 1. Leaf pieces of Nicotiana tabacum are dipped in a suspension of bacteria (Agrobacterium). Co-cultivation is then carried out for 3 days to facilitate transformation of the plant cells. Then, the leaf pieces are transferred to Petri dishes containing a cytokinin glucuronide as well as antibiotics (to avoid bacterial proliferation) including optionally kanamycin (see Examples 9 and 10, where the desired co-introduced nucleotide sequence is a NPT gene (kanamycin gene resistance) to confirm the introduction of that gene). Thus, the cytokinin glucuronide is used as a positive selective agent while the GUS gene is used as a marker gene for positive selection using its capacity to metabolize a compound (the cytokinin glucuronide) which is not usually metabolised. The leaf pieces are then sub-cultivated in the same medium, but without cytokinin glucuronides.

Regenerated shoots are transferred to other containers. After 2 weeks, the X-Gluc assay is performed on the green shoots. These shoots are the transformed ones. X-Gluc is only used to confirm the incorporation into the cell genome of the GUS gene. It plays no role at all in the selection. It is the cytokinin glucuronide, not X-Gluc, which acts as a positive selective agent in that it provides the transformed cells with a selective advantage compared to the non-transformed cells. The transformed cells remain green and healthy, because they are able to cleave active cytokinin from cytokinin glucuronide and use that cytokinin to their selective advantage which allows them to be then readily identified and isolated from the non-transformed cells (see the sentence bridging pages 6 and 7 in the patent specification). This exemplifies exactly the method which is claimed.

35. It is further argued by the opponents-appellants that, given the evident difficulties in performing what was claimed in respect of a GUS-based system in connection with the supposed pH-dependency of native GUS expression, undue difficulties could be expected for other systems falling under the broad terms of claim 1. It had to be presumed that other systems would have had their own problems and specific requirements which were not disclosed.
36. In the description (see paragraphs 0046 to 0051 on pages 7 and 8 in the patent specification) it has been acknowledged that the presence of native GUS activity in certain plant cells may be a problem and a choice of solutions to reduce accordingly any native β -glucuronidase activity therein has been proposed. The

- opponents have not provided any evidence that such solutions are inoperative.
37. The use of a preferred co-introduced (marker) nucleotide sequence (GUS-gene) has been illustrated in the experimental part of the description (see point 34, supra). Thereby, the requirements of Rule 27(1)(e) EPC are met. Merely putting forward unsupported allegations that the use of other co-introduced (marker) nucleotide sequences may be associated with problems that could not be solved without undue burden by the skilled person is not sufficient to cast doubts on the sufficiency of the general teaching of the patent specification.
38. Nevertheless, although the general feasibility of the concept of the invention as outlined in claim 1 over a broad range of applications is not put in doubt by the allegations put forward by the opponents-appellants, different considerations apply to individualised specific embodiments which are identified in the dependent claims.
- 38.1 Two specific preferred co-introduced marker nucleotide sequences are mentioned in claim 3, namely **mannose-6-phosphate isomerase** and **UDP-galactose-epimerase** which are only merely referred to in the description (see lines 52 to 54 on page 5 in the patent specification) without any details or support by any citation of the state of the art. This places an undue burden on the skilled person as putting specific embodiments into practice presupposes having easy access to the starting materials. In the absence of adequate information in terms either of a

description or of appropriate references, the skilled person is left to his or her own resources when wishing to put specific embodiments into practice. The same applies to the use of "an opine metabolism gene", also mentioned in claim 3, which is envisaged in the description (see Example 18 on pages 45 and 46 in the patent specification) only as a speculative option.

38.2 Moreover, in the board's judgement, it is unclear how **the expression product of co-introduced nucleotide sequence which encodes a permease or which is a transport gene**, both nucleotide sequences being also mentioned in claim 3, may metabolise a compound so as to provide the transformed cells with a physiological advantage. In fact, the actual function of a permease and of the polypeptide encoded by a transport gene is not to metabolise such a compound but to allow it to cross the cell membranes (see lines 28 to 30 on page 6 in the patent specification) and to transport it into the transformed cells, respectively. Thus, the obscurity of these embodiments of claim 3 is such that it amounts to an objection under Article 83 EPC as the skilled person has to rely on his or her own resources when trying to put these particular embodiments into practice. The same remarks and conclusion apply ipso facto to the subject-matter of dependent claim 5.

38.3 Finally, similar remarks and conclusions concern dependent claim 6 which relates to a selection method in which it is not the metabolized compound that is responsible for the provision of a physiological advantage to the transformed cells but rather the blockade of the metabolism synthesis of the said

compound in these cells (see lines 48 and 49 on page 6 in the patent specification).

39. Therefore, it is the Board's judgment that whereas the invention as generally outlined in claim 1 can be considered to enjoy sufficient technical support in the patent specification, the requirements of Article 83 EPC are not met insofar as the subject-matter of claims 3, 5 and 6 is concerned. For these reasons, the first auxiliary request as a whole cannot be allowed.

Second auxiliary request

40. The second auxiliary request differs from the first only in that (i) claim 3 has been limited to the exemplified embodiment, namely to a method according to either of claims 1 or 2, wherein the said co-introduced expressible β -glucuronidase when the compound is a cytokinin glucuronide, and (ii) claims 5 and 6 have been deleted.
41. For the reasons given at points 19 to 23 (see supra) with respect to the first auxiliary request the requirements of Articles 123(2) and 84 EPC are met. As the claims (see claims 5 and 6) or parts thereof (see claim 3) of the first auxiliary request, in respect of which the disclosure of the invention was considered to be insufficient (see points 38.1 to 38.3 supra), are no longer present in this request, the requirements of Article 83 EPC are also met. Furthermore, as the subject-matter of claim 1 involves an inventive step (see points 24 to 32, supra), claims 2 to 4 being dependent on claim 1, the second auxiliary request as a whole meets the requirement of Article 56 EPC.

42. For these reasons, the second auxiliary request represents a valid basis for the maintenance of the patent in an amended form.

Order

For these reasons it is decided that:

1. The decision under appeal is set aside.

2. The case is remitted to the first instance with the order to maintain the patent on the basis of the claims 1 to 4 of the second auxiliary request filed during the oral proceedings and a description to be adapted thereto.

The Registrar:

The Chairman:

A. Wolinski

L. Galligani