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(73) **Īpašnieks(i):**
AKTIESELSKABET DE DANSKE SPRITFABRIKKER (DANISCO A/S);
Langebrogade 1, DK-2300 Copenhagen S, DK

(72) **Izgudrotājs(i):**
Kirsten GAUSING (DK),
Jette D. KREIBERG (DK)

(74) **Pilnvarotais vai pārstāvis:**
Ņina DOLGICERE,
Patentu aģentūra "KDK",
a/k 185, Rīga LV-1084, LV

(54) **Virsraksts:** **Kartupeļu alfa-amilāzes gēns**

(57) **Kopsavilkums:** Ar vektora palīdzību ievada kartupeļa, piem. šķirnes Dianella, auga genomā DNS nukleotīdu sekvenci, subekvenci vai tās homologu, kas kodē α-amilāzi. Iegūst ģenētiski modificētus augus ar pazeminātu α-amilāzes aktivitāti (derīgi kartupeļu čipšu ražošanai) vai ar paaugstinātu α-amilāzes aktivitāti (derīgi spirta ražošanai).

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KARTUPEĻU α-AMILĀZES GĒNS

Patenta formula

1. DNS fragments, kas satur kādu no 1., 2., 3., 4. vai 5.zīmējumā parādītajām nukleotīdu sekvencēm, tās subsekvenci, kura satur vismaz 15 nukleotīdus un hibridizējas vismaz ar vienu no minētajām nukleotīdu sekvencēm augstas stringences hibridizācijas apstākļos: hibridizācija 67 °C 2xSSC un pēdējā mazgāšana 67 °C 1xSSC, vai vidēji augstas stringences hibridizācijas apstākļos: hibridizācija 67 °C 3xSSC un pēdējā mazgāšana 67 °C 1xSSC, vai satur minētās nukleotīdu sekvences homologu, kas kodē polipeptīdu, kurš ir identisks minētās nukleotīdu sekvences kodētajam polipeptīdam un hibridizējas vismaz ar vienu no minētajām nukleotīdu sekvencēm iepriekš definētās augstas vai vidēji augstas stringences hibridizācijas apstākļos.

2. DNS fragments saskaņā ar 1.punktu, kas satur nukleotīdu sekvenci, kura ir:
(a) nukleotīdu sekvence, kas 1.zīmējumā parādītajā nukleotīdu sekvencē sākas ar nukleotīdu 541, beidzas ar nukleotīdu 1761 un kodē kartupeļu α-amilāzes prekursoru, vai tās subsekvence vai homologs.

(b) nukleot'odu sekvence, kas 1.zīmējumā parādītajā nukleotīdu sekvencē sākas ar nukleotīdu 596, beidzas ar nukleotīdu 1761 un kodē kartupeļu α-amilāzi, vai tās subsekvence vai homologs.
(c) nukleotīdu sekvence, kas 2.zīmējumā parādītajā nukleotīdu sekvencē sākas ar nukleotīdu 2, beidzas ar nukleotīdu 1219 un kodē kartupeļu α-amilāzes prekursoru, vai tās subsekvence vai homologs.
(d) nukleotīdu sekvence, kas 2.zīmējumā parādītajā nukleotīdu sekvencē sākas ar nukleotīdu 53, beidzas ar nukleotīdu 1219 un kodē kartupeļu α-amilāzi, vai tās subsekvence vai homologs.
(e) nukleotīdu sekvence, kas 3.zīmējumā parādītajā nukleotīdu sekvencē sākas ar nukleotīdu 6, beidzas ar nukleotīdu 1052 un kodē daļu no kartupeļu α-amilāzes, vai tās subsekvence vai homologs.
(f) nukleotīdu sekvence, kas 4.zīmējumā parādītajā nukleotīdu sekvencē sākas ar nukleotīdu 3, beidzas ar nukleotīdu 647 un kodē daļu no kartupeļu α-amilāzes, vai tās subsekvence vai homologs.
(g) 1.zīmējumā parādītie nukleotīdi 1330-1624 un
(h) 4.zīmējumā parādītie nukleotīdi 387-591.

3. DNS fragments saskaņā ar 1.punktu, kas satur nukleotīdu sekvenci, kura ir:
(a) 1.zīmējumā parādītā nukleotīdu sekvence, ietverta cDNS klonā AmyZ3, kas iekļauts E.coli K-12, kura deponēta kā DSM 5275 (DSM-Vācijas mikroorganismu kolekcija), vai tās subsekvence vai homologs.
(b) 1.zīmējumā parādītā nukleotīdu sekvence, ietverta cDNS klonā AmyZ4, kas iekļauts E.coli K-12, kura deponēta kā DSM 5276, vai tās subsekvence vai homologs.
(c) 3.zīmējumā parādītā nukleotīdu sekvence, ietverta cDNS klonā AmyZ1, kas iekļauts E.coli K-12, kura deponēta kā DSM 5882, vai tās subsekvence vai homologs.
(d) 4.zīmējumā parādītā nukleotīdu sekvence, ietverta cDNS klonā AmyZ6, kas iekļauts E.coli K-12, kura deponēta kā DSM 5883; vai
(e) 2.zīmējumā parādītā nukleotīdu sekvence, ietverta cDNS klonā AmyZ7, kas iekļauts E.coli K-12, kura deponēta kā DSM 5884, vai tās subsekvence vai homologs.

4. Divdīgļlapju auga DNS fragments ar GC saturu 35-50%, vēlams 40-50% robežās-rēķinot kā kodējošā reģiona vidējo, kas hibridizējas ar DNS sekvenci saskaņā ar jebkuru punktu 1.-3. augstas stringences hibridizācijas

apstākļos: hibridizācija 67 °C 2xSSC un pēdējā mazgāšana 67 °C 1xSSC, vidēji augstas stringences hibridizācijas apstākļos: hibridizācija 67 °C 3xSSC un pēdējā mazgāšana 67 °C 1xSSC, vai zemas stringences hibridizācijas apstākļos kā, piemēram, 6xSSC 67 °C un mazgāšana 4xSSC 67 °C.

5. DNS fragments saskaņā ar jebkuru punktu 1.-4., kas ir pilnīgs α-amilāzes gēns ar regulātorreģioniem, promoterreģioniem, kodējošiem reģioniem, transkribētiem nekodējošiem reģioniem, ieskaitot intronus un transkripcijas terminācijās reģionus, vai tā daļa.

6. DNS fragments saskaņā ar 5.punktu, kaş ir α-amilāzes pseidogēns.

7. DNS fragments saskaņā ar jebkuru punktu 1.-6., kas iegūts no divdīgļlapju auga, sevišķi no Solanaceae dzimtas auga, vēlams Solanum ģints, vēlams Solanum tuberosum.

8. DNS fragments saskaņā ar jebkuru punktu 1.-5. vai 7.punktu, kas kodē polipeptīdu ar α-amilāzes aktivitāti.

9. DNS fragments saskaņā ar jebkuru punktu 1.-8., kas ir cDNS, genomiska DNS, sintētiska DNS vai to kombinācija.

10. DNS fragments, kas kodē saplūsmes proteīnu, kurš satur DNS fragmentu saskaņā ar jebkuru punktu 1.-9. pareizā nolasīšanas kārtībā, ar mazākais vienu citu DNS fragmentu, kurš kodē otru polipeptīdu vai tā daļu, pie kam otrs DNS fragments ir, piemēram, signālsekvence, kura kodē prokariotisko signālpeptīdu.

11. Ar DNS fragmentu kodētais polipeptīds saskaņā ar jebkuru punktu 1.-10., kas nesatur kopā ar polipeptīdiem dabā sastopamos fermentus.

12. Polipeptīds, kas satur jebkurā zīmējumā 1.-4. parādīto aminoskābju sekvenci, tās subsekvenci ar α-amilāzes aktivitāti un vismaz vienu aktīvu centru, vai polipeptīdu, kas iegūts no divdīgļlapju auga un uzrāda vismaz ap 70% homoloģiju ar minēto aminoskābju sekvenci.

13. Polipeptīds saskaņā ar 12.punktu, kas satur 1. vai 2.zīmējumā parādīto aminoskābju sekvenci, pie kam polipeptīdam ir tādas pašas fermentu īpašības kā 1. vai 2.zīmējumā parādītajam polipeptīdam.

14. DNS fragments, kas kodē mRNS molekulu, kura spēj hibridizēties ar mRNS, kura transkribēta no DNS fragmenta saskaņā ar jebkuru punktu 1.-10., tādējādi inhibējot tā translāciju.

15. DNS fragments, komplementārs attiecībā pret DNS fragmentu saskaņā ar jebkuru punktu 1.-10., kur DNS komplementāro fragmentu regulē ar promoteru, kas spēj iniciēt mRNS molekulas sintēzi, un šī mRNS molekula hibridizējas ar mRNS, kas transkribēta no DNS fragmenta saskaņā ar jebkuru punktu 1.-10.

16. DNS fragments saskaņā ar 14. vai 15.punktu, kas kodē anti-AmyZ4 vai anti-AmyZ6 mRNS.

17. Vienas virknes DNS vai RNS fragments, kas ir komplementārs ikvienai DNS fragmenta saskaņā ar jebkuru punktu 1.-10. virknei.

18. Vektors, kas spēj replicēties saimniekorganismā un kurš nes DNS fragmentu saskaņā ar jebkuru punktu 1.-10. vai 14.-17.

19. Saimniekorganisms, kas satur vektoru saskaņā ar 18.punktu.

20. Saimniekorganisms saskaņā ar 19.punktu, kas spēj veikt iekļautā DNS fragmenta saskaņā ar jebkuru punktu 1.-10. vai 14.-17. replikāciju vai ekspresiju.

21. Saimniekorganisms, kas nes DNS fragmentu saskaņā ar jebkuru punktu 1.-10. vai 14.-17., pie kam DNS fragments ir organisma genoma daļa.

22. Saimniekorganisms saskaņā ar 21.punktu, kas spēj veikt iekļautā DNS fragmenta replikāciju vai ekspresiju.

23. Saimniekorganisms saskaņā ar jebkuru punktu 19.-22., kas ir šūnu līnija, vēlams auga šūnu līnija, augs vai mikroorganisms, piemēram, Escherichia ģints baktērija, vēlams E.coli vai no Bacillus ģints, vēlams

B.subtilis, raugs, vēlams no Saccharomyces ģints, vēlams S.cereviciae, vai sēne, vēlams no Aspergillus ģints.

24. E.coli baktērija, kas izvēlēta no:
(a) E.coli K-12 ar iekļautu plazmīdu pAmyZ3, deponēta kā DSM 5275,
(b) E.coli K-12 ar iekļautu plazmīdu pAmyZ4, deponēta kā DSM 5276,
(c) E.coli K-12 ar iekļautu plazmīdu pAmyZ1, deponēta kā DSM 5882,
(d) E.coli K-12 ar iekļautu plazmīdu pAmyZ6, deponēta kā DSM 5883, vai
(e) E.coli K-12 ar iekļautu plazmīdu pAmyZ7, deponēta kā DSM 5884.

25. Ģenētiska konstrukcija, ar DNS fragmentu saskaņā ar jebkuru punktu 1.-10. kodētas mRNS molekulas translācijas aizkavēšanai; minētā konstrukcija satur:
(1) regulējošu sekvenci, kas ir funkcionāli pievienota
(2) DNS fragmentam saskaņā ar jebkuru p.14.-17., kas kodē RNS molekulu, kura spēj inhibēt DNS fragmenta saskaņā ar jebkuru punktu 1.-10. translāciju;
(3) transkripcijas terminācijas DNS sekvenci, kas funkcionāli pievienota DNS fragmentam (2).

26. Ģenētiska konstrukcija polipeptīda saskaņā ar jebkuru punktu 11.-13. iegūšanai, pie kam minētā konstrukcija satur:
(a)
(1) regulējošu sekvenci, kas funkcionāli pievienota
(2) DNS fragmentam saskaņā ar jebkuru punktu 1.-10., kas kodē polipeptīdu un
(3) transkripcijas terminācijas DNS sekvenci, kas funkcionāli pievienota DNS fragmentam (2);

(b)
(1) regulējošu sekvenci, kas funkcionāli pievienota
(2) DNS fragmentam saskaņā ar 5.punktu un satur α-amilāzes gēnu bez promotera; vai

(c)
(1) regulējošu sekvenci, kas funkcionāli pievienota
(2) DNS fragmentam saskaņā ar 5.punktu, kas satur α-amilāzes gēnu bez promotera, vai transkripcijas terminācijas sekvenci un

(3) transkripcijas terminācijas sekvenci, kas funkcionāli pievienota DNS fragmentam (2).

27. Ģenētiskā konstrukcija saskaņā ar 25. vai 26. punktu, kurā regulējošā sekvence ir auga promoters, vēlams-uzbūves vai regulējams auga promoters, pie kam uzbūves promoters ir, piemēram, NOS promoters, ubikvitīna promoters vai auga vīrusa promoters, vēlams CaMV promoters, un regulējamais auga promoters ir regulējams, piemēram, vismaz ar vienu faktoru, kas izvēlēts no attīstības, ķīmisko, fizikālo vai fizioloģisko faktoru grupas.

28. Ģenētiskā konstrukcija saskaņā ar 27.punktu, kurā auga promoters ir auga α-amilāzes promoters, vēlams kartupeļu α-amilāzes promoters, vai patatinpromoters.

29. Ģenētiskā konstrukcija saskaņā ar jebkuru punktu 25.-28., kurā transkripcijas terminācijas sekvence ir auga transkripcijas terminācijas sekvence.

30. Ģenētiski modificēts augs, kas savā genomā satur ģenētisku konstrukciju saskaņā ar jebkuru punktu 25.-29.

31. Ģenētiski modificēts augs saskaņā ar 30.punktu, kas savā genomā satur vismaz vienu ģenētisku konstrukciju saskaņā ar 25.punktu, pie kam minētajam augam ir pazemināta α-amilāzes aktivitāte, salīdzinot ar atbilstošu nemodificētu augu.

32. Ģenētiski modificētais augs saskaņā ar 30.punktu, kas savā genomā satur vismaz vienu ģenētisku konstrukciju saskaņā ar 26.punktu, pie kam minētajam augam ir paaugstināta α-amilāzes aktivitāte, salīdzinot ar atbilstošu nemodificētu augu.

33. Ģenētiski modificētais augs saskaņā ar 30.punktu, kurā α-amilāzes aktivitāte ir regulējama zemās temperatūrās, savā genomā satur vismaz vienu ģenētisku konstrukciju saskaņā ar 25.punktu, kā arī promoteru, kas temperatūras apmēram zem 8 $^{\circ}C$ aktivē konstrukcijā esošā DNS fragmenta transkripciju.

34. Ģenētiski modificētais augs saskaņā ar 31. vai 33.punktu, kurā ģenētiskā konstrukcija ir aktīva tajos pašos audos vai šūnās, kuros ir veikta vismaz viena α-amilāzes gēna ekspresija.

35. Ģenētiski modificētais augs saskaņā ar jebkuru punktu 30.-34., kas ir divdīgļlapju augs, piemēram, Solanaceae dzimtas augs, vēlams Solanum ģints, labāk - Solanum tuberosum.

36. Vektoru sistēma, kas satur vismaz vienu ģenētisku konstrukciju nesošu vektoru saskaņā ar jebkuru punktu 25.-29. un kura spēj introducēt ģenētisko konstrukciju auga genomā, vēlams Solanaceae dzimtas augā, vēlams Solanum ģints - labāk Solanum tuberosum, pie kam minētā vektoru sistēma ir, piemēram, bināra vektoru sistēma.

37. Vektoru sistēma saskaņā ar 36.punktu, kas satur virulences funkciju, kura spēj inficēt augu, un vismaz vienu T-DNS sekvences robežda|u, kura novietota uz tā paša vektora kā ģenētiskā konstrukcija, pie kam minētā vektoru sistēma satur, piemēram, Agrobacterium tumefaciens Ti vai Ri plazmīdu vai Agrobacterium rhizogenes Ri plazmīdu.

38. Mikroorganisms, kas spēj inficēt augu, vēlams Agrobacterium spp., piemēram, Agrobacterium tumefaciens celms, un satur vektoru saskaņā ar 36. vai 37. punktu.

39. Ģenētiski modificēta auga saskaņā ar jebkuru punktu 30.-35. veidošanas paņēmiens, kurā ģenētisko konstrukciju uz auga genomu pārvieto tā, lai iegūtu konstrukciju saturošu ģenētisko materiālu un beidzot to reģenerētu ģenētiski modificētā augā.

40. Paņēmiens sakaņā ar 39.punktu, ar kuru ģenētisko konstrukciju pārvieto uz augu vai tā daļu ar mikroorganisma palīdzību saskaņā ar 38.punktu, tiešu injekciju vai elektroporāciju.

41. DNS fragments saskaņā ar jebkuru punktu 1.-10., kas ir iezīmēts, piemēram, ar fluoforiem, radioaktīviem izotopiem un kompleksveidojošiem aģentiem, kā piemēram, biotīnu.

42. Paņēmiens α-amilāzes mRNS daudzuma noteikšanai organismā, vēlams augā, labāk - divdīgļlapju augā, pie kam no organisma iegūto nūkleīnskābi saturošo paraugu hibridizē ar DNS fragmentu saskaņā ar jebkuru punktu 1.-10. vai 41. punktu denaturētā veidā, vai ar tā RNS kopiju labvēlīgos apstākļos hibridizācijai starp denaturēto DNS fragmentu vai RNS kopiju un parauga nukleīnskābi un nosaka hibridizētās nukleīnskābes daudzumu.

43. Paņēmiens α-amilāzes gēna vai α-amilāzes cDNS izolēšanai no organisma genomiskās vai cDNS gēnu bankas, pie kam organisms, vēlams, ir augs, labāk - divdīgļlapju augs; no gēnu bankas saņemto nukleīnskābi saturošo paraugu hibridizē ar DNS fragmentu saskaņā ar jebkuru punktu 1.-10. vai 41.punktu denaturētā veidā, vai ar tā RNS kopiju labvēlīgos apstākļos hibridizācijai starp DNS fragmentu vai RNS kopiju un parauga nukleīnskābi un izolē hibridizēto klonu tā, lai iegūtu minētā organisma α-amilāzes gēnu vai cDNS.

44. Paņēmiens α-amilāzes gēna vai tā daļas klātbūtnes detektēšanai organismā, vēlams augā - labāk divdīgļlapju augā, ietver parauga, kas satur DNS fragmentu ar α-amilāzes gēnu vai tā daļu fragmenta sastāvā, amplikāciju, izmantojot DNS fragmentu saskaņā ar jebkuru punktu 1.-10., atbilstoši polimerāzes ķēdes pieaudzēšanas tehnikai.

45. Augu barības vielu tendences veidot reducējošos cukurus novērtēšanas paņēmiens, kas satur α-amilāzes mRNS vai gēna daudzuma noteikšanu auga paraugā saskaņā ar 42.punktā definēto paņēmienu un daudzuma korelēšanu ar iepriekš pieņemto standartu.

46. Paņēmiens saskaņā ar jebkuru punktu 42.-45., kurā organisms ir divdīgļlapju augs, vēlams Solanum - labāk Solanum tuberosum, vai S.melongena (aubergine), Lycopersicon, vēlams Lycopersicon esculentum (tomāts), Capsicum, vēlams Capsicum annuum (pipars), Nicotiana, vēlams Nicotia tabacum (tabaka), Jpomoea, vēlams Jpomoea batatus (saldais kartupelis), Brassica, Medicago, Trifolium spp. (āboliņš), Glycine max (sojaspupa), Arachis, vai sakņaugu kultūra, kā Beta, vēlams Beta vulgaris (cukurbiete).

47. Paņēmiens DNS fragmentu saskaņā ar jebkuru punktu 1.-10. vai tā homologu saturošu alēļu analīzei organismā, kurā veic minētā organisma nukleīnskābes hibridizāciju ar DNS fragmentu saskaņā ar jebkuru punktu 1.-10. un hibridizācijas paraugu novērtējumu saskaņā ar restrikcijas fragmentu garuma polimorfisma (RFLP) analīzes principiem.

POTATO ALPHA-AMYLASE GENE

In one aspect, the present invention relates to a DNA fragment comprising a nucleotide sequence substantially as shown in Fig. 1, 2, 3, 4 or 5 or a subsequence or analogue thereof. The nucleotide sequence shown in Fig. 1 was constructed on the basis of two potato α-amylase cDNA clones by hybridization with a barley α-amylase gene as described in Example 5 and subsequent sequencing (Example 6). The cDNA clones were obtained from a cDNA library constructed from polyA-rich potato RNA as described in Example 4. The potato from which the RNA had been isolated was a potato of the variety Dianella. The nucleotide sequences shown in Figures 1 and 2 encode one type of potato α-amylase and the nucleotide sequences shown in Figures 3-4 partially encode a second type of α-amylase. The corresponding amino acid sequences are also shown in Figures 1-4, respectively. All four α-amylase amino acid sequences are novel and unique. Fig. 5 shows the nucleotide sequence of a partial potato α-amylase cDNA similar to the sequences shown in Figures 1-2, the sequence being the product of a pseudo gene which does not encode an α-amylase protein.

In another aspect, the present invention relates to an α-amylase gene comprising a DNA fragment substantially as shown in Fig. 1-5 or a subsequence or analogue thereof. The term "gene" is used in the present context to indicate a DNA sequence which is involved in producing a polypeptide chain and which includes regions preceding and following the coding region (leader and trailer sequences) as well as intervening sequences, the so-called introns, which are placed between individual coding segments (so-called exons) or in the leader or trailer region. The leader region comprises a regulatory sequence which controls the expression of the gene at the level of transcription and translation. The regulatory sequence includes a promoter. The trailer region comprises sequences which are involved in termination of transcription of the gene and optionally sequences responsible for polyadenylation of the transcript and the 3' untranslated region.

The term "subsequence" is used in the present context to indicate a nucleotide sequence which is derived from a DNA fragment or gene of the invention and which has retained a characteristic nucleotide sequence thereof. Typically, the subsequence is a part of a nucleotide sequence shown in Fig. 1-5, i.e. being a number of nucleotides shorter than one of these nucleotide sequences, the subsequence being either a consecutive stretch of nucleotides taken from a nucleotide sequence shown in Fig. 1-5 or being composed of one or more separate nucleotides or nucleotide sequences of a nucleotide sequence shown in Fig. 1-5.

In the present specification and claims, the term "subsequence" designates a sequence which preferably has a size of at least 15 nucleotides, more preferably at least 16 nucleotides, still more preferably at least 18 nucleotides, even more preferably at least 20 nucleotides and most preferably at least 50 nucleotides. It is well known that small fragments are useful in PCR techniques. In particularly preferred embodiments, the subsequence is one hybridizing to any of the DNA sequences referred to above and/or encoding a polypeptide displaying α-amylase activity. Particularly preferred DNA fragments and subsequences according the invention are derived from dicotyledonous plants. The DNA fragments according to the invention preferably have a GC content, calculated as an average for the coding region, in the range of 35-50%, preferably 40-50%.

The term "analogue" with regard to the DNA fragments of the invention is intended to indicate a nucleotide sequence which encodes a polypeptide identical or substantially identical to the polypeptide encoded by a DNA fragment of the invention or a subsequence thereof. It is well known that the same amino acid may be encoded by various codons, the codon usage being related, *inter alia*, to the preference of the organism in question expressing the nucleotide sequence. Thus, one or more nucleotides or codons of the DNA fragment of the invention may be exchanged by others which, when expressed, result in a polypeptide identical or substantially identical to the polypeptide encoded by the DNA fragment in question. The sequences in Figures 1, 2 and 5 represent an example of analogous sequences; the sequences in Figures 3 and 4 represent another example. Furthermore, the term "analogue" is intended to allow for variations in the sequence such as substitution, insertion (including introns), addition or rearrangement of one or more nucleotides, which variations do not have any substantial effect on the polypeptide encoded by the DNA fragment or a subsequence thereof. The term "substitution" is intended to mean the replacement of one or more nucleotides in the full nucleotide sequence with one or more different nucleotides, "addition" is understood to mean the addition of one or more nucleotides at either end of the full nucleotide sequence, "insertion" is intended to mean the introduction of one or more nucleotides within the full nucleotide sequence, "deletion" is intended to indicate that one or more nucleotides have been deleted from the full nucleotides sequence whether at either end of the sequence or at any suitable point within it, and "rearrangement" is intended to mean that two or more nucleotides residues have been exchanged with each other.

The terms "fragment", "sequence", "subsequence" and "analogue", as used in the present specification and claims with respect to fragments, sequences, subsequences and analogues according to the invention should of course be understood as not comprising these phenomena in their natural environment, but rather, e.g., in isolated, purified, *in vitro* or recombinant form.

In the present context, the conventional abbreviations for nucleotides are used, i.e. A represents adenine, T represents thymidine, G represents guanine, and C represents cytosine.

The DNA fragment of the present invention comprising a nucleotide sequence substantially as shown in Fig. 1-5 may be used for diagnosis, e.g. for RFLP analysis used for detecting the organization of genes encoding α-amylase present in various organisms, preferably plants (see Example 28), and/or for identifying tissues of the organism containing a high amount of α-amylase genes and/or messenger RNA and tissues which do not contain any such genes and/or mRNA. The DNA fragments of the invention may also be used for screening of breeding material for its content of α-amylase messengers, thereby allowing the determination at an early stage of the tendency of the plant breeding material to form reducing sugars, the determination being based on a correlation between α-amylase activity and the amount of reducing sugars produced by a given plant. The present inventors were the first to recognize that such a correlation exists. The early screening of breeding material has a number of advantages and constitutes a novel and very useful approach to characterization of potato breeding material with respect to starch metabolism.

Furthermore, the DNA fragments shown in Figures 1 and 2 may be used for preparing a polypeptide, e.g. a potato α-amylase, by use of recombinant DNA techniques. The polypeptide produced constitutes an aspect of the present invention and may be used in the same manner as conventional α-amylase, e.g. in brewing or as a constituent in detergent compositions. As explained below, the DNA fragments shown in Fig. 3 and 4 represent only partial nucleotide sequences of potato α-amylase messenger RNA and partial amino acid sequences of potato α-amylase precursor. However, although the full length sequences have not yet been deduced, the gene portions which are lacking may be obtained from cDNA or genomic clone libraries isolated by hybridization of α-amylase cDNA clones to the libraries.

DETAILED DISCLOSURE OF THE INVENTION

As explained above, the nucleotide sequences shown in Fig. 1-5 were obtained from a potato cDNA library. More specifically, the nucleotide sequence was obtained from a cDNA library prepared from polyA-rich RNA isolated from sprouts of a potato (*Solanum tuberosum*) of the variety Dianella, as explained below under "Materials and Methods". In the present context, "potato" and "*Solanum tuberosum*" are used interchangeably. The polypeptides encoded by the nucleotide sequences shown in Fig. 1-4 have substantially the amino acid sequences of two different types of α-amylases from *Solanum tuberosum* of the variety Dianella. In particular, the nucleotide sequences of Fig. 1 and 2 encode one type of α-amylase, while the nucleotide sequences of Fig. 3 and 4 encode another type of α-amylase.

The nucleotide sequence shown in Fig. 1 contains one long open reading frame of 407 codons, starting at nucleotide 542 and terminating at nucleotide 1761, the reading frame encoding an α-amylase precursor. The α-amylase precursor comprises a signal peptide, presumably encoded by nucleotides 541-595 and a mature α-amylase enzyme, probably encoded by nucleotide 596 to 1761.

Furthermore, the nucleotide sequence shown in Fig. 1 comprises non-coding regions, i.e. a 5' untranslated region, a so-called 5' leader sequence, which comprises an intron sequence of 128 nucleotides (nucleotides 297-424) and a 3' untranslated region. The nucleotide sequence shown in Fig. 1 is further discussed in Example 8.

The nucleotide sequence shown in Fig. 2 contains one long open reading frame starting at nucleotide 2 and terminating at nucleotide 1219, which reading frame encodes an α-amylase precursor. The mature α-amylase enzyme is presumably encoded by nucleotides 53 to 1219.

As shown in the Examples, different α-amylase cDNA clones were obtained from the potato cDNA library, one of which did not contain an intron (Amy4) and one of which (Amy3) contained an intron of a length of 128 nucleotides. The distribution of introns in α-amylase genes, especially potato α-amylase genes, is not known. It has been demonstrated that the α-amylase genes are clustered in a gene family, possibly with only one gene for each of the two types of α-amylase genes characterized represented by different alleles (Example 16). Within a gene family, a high degree of homology between coding regions of the genes is expected, whereas less homology is expected between non-coding regions. For different alleles of the same gene, the homology is expected to be extensive throughout the alleles.

The sequence of events leading to the elucidation of the nucleotide sequences shown in Fig. 1-5 is described in Examples 2-6. From the explanation given therein, it is evident that isolation of cDNA clones containing nucleotide sequences substantially as shown in Fig. 1-5 required a great effort regarding the design of the experiments. Thus, it is evident from Example 5 that the frequency of positive clones isolated from the potato α-amylase cDNA library was very low, i.e. approximately 0.008%, which shows that for the construction of a potato cDNA library it was important to choose a tissue in which a sufficiently high amount of α-amylase mRNA is present. Furthermore, the preparation of useful barley α-amylase probes as described in Example 2 was essential, especially with regard to constructing a probe showing optimal hybridization properties to the cDNA clones obtained from potato α-amylase messenger RNA. Such a probe was constructed, which is shown by the fact that all clones hybridizing to the probe were clones having the desired α-amylase nucleotide sequence. Furthermore, it was important to choose suitable hybridization conditions which do not result in too many positive sequences (resulting in "black" filters from which no clear results can be obtained) or in filters which do not result in any hybridization at all. The hybridization conditions allowed isolation of two types of potato α-amylase cDNA clones that are so different that they do not cross-hybridize under normal conditions. Furthermore, in the hybridization DNA from *E. coli* was used as a carrier instead of salmon sperm DNA, which is the conventional carrier used. The use of *E. coli* DNA is very advantageous, since background signals are minimized or substantially avoided.

It was found that the fragments showing the highest degree of hybridization to barley α-amylase genes were DNA fragments comprising nucleotides 1330-1624 as shown in Fig. 1 or nucleotides 387-591 as shown in Fig. 4. It is therefore expected that these or similar fragments may be of particular value in the isolation of α-amylase genes from various dicotyledonous plants other than potato.

The polypeptide expressed from a DNA fragment comprising nucleotides 541-1761 of the nucleotide sequence shown in Fig. 1 or a subsequence or analogue thereof is an α-amylase precursor, i.e. comprising the mature α-amylase enzyme as well as a signal peptide separated by a cleavage or processing site. The signal peptide serves to mediate the transport of the precursor across membranes, e.g. out of the cell or tissue in which it is produced. As described in Example 13, the structure of the signal peptide is atypical, possibly because of an unusual transport mechanism. The α-amylase precursor also constitutes an aspect of the present invention.

Nucleotides 596-1761 of the nucleotide sequence shown in Fig. 1 probably encode a mature α-amylase enzyme, i.e. an enzyme without a signal peptide. While it is contemplated that the α-amylase enzyme in most cases will be prepared in a precursor form, i.e. containing a signal peptide which may subsequently be cleaved off by processing of the precursor by the organism producing it, thereby resulting in the mature α-amylase enzyme, the mature α-amylase may also be produced directly. Thus, in a further aspect, the present invention relates to a DNA fragment comprising substantially the nucleotide sequence starting at nucleotide 596 and terminating at nucleotide 1761 of the nucleotide sequence shown in Fig. 1 or a subsequence or analogue thereof.

Similarly, the present invention relates to a DNA fragment comprising substantially a nucleotide sequence starting at nucleotide 53 and terminating at nucleotide 1219 of the nucleotide sequence shown in Fig. 2 encoding a potato α-amylase, or a subsequence or analogue thereof, a DNA fragment comprising substantially a nucleotide sequence starting at nucleotide 6 and terminating at nucleotide 1052 of the nucleotide sequence shown in Fig. 3 encoding a partial potato α-amylase, or a subsequence or analogue thereof, and a DNA fragment comprising substantially a nucleotide sequence starting at nucleotide 3 and terminating at nucleotide 647 of the nucleotide sequence shown in Fig. 4 encoding a partial potato α-amylase, or a subsequence or analogue thereof.

The invention thus relates to a DNA fragment which is homologous to a nucleotide sequence shown in Fig. 1-5 or an analogue or subsequence thereof, i.e. one which hybridizes to the DNA sequence under conventional hybridization conditions (12), or under low stringency conditions such as 6 x SSC and 67 °C and subsequent wash at 4 x SSC and 67 °C, and which preferably has a GC content in the range of about 35-50%, more preferably about 40-50%. The term "homology" is used here to denote the presence of any degree of complementarity between a given probe and the nucleic acid species being analyzed. The minimum degree of homology which is detectable is a function of the experimental conditions employed during hybridization and of characteristics of the probe and the nucleic acid species being analyzed. Thus, the size of the probe as well as the complexity of the nucleotide sequence being isolated, e.g. with respect to purity, content of other nucleic acid sequences than the ones related to α-amylase genes, etc. are of importance. As explained in the Examples, sequences homologous to the nucleotide sequences shown in Fig. 1-4 encoding a potato α-amylase are found in other organisms than the one analyzed, e.g. in other potato varieties or in other dicotyledonous plants of the types disclosed below and should also be considered to be within the scope of the present invention. Thus, corresponding nucleotide sequences encoding α-amylase from other dicotyledonous plants such as other plants of the family *Solanaceae*, e.g. other potato varieties, may easily be isolated by hybridization under the conditions stated above to a DNA fragment of the invention comprising substantially a nucleotide sequence as shown in Fig. 1 or an analogue or subsequence thereof.

Further, the invention relates to a DNA fragment which is an α-amylase pseudo-gene. The term "α-amylase pseudo-gene" refers to an originally active α-amylase gene which has been mutated in such a way, e.g. by substitutions or deletions, that it is no longer active, but still can be identified as an α-amylase gene by hybridization to a DNA fragment comprising a nucleotide sequence substantially as shown in Fig. 1-4, or a subsequence or analogue thereof. The cDNA sequence shown in Fig. 5 is an example of the product of an active pseudo-gene.

The α-amylase pseudo-genes will have utilities as explained below for the genes proper.

As described below in the examples, the DNA fragments of the invention may be isolated using a method comprising the steps of:

preparing a cDNA library from poly A-rich RNA isolated from a tissue of a dicotyledonous plant, preferably from poly A-rich RNA from a tissue of a potato plant, in a vector, preferably a λ vector, preferably the λ-ZAP vector;

screening said cDNA library by hybridization with a probe comprising a barley α-amylase gene sequence, under hybridization conditions allowing detection of hybridization without creating an excessive background hybridization, but at the same time allowing the detection of weak hybridization; and

isolating recombinant clones which hybridize with the probe, obtaining the DNA fragment from the isolated clones, and optionally producing a subsequence of the DNA fragment by digestion with a restriction enzyme.

The DNA fragments of the invention may be used for isolating similar DNA fragments from various dicotyledonous plants using a method comprising the steps of:

preparing a cDNA library from poly A-rich RNA isolated from a tissue of a dicotyledonous plant, preferably from poly A-rich RNA from a tissue from a potato plant, in a vector, preferably a λ vector, preferably the λ-ZAP vector;

screening said cDNA library by hybridization with a probe comprising a DNA fragment obtainable as

described above; and
isolating recombinant clones which hybridize with the probe, obtaining the DNA fragment from the isolated clones, and optionally producing a subsequence of the DNA fragment by digestion with a restriction enzyme.

The DNA fragments described above may be produced by various methods, e.g. by building the oligonucletoide sequence of the fragment by oligonucleotide synthesis. In the case of DNA fragment of the invention which is part of a fragment encoding a fusion protein, the fragment may be produced by ligating a DNA fragment of the invention obtainable as described above to a second DNA fragment encoding a second polypeptide or part thereof, preferably using a DNA ligase.

As it will be apparent from the above disclosure, the nucleotide sequences shown in Fig. 1-5 were derived from a dicotyledonous plant, namely a potato of the variety Dianella. Although nucleotide sequences which may be isolated from other plants, especially other potato varieties than the variety Dianella, may not have the same sequence, they are expected to be homologous to a DNA fragment of the invention to an extent which allows them to hybridize to a DNA fragment comprising a nucleotide sequence substantially as shown in Fig. 1-5 under the hybridization conditions specified above. As mentioned above, such nucleotide sequences constitute an aspect of the present invention. Typically, nucleotide sequences hybridizing to the DNA fragments of the invention are found in plants of the family *Solanaceae*, in particular of the genus *Solanum*, especially *Solanum tuberosum*, or *S. melongena* (aubergine). Other examples are plants of the genus *Lycopersicon*, e.g. *Lycopersicon esculentum* (tomato), the genus *Capsicum*, e.g. *Capsicum annuum* (pepper), the genus *Nicotiana*, e.g. *Nicotiana tabacum* (tobacco), the genus *Ipomoea*, e.g. *Ipomoea batatus* (sweet potato), the genus *Brassica*, e.g. *Brassica napus* (rapeseed), the genus *Medicago*, especially *Medicago sativa* (lucerne), the genus *Trifolium* spp. (clover), *Glycine max* (soya bean), the genus *Arachis*, especially *Arachis hypogaea* (peanut), *Phaseolus* spp., *Vicia* spp., *Vigna* spp. (beans), the genus *Pisum*, especially *Pisum sativum*, root crops such as of the genus *Beta*, especially *Beta vulgaris* (sugar beet) and the genus *Daucus*, especially *Daucus carota* (carrots).

A DNA fragment of the invention may comprise one or more second nucleotide sequences encoding a second polypeptide or part thereof so as to encode a fusion protein, at least part of the DNA fragment of the invention being expressed in conjunction with another DNA fragment or gene. For certain purposes, it may be desirable that the polypeptide encoded by a DNA fragment of the invention is in the form of a fusion protein, i.e. a protein in which a polypetide having an amino acid sequence substantially as shown in Fig. 1-4 or a fragment or an analogue thereof is fused to a second polypeptide. It may be advantageous that the second polypeptide is one which contributes to the properties of the fusion protein, e.g. by modifying, e.g. decreasing or increasing, the activity, e.g. the enzymatic activity of a polypeptide of the invention. Also, a fusion protein may have two or more enzymatic activities, one being an α-amylase activity and the other(s) being an α-amylase activity or an enzymatic activity which, e.g., is normally used together with an α-amylase. Thus, as explained above for the production of alcohol, several different enzymes are used, i.e. one or more cellulases and β-glucosidases. Thus, a fusion protein having α-amylase activity together with cellulase and/or β-glucosidase activity may be advantageous for the production of alcohol. Furthermore, the DNA fragments of the invention or part thereof may be fused to a polypeptide involving transport out of cells or tissues, e.g. a so-called exoenzyme. Signal peptides involving transport will be discussed in further detail below.

The fusion protein may function when expressed in a higher organism such as a plant, but may also be useful in expression from lower organisms such as bacteria or yeasts. Thus, when the polypeptide is to be produced by use of recombinant DNA techniques, e.g. as described below, the recovery or isolation of the polypeptide may be easier and more economical when the polypetide to be recovered is a fusion protein. The second polypeptide to which the polypeptide defined above is fused may be one containing antigenic determinants, against which antibodies may be raised, which antibodies may be used for the recovery of the fusion product, e.g. by using the principles of chromatography such as affinity chromatography, in which the polypeptide may be recovered by means of immobilized antibodies. Procedures for isolating the polypeptide will be dealt with in further detail in the following.

As explained above and in the following examples, the nucleotide sequences shown in Fig. 1-5 were obtained from a cDNA library constructed from mRNA isolated from the potato variety Dianella. Other sequences, either subsequences, analogues or homologues to the DNA fragment comprising a nucleotide sequence as shown in Fig. 1-5, in accordance with the definitions given above, obtained from another organism or the same organism and encoding another α-amylase than the ones outlined in Fig. 1-5, may be obtained in the same manner. Homologous nucleotide sequences may be obtained from complementary cDNA obtained by preparing a cDNA library on the basis of mRNA from an organism producing α-amylase, e.g. by employing a method similar to the one used to obtain the nucleotide sequences shown in Fig. 1-5.

Alternatively, the nucleotide sequence may be derived from the genome of an organism producing α-amylase by screening for genomic sequences hybridizing to a DNA fragment of the invention, e.g. as described below. This may be accomplished by establishing a genomic library of the plant, e.g. potato, and screening for α-amylase clones by hybridization with a DNA fragment of the invention. DNA fragments of the invention comprising a nucleotide sequence substantially as shown in Fig. 1-5 or a subsequence or analogue thereof may, however, also be synthetic DNA prepared by DNA synthesis in accordance with well known methods.

Most eucaryotic genes contain introns which, as explained above, vary in both size, composition and position between genes belonging to the same gene family. Thus, α-amylase genes or DNA fragments constituting parts thereof may contain introns of varying size, composition and position. These introns may be different from the introns shown in Fig. 1 and thus, the invention also relates to a DNA fragment comprising a nucleotide sequence as shown in Fig. 1 or an analogue or subsequence thereof, especially the part of the sequence encoding a mature α-amylase, i.e. corresponding to nucleotides 596-1761 of the sequence shown in Fig. 1, which DNA fragment further includes one or more non-coding regions such as one or more regulatory sequences and/or introns. In the present context, the term "intron" is used in its conventional meaning, i.e. as a DNA segment which is transcribed, but not translated, since it is removed from within the transcript by splicing together RNA sequences transcribed from the DNA sequences on either side of the intron. An intron may be of a wide variety of sizes and is typically of a size of about 50-500 nucleotides in plants. A DNA fragment of the invention may also contain one or more 5' or 3' untranslated regions which always have to be present in order to obtain expression of the gene or DNA sequence position between the regions. The 5' and 3' untranslated regions are specific for the organism which expresses the DNA fragment or gene and accordingly, the α-amylase encoding sequence should be preceded and followed by 5' and 3' untranslated regions, respectively, which allow for expression in the organism. The untranslated region may be one which is naturally present in the sequence or one which synthetically has been introduced instead of or in addition to the 5' and/or 3' untranslated regions shown in Fig. 1-4, which regions may vary in composition and/or length, preferably so as to comply with specific requirements of the organism expressing a DNA fragment of the invention.

As explained above, the nucleotide sequence shown in Fig. 1 contains a sequence encoding a signal peptide which is atypical in composition and length. It may furthermore be noted that AmyZ3/4 and AmyZ7 encode identical signal peptides, with the exception that AmyZ7 lacks a Met initiation codon. The nature of a signal peptide used by a given organism depends, *inter alia*, on the organism and the part thereof, e.g. the specific cell or tissue, in which the polypeptide (precursor) is produced and to which part of the same cell or another location in the organism the polypeptide optionally is to be transported. Thus, a DNA fragment comprising the nucleotide sequence shown in Fig. 1 or a subsequence or analogue thereof, especially nucleotides 596-1761 of the sequence, may have another signal peptide than that of the nucleotide sequence shown in Fig. 1, resulting in a precursor protein different from the one encoded by the nucleotide sequence shown in Fig. 1. The signal peptide may be chosen so as to comply with specific requirements of the organism which is to produce the precursor, including an optional subsequent transport of the precursor within the organism. Typical signal peptides have a core of hydrophobic amino acids and thus, a DNA fragment of the present invention preferably contains a stretch of codons encoding hydrophobic amino acids.

The expression of genes in all organisms is subjected to regulation, which in plants generally has been found to be very complicated, requiring that a network of different regulatory systems and factors function together. Although very little is known about the regulation of plant genes at present, various regulatory mechanisms have been elucidated, two important types of regulation being tissue specific regulation and developmental regulation. To alter the expression of a DNA fragment of the invention or a gene comprising said DNA fragment, a regulatory sequence may be functionally connected to the DNA fragment or gene so as to obtain expression of said fragment or gene under the control of the inserted regulatory sequence. Accordingly, a DNA fragment comprising a nucleotide sequence substantially as shown in Fig. 1-4 or a subsequence or analogue thereof which further comprises one or more regulatory sequences is within the scope of the present invention. Typically, the regulatory sequence is a promoter which may be constitutive or regulatable. The term "promoter" is intended to mean a short DNA sequence to which RNA polymerase binds prior to transcription of the DNA to which the promoter is functionally connected, allowing transcription to take place. However, in its broader scope, the term "promoter" includes the RNA polymerase binding site as well as regulatory sequence elements located within several hundreds of base pairs, occasionally even further away, from the transcription start site. A "constitutive promoter" is a promoter which is subjected to substantially no regulation such as induction or repression, but which allows for a steady and substantially unchanged transcription of the DNA sequence to which it is functionally bound in all active cells of the organism provided that other requirements for the transcription to take place is fulfilled.

A "regulatable promoter" is a promoter whose function is regulated by one or more factors. These factors may either be ones which by their presence ensure expression of a DNA fragment of the invention or may, alternatively, be ones which suppress the expression of said DNA fragment so that their absence causes the DNA sequence to be expressed. Thus, the promoter and optionally its associated regulatory sequence may be activated by the presence or absence of one or more factors to affect transcription of the DNA fragment comprising a nucleotide sequence substantially as shown in Fig. 1 or an analogue or subsequence thereof.

Other types of regulatory sequences are upstream and downstream sequences involved in control of termination of transcription and removal of introns, as well as sequences responsible for polyadenylation, and initiation of translation. When the regulatory sequence is to function in a plant such as a dicotyledonous plant, e.g. a potato plant, such a regulatory sequence is preferably one which is derived from a dicot plant, e.g. derived from an α-amylase gene.

Factors regulating promoter activity may vary depending, *inter alia*, on the kind of promoter employed as well as on the organism in which it is to function. Tissue specific regulation may be regulated by certain intrinsic factors which ensure that genes encoding proteins specific to a given tissue are expressed. Such factors are known to exist in mammals and plants so as to allow for specific tissues to develop, and a low number of tissue specific sequences have been described. Among these is the patatin promoter which is known to function in tubers and to a lesser degree in leaves of potato plants. The patatin promoter is a strong promoter and is further described in (44).

Another type of promoter which has been elucidated is a promoter derived from a plant virus, e.g. a cauliflower mosaic virus (CaMV), a strong constitutive promoter.

Other promoters may be derived from the Ti-plasmid such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter, and promoters from other open reading frames in the T-DNA such as ORF7.

Generally, in order to ensure that the promoters are accepted by the host organism it is advantageous to use a promoter which is isolated from the host organism in question, although a promoter from the same subclass of organisms, e.g. dicots, as a rule functions accurately and efficiently. The regulatory sequence may be an α-amylase promoter, i.e. a promoter which is naturally found in connection with α-amylase genes and involved in the transcription thereof. An α-amylase promoter may be obtained from an isolated α-amylase gene, which may be obtained by hybridization, e.g. as explained in further detail below, to a DNA fragment of the invention comprising a nucleotide sequence substantially as shown in Fig. 1-5 or a subsequence of analogue thereof. Typically, the α-amylase promoter should be obtained from the same organism as the one in which it is to function, but could also be obtained from another organism, preferably of the same subclass. Optionally, and if desired, the natural promoter may be modified for the purpose, e.g. by modifications of the promoter nucleotide sequence so as to obtain a promoter functioning in another manner than the natural promoter, e.g. being weaker or stronger. Thus, an α-amylase promoter would be suitable for the construction of a transgenic potato plant (which will be discussed in further detail below).

A certain basic α-amylase activity is needed to allow a given plant to grow and develop and to perform its essential metabolical functions. Thus, when designing new plant constructs, especially new potato plant constructs, in which a reduced α-amylase activity is desirable, these constructs are preferably designed so as to allow for a lower expression of α-amylase compared to the expression in the natural plant, while maintaining an expression level which is sufficient to ensure that the metabolic functions essential for growth and development of the plant can be carried out. This may, for instance, apply in the construction of transgenic potatoes for use in the production of potato chips.

A method of decreasing the expression of the intrinsic α-amylase in a plant is at the level of translation. This may be done by providing an anti-sense RNA capable of inhibiting the translation of an mRNA encoded by the intrinsic α-amylase genes, the anti-sense RNA comprising a nucleotide sequence substantially in the opposite orientation as those shown in Fig. 1-5 or a subsequence or analogue thereof. The terms "intrinsic α-amylase" and "intrinsic α-amylase genes", respectively, refer to the naturally abundant α-amylase enzyme and α-amylase genes in plants, of which there may be several.

As explained in further detail below (see Example 7), the nucleotide sequences shown in Fig. 1-5 represent two distinctly different types of cDNA clones. One type is represented by clones AmyZ3/4, AmyZ7 and AmyZ2 (Fig. 1, 2 and 5: "AmyZ3/4 *type*"), and the other type is represented by clones AmyZ1 and AmyZ6 (Fig. 3 and 4; "AmyZ1 *type*"). The nucleotide sequence homology between clones of the two types is low, about 55-60%. Therefore, translation of mRNA from either of the two types may be prevented independently of the other, since the two types are so different. (On the other hand, the homology within each of the types is very high, well over 90%). In order to prevent translation of mRNA from both types of

DNA, constructs must be prepared with both types of anti-messenger genes (see Example 24). For this purpose, the fact that clones AmyZ1 and AmyZ6 are not full length is of relatively little importance.

Thus, in one aspect, the present invention provides a DNA fragment which encodes an anti-sense RNA, i.e. a mRNA molecule capable of hybridizing to a mRNA transcribed from the intrinsic α-amylase genes, thereby inhibiting the translation thereof. The expression of the nucleotide sequence coding for the anti-sense RNA may be either constitutive or regulated.

Preferably, the DNA fragment encoding an antisense RNA molecule is substantially complementary to a DNA fragment of the invention so as to provide an efficient hybridization of the two RNA molecules, thereby ensuring an efficient inhibition of the translation of an α-amylase mRNA to α-amylase. The DNA fragment encoding an antisense RNA molecule is preferably of a size which provides a sufficient degree of homology between the antisense RNA and the mRNA to which it is to hybridize, e.g. the complementary strand of a complete sequence of Fig. 1-5. Generally, the DNA fragment should be as long as possible, resulting in a high probability of collision and thus hybridization between the RNA transcribed therefrom and the mRNA to which it is to hybridize. Antisense regulation in plants is further discussed in references (59), (60) and (61).

As mentioned above, the present invention also relates to polypeptides encoded by a DNA fragment as defined above, i.e. a DNA fragment comprising a nucleotide sequence substantially as shown in any of Figures 1-4 or a subsequence or analogue thereof or a DNA fragment hybridizing therewith under the above specified hybridization conditions. Also an analogue or a fragment of said polypeptide is within the scope of the present invention.

The term "analogue" with regard to the polypeptides of the invention is used in the present context to indicate a protein or polypeptide with a similar amino acid composition and sequence as an amino acid sequence of α-amylase from *Solanum tuberosum* shown in Fig. 1 or 2, allowing for minor variations in the amino acid sequence, e.g. substitution, deletion, addition, insertion or rearrangement of one or more amino acids in the polypeptide which do not have any substantial adverse effect on the enzymatic properties of the polypeptide as compared to the polypeptides shown in Fig. 1 and 2. The terms "substitution", "insertion", "addition" and "rearrangement" are explained above in connection with the explanation of the DNA fragments of the invention and are to be understood accordingly. Variations in the carbohydrate moieties, etc. which do not have an adverse effect on the enzymatic properties of the analogue are also comprised by the term "analogue". The term "analogue" covers the term "homologue" in its conventional meaning, i.e. a protein or polypeptide which is developmentally related. The analogous polypeptide or protein may be derived from another organism than *Solanum tuberosum*, e.g. from another dicotyledonous plant, or may be partially or completely of synthetic origin. The term is further intended to mean any enzymatic subsequence, functional equivalent or derivative of an amino acid sequence shown in Fig. 1 or 2.

The term "enzymatic subsequence" is intended to indicate an amino acid sequence comprising at least one active site of the α-amylase enzyme. By the term "active site" is meant the part of the amino acid sequence which directly or indirectly is involved in the conversion of starch to reducing sugar, i.e. in the hydrolysis of α-1,4 linkages in the starch.

The term "functional equivalent" is intended to include all enzymatically active substances with the ability to convert starch to reducing sugars such as glucose and fructose in a manner similar to α-amylase, i.e. to hydrolyze α-1,4-linkages. The functional equivalent may be derived from an organism different from *Solanum tuberosum*, e.g. from another dicotyledonous plant, or may partially or completely be of synthetic origin. It should be understood that the similarities between the amino acid sequences of α-amylase from *Solanum tuberosum* shown in Fig. 1 and 2 and the functional equivalent are qualitative rather than quantitative, relating to the nature rather than the level of activity of the functional equivalent.

The present invention also relates to polypeptides derived from a dicotyledonous plant showing a high degree of homology, typically at least about 60%, for example at least about 70%, with an amino acid sequence of a potato α-amylase shown in any of Figures 1-4. The term "homology" is intended to indicate the presence of any degree of similarity between a given amino acid sequence and an amino acid sequence shown in Fig. 1-4. It will be understood that the homology is dependent on the number of amino acid residues to be compared as well as the parts of the amino acid sequence which is actually compared. The term "degree of homology" is to be understood as the fraction of identical amino acid residues in the amino acid sequences to be compared. In the present context, the degree of homology between any two amino acid sequences is the number of identical amino acid residues at corresponding positions in the two amino acid sequences to be compared as compared to the total number of amino acid residues compared in the two amino acid sequences. The degree of homology between two amino acid sequences should be determined for the greatest possible overlap between the two amino acid sequences, i.e. the amino acid sequences to be compared should be placed so as to allow for the greatest possible overlap between identical amino acid residues, resulting in the maximal degree of homology obtainable between the two amino acid sequences to be compared.

The dicotyledonous plant from which the polypeptide is derived is preferably a member of the family *Solanaceae*, in particular of the genus *Solanum*, as a high degree of homology between polypeptides from these plants is expected. Thus, α-amylases from any potato plant are expected to be homologous with the polypeptides of the invention. Also, related plants of the family *Solanaceae*, such as plants of the genus *Capsicum*, e.g. *Capsicum annuum*, *Lycopersicon*, e.g. *Lycopersicon lycopersicum*, and *Nicotiana*, e.g. *Nicotiana tabacum*, as well as dicotyledonous plants from other families, are expected to contain polypeptides homologous to the polypeptides of the invention, i.e. α-amylase.

Polypeptides homologous to the amino acid sequences substantially as shown in Fig. 1-4 or a fragment or analogue thereof may be obtained by isolating genes or messenger RNAs encoding such polypeptides using a DNA fragment of the invention and preparing the polypeptides on the basis of the knowledge of such genes or mRNAs, such as will be explained in further detail below. In particular, it is expected that DNA fragments of the invention, particularly fragments comprising a nucleotide sequence as shown in Fig. 1 or 3, can be used for isolating corresponding DNA fragments from plants closely related to potato, for example tomato (*Lycopersicon*). By preparing an α-amylase in the manner shown below, i.e. by use of recombinant DNA techniques or by liquid or solid phase polypeptides synthesis, it is possible to obtain a polypeptide of the invention in a substantially pure form, which is advantageous for certain purposes.

In the present context, the term "substantially pure" is understood to mean that the polypeptide in question is substantially free from other components, e.g. other components which may influence the enzymatic activity of the polypeptide, which other components may result from the production and/or recovery of the polypeptide or otherwise may be found together with the polypeptide.

Polypeptides of the invention may be produced by any convenient method, e.g. a method involving the use of recombinant DNA technology or by solid or liquid phase peptide synthesis. Substantially pure polypeptides of the invention having substantially an amino acid sequence shown in Fig. 1-4, or an analogue or α-amylase related fragment thereof, the polypeptide or analogue or fragment thereof being substantially free of naturally co-occuring enzymes, may be prepared by a method which comprises:

a) inserting a DNA fragment as defined above, i.e. a DNA fragment comprising a nucleotide sequence substantially as shown in Fig. 1 or an analogue or subsequence thereof, optionally in a suitable modified form, in an expression vector,

b) transforming a suitable host microorganism with the vector produced in step a),

c) cultivating the microorganism produced in step b) under suitable conditions or expressing the polypeptide, and

d) harvesting the polypeptide from the culture.

The method may optionally comprise a further step in which the polypeptide produced is subjected to one or more modifications.

In step a) of the method, the modification of the sequence optionally carried out may be performed before or after the sequence has been inserted in the vector. The modification may be carried out in order to adapt the polypeptide expressed from the DNA sequence to a given purpose, e.g. to modify one or more active sites of polypeptide as discussed above. The modification may comprise substitution, addition, insertion or deletion of one or more nucleotides in the sequence or a combination thereof, in accordance with the explanation given above of substitution, addition, insertion, rearrangement or deletion of amino acid residues in the amino acid sequence.

The transformation in step b) of the method may be carried out by standard procedures, such as disclosed in Maniatis et al. (12). The cultivation of the host microorganism in step d) of the method may be carried out in a culture medium conventionally used for fermentation purposes and suited to the host organism in question, and under conditions of pH, temperature, aeration, etc. suited to the type of microorganism in question, e.g. as disclosed in Maniatis et al. (12).

In step d) of the method, the harvesting of the polypeptide or an analogue or fragment thereof may proceed by well-known methods such as by precipitation, gel filtration, ion exchange, HPLC reverse phase chromatography or immunoaffinity chromatography.

The polypeptide produced may be subjected to one or more modifications, e.g. in order to modify one or more active sites thereof, or in order to remove undesired parts of the polypeptide, e.g. a signal sequence, a part of a fusion protein, etc. The modification may be performed in accordance with well-known methods, for instance by thermal treatment, treatment with a chemical such as formaldehyde, glutaraldehyde or a suitable proteolytic enzyme, e.g. trypsin, or substitution, addition, insertion or deletion of one or more amino acids in the polypeptide as explained above.

In an alternative method, the polypeptide or an analogue or fragment thereof may be prepared by the well-known methods of liquid or solid phase peptides synthesis.

Alternatively, when producing a polypeptide of the invention by use of recombinant DNA techniques such as will be explained in more detail below, the signal sequence mediates transport out of the cell and the fermentation medium containing the recombinant polypeptide may be used as it is prior to any recovery or isolation of the α-amylase enzyme.

In a further aspect, the present invention relates to a vector which is capable of replicating in a host organism and which carries a DNA fragment as described above, i.e. a DNA fragment comprising a nucleotide sequence substantially as shown in Fig. 1-5 or a subsequence or analogue thereof or an α-amylase gene comprising said DNA fragment or a DNA fragment encoding an antisense RNA molecule capable of hybridizing to a mRNA transcribed from a DNA fragment of the invention, The vector may either be one which is capable of autonomous replication, such as a plasmid, or one which is replicated with the host chromosome, such as a bacteriophage or integrated into a plant genome via the border sequences of Ti vectors. For production purposes, the vector is an expression vector capable of expressing the DNA fragment in the organism chosen for the production. Thus, the expression vector is a vector which carries the regulatory sequences necessary for expression such as the promoter, an initiation signal and a termination signal, etc. The vector may also be one used for diagnosis of α-amylase genes or messengers from potato and other organisms for which purpose expression is not required. Methods of diagnosis are further explained below.

In a still further aspect, the present invention relates to an organism which carries and which is capable of replicating or expressing an inserted DNA fragment as defined above, i.e. a DNA fragment comprising a nucleotide sequence substantially as shown in Fig. 1-5 or a subsequence or analogue thereof or an α-amylase gene or pseudogene comprising said DNA fragment or a DNA fragment encoding an antisense RNA molecule capable of hybridizing to a mRNA transcribed from a DNA fragment of the invention.

The term "inserted" indicates that the DNA fragment (or subsegment or analogue, or gene or pseudogene) has been inserted into the organism or an ancestor thereof by means of genetic manipulation, in other words, the organism may be one which did not naturally or inherently contain such a DNA fragment in its genome, or it may be one which naturally or inherently did contain such a DNA fragment, but in a lower number so that the organism with the inserted DNA fragment has a higher number of such fragments than its naturally occurring counterparts.

The DNA fragment carried by the organism may be part of the genome of the organism, or may be carried on a vector as defined above which is harboured in the organism. The DNA fragment may be present in the genome or expression vector as defined above in frame with one or more second DNA fragments encoding a second polypeptide or part thereof so as to encode a fusion protein, e.g. as defined above.

The organism may be a higher organism such as a plant, preferably a dicotyledonous plant such as a plant of the family *Solanaceae*, or a lower organism such as a microorganism. A lower organism such as a bacterium, e.g. a gram-negative bacterium such as a bacterium of the genus *Escherichia*, e.g. *E. coli*, or a gram-positive bacterium such as of the genus *Bacillus*, e.g. *B. subtilis*, or a yeast such as of the genus *Saccharomyces* or a fungus, e.g. of the genus *Aspergillus*, is useful for producing a recombinant polypeptide as defined above. As most organisms inherently produce α-amylase, it may be desirable to modify, i.e. increase, reduce or destroy the inherent α-amylase production, so that the inherent α-amylase production will not influence the preparation of an α-amylase according to the present invention. However, it may be advantageous that the microorganism used for a given recombinant production also produces its natural α-amylase, for instance to obtain an additive effect between the two α-amylases produced. The recombinant production will be explained in further details below.

Examples of such microorganisms are the *E. coli* K-12 strain harbouring the plasmid pAmyZ3 which has been deposited at Deutsche Sammlung von Mikroorganismen (DSM) on 4 April, 1989 under the accession number DSM 5275, the *E. coli* K-12 strain harbouring the plasmid pAmyZ4 which has been deposited at DSM on 4 April, 1989 under the accession number DSM 5276, the *E. coli* harbouring the plasmid pAmyZ1 which has been deposited at DSM on 18 April, 1990 under the accession number DSM 5882, the *E. coli* harbouring the plasmid pAmyZ6 which has been deposited at DSM on 18 April, 1990 under the accession number DSM 5883, and the *E. coli* harbouring the plasmid pAmyZ7 which has been deposited at DSM on 18 April, 1990 under the accession number DSM 5884.

Also, the organism may be a cell line, e.g. a mammalian cell line, or, more preferably, a plant cell line. Most preferably, the organism is a plant, i.e. a genetically modified plant such as will be discussed in further detail below.

In a further aspect, the present invention relates to a genetic construct useful for inhibiting the translation of a mRNA molecule encoded by a DNA fragment as defined above, comprising a nucleotide sequence substantially as shown in Fig. 1-5 or a subsequence or analogue thereof, which construct comprises 1) a regulatory sequence functionally connected to 2) a DNA fragment as defined above encoding a RNA molecule capable of inhibiting the translation of the intrinsic α-amylase mRNA, the DNA fragment comprising a nucleotide sequence substantially as shown in Fig. 1-4 in the opposite orientation or an analogue or subsequence thereof, and 3) a transcription termination DNA sequence, functionally connected to the DNA fragment of 2). The principle of this genetic construct is the construction of a genetically modified plant in which the α-amylase production is reduced so as to obtain a plant having a reduced α-amylase activity. As an example, the genetic construct will be useful in constructing a genetically modified potato plant with a reduced α-amylase activity which results in a reduced amount of reducing sugars in the plant, i.e. a potato plant useful as a raw material for chips production. When the genetic construct is to be used in the construction of a potato plant for this purpose, it is preferred that the DNA fragment is based on a nucleotide sequence which is complementary to a sequence which is well-expressed in potato tubers or one which shows a sufficiently high degree of homology (i.e. hybridizes under the conditions outlined above) to an α-amylase messenger from the tuber, typically one which is normally expressed in another tissue than the tuber, such as in a shoot or leaf. Alternatively, the nucleotide sequence may be transcribed from a pseudo-gene.

The conditions under which the genetic construct is used should ensure that the antisense RNA-molecule expressed from the genetic construct is allowed to hybridize to an mRNA transcribed from the intrinsic α-amylase genes. Preferably, the genetic construct is active in the same cell or tissue where the intrinsic α-amylase genes are active. Preferably, the genetic construct is stably integrated into the genome to ensure that it is inherited from generation to generation. Useful genetic constructs for reducing the amount of reducing sugars in a potato plant are described in further detail in Example 24.

As discussed above, it may be advantageous to construct an organism with an increased α-amylase activity as compared to the organism in its natural form. Preferably, the organism is a plant, especially a potato plant. A potato plant with an increased α-amylase activity as compared to the potato in its natural form may be used as a raw material for the production of spirits, because an increased α-amylase activity will lead to an increased amount of reducing sugars in the potato, which is advantageous for the spirits production. Accordingly, the present invention relates to a genetic construct useful for producing a polypeptide as defined above, i.e. a polypeptide encoded by a DNA fragment of the invention comprising a nucleotide sequence substantially as shown in Fig 1 or 2 or a subsequence or analogue thereof or an α-amylase gene, which construct comprises 1) a regulatory sequence functionally connected to 2) a DNA fragment as defined above encoding the polypeptide, and 3) a transcription termination DNA sequence functionally connected to the DNA fragment of 2).

The genetic construct useful for producing a polypeptide of the invention, e.g. an α-amylase or a part thereof, is preferably used in the construction of a plant having an increased α-amylase activity as compared to a plant not containing the genetic construct.

The regulatory sequence contained in the above-defined genetic constructs is preferably a plant promoter such as a constitutive or regulatable plant promoter. The promoter may be of the type discussed above, and when the genetic construct is to be used in a genetically modified potato plant, the promoter is preferably a plant promoter, which may be the CaMV promoter or the NOS promoter, the α-amylase promoter or the promoter from a potato poly-ubiquitin gene.

The genetic construct may further be provided with a marker which allows for the selection of the genetic construct in a plant cell into which it has been transferred. Various markers exist which may be used in plant cells, particularly markers which provide for antibiotic resistance. These markers include resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

As mentioned above, the genetic construct is preferably to be used in modifying a plant. Accordingly, the present invention also relates to a genetically modified plant comprising in its genome a genetic construct as defined above. The genetic construct may be one which is active in reducing the α-amylase activity or one which is involved in increasing the α-amylase activity of the plant. The invention thus further relates to a genetically modified plant which has an increased or decreased α-amylase activity compared to a corresponding non-modified plant. It will furthermore often be desired that a genetically modified plant of the invention is one in which the α-amylase activity may be regulated by various factors, for example by factors such as temperature. For example, when the plant in question is a potato plant, it will for certain purposes be desirable that the α-amylase activity may be regulated, e.g. reduced, at relatively low temperatures, so that potatoes may be stored at low temperatures without the conversion of starch to reducing sugars. Thus, the invention further relates to a genetically modified plant in which the α-amylase activity may be regulated at low temperatures, in particular at temperatures of below about 8 °C.

A genetically modified plant may also be one which produces antisense mRNA, which reduces starch decomposition by hybridizing to an mRNA which is transcribed by an α-amylase gene. The invention therefore further relates to a genetically modified plant, in particular *Solanum tuberosum*, which produces mRNA which is capable of hybridizing to an mRNA transcribed from a DNA fragment as shown in Fig. 1-4, thereby inhibiting the translation thereof and reducing starch decomposition compared to a corresponding non-modified plant.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material. Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. Thus, in another aspect, the present invention relates to a vector system which carries a genetic construct as defined above and which is capable of introducing the genetic construct into the genome of a plant such as a plant of the family *Solanaceae*, in particular of the genus *Solanum*, especially *Solanum tuberosum*. The vector system may comprise one vector, but comprises preferably two vectors; in the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in "Binary vectors" (53).

One extensively employed system for transformation of plant cells with a given genetic construct, e.g. as described above, is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* (42, 54).

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above. Non-limiting examples of such Ti plasmids are given in Example 24 and 25 below, a further example being pGV3850.

The DNA fragment of the invention to be inserted into the Ti-plasmid should preferably be inserted between the terminal sequences of the T-DNA or adjacent to a T-DNA sequence so as to avoid that the sequences immediately surrounding the T-DNA borders are disrupted, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

As will be understood from the above explanation, the vector system of the invention is preferably one which contains a virulence function capable of infecting the plant and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct. Furthermore, the vector system is preferably an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants; many vector systems exist which are based on these plasmids or derivatives thereof.

In the construction of a transgenic plant using a vector it is preferred that the genetic construct to be inserted in the plant is first constructed in a microorganism in which the vector can replicate and which is easy to manipulate. An example of a useful microorganism is *E. coli*, but other microorganisms having the above properties may be used. When a vector of a vector system as defined above has been constructed in *E. coli*, it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*.

The Ti-plasmid harboring the DNA fragment of the invention is thus preferably transferred into a suitable *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harboring the DNA fragment of the invention, which DNA is subsequently transferred into the plant cell to be modified. This transformation may be performed in a number of ways, e.g. as described in (53) and in EP 0 122 791. However, for potato and other Solanaceae, infection of leaf pieces using *A. tumefaciens* is most often employed (74).

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in (54).

As will be understood from the introductory part of the present specification, the DNA fragment of the present invention may be used for diagnostic purposes, which will be further explained in the following.

Various types of diagnosis may be performed by use of the DNA fragment of the invention. In a given sample, α-amylase messenger RNA's may be qualitatively as well as quantitatively determined by hybridization to the DNA fragment of the invention under conditions suited for said hybridization. Furthermore, genes encoding α-amylase present in an organism such as a plant may be identified and isolated by use of the DNA fragment of the invention, e.g. by screening a gene library of such an organism.

When the DNA fragment is to be employed for diagnostic purposes, it will often be useful to provide it with a label which may be used for detection. Thus, in a further aspect the present invention relates to a DNA fragment as defined above which is provided with a label. Preferably, the label is selected from the group consisting of fluorophores, radioactive isotopes, isotopes and complexing agents such as biotin.

Examples of radioactive isotopes useful as label substances are H^3, C^{14}, S^{35} and P^{32}. The radioactivity emitted by these isotopes may be measured in a gamma-counter, a scintillation counter or by radioautography followed by densitometry in a manner known *per se*.

Complexing agents may be e.g. Protein A, biotin (which forms a complex with avidin and streptavidin) or lectin. In this case, the complex is not in itself directly detectable, necessitating labelling of the substance with which the complexing agent forms a complex. Any of the labels disclosed above may be used for this purpose.

In a further aspect, the present invention relates to a method of isolating an α-amylase gene or messenger from an organism, e.g. a plant, in particular a dicotyledon, the method comprising hybridizing a nucleic acid containing sample obtained from a gene library or cDNA library from the organism with a DNA fragment of the invention comprising a nucleotide sequence substantially as shown in Fig. 1-5 or a subsequence or analogue thereof, optionally in a labelled form, in a denatured form or an RNA copy thereof under conditions favorable to hybridization between the DNA fragment or RNA copy and the nucleic acid of the sample, and recovering the hybridized clone so as to obtain an α-amylase gene or cDNA of the organism.

In a still further aspect, the invention relates to a method of quantifying the amount of an α-amylase messenger present in different tissues in an organism, e.g. a plant, in particular a dicotyledon, the method comprising hybridizing a nucleic acid containing sample obtained from the organism with a DNA fragment of the invention comprising a nucleotide sequence substantially as shown in Fig. 1-4 or a subsequence or analogue thereof, optionally in labelled form, in denatured form or an RNA copy thereof under conditions favorable to hybridization between the denatured DNA fragment or RNA copy and the RNA of the sample and determining the amount of hybridized nucleic acid (40).

Another approach for detecting the presence of a specific α-amylase gene, e.g introduced by the genetic methods described previously, or a part thereof in an organism, e.g. a plant, in particular a dicotyledon, is to employ the polymerase chain reaction (5), i.e. using a method in which the DNA fragment which comprises the α-amylase gene or part thereof in a sample is subjected to multiple fold amplification.

Based on the observations that reducing sugar content in stored potatoes is correlated with α-amylase activity as described in the Examples, and that the correlation extends to shoots and presumably to other parts such as the leaves, potato varieties can be screened for their tendency to accumulate sugar in stored potatoes already at a stage when young plantlets have formed a few leaves, i.e. at a very early stage. This may, e.g., be performed by spotting RNA extracts from 0.1 to 0.5 grams of leaves (e.g. obtained as described below in "Materials and Methods") on filters suitable for hybridization and hybridizing with a DNA fragment of the invention, optionally provided with a label such as biotin or an radioactive isotope. As a reference, hybridization with similarly labeled ubiquitin coding regions from any organism, e.g. barley, may be used, as ubiquitin sequences are extremely well conserved and constitutively expressed in different plant tissues. The results are compared with similar dot blots obtained from analysis of potato varieties with known sugar characteristics, e.g. from the four potato varieties described in "Materials and Methods". A dot blot analysis as described above may be carried out on a large amount of breeding material and lead to early determination of the sugar characteristics of a given potato variety.

Restriction fragment length polymorphisms (RFLP) are increasingly used to follow specific alleles of genes in various organisms. The alleles are either themselves followed or they are used as markers (unlinked or linked) in crosses involving other characteristics, e.g. pathogen resistance and morphological characteristics such as tuber colour. So far, the method has primarily been employed in humans, but it has also been employed in plants, e.g. in potatoes (27). It is contemplated that a DNA fragment of the invention may be useful in RFLP-analysis of α-amylase genes, especially in dicotyledonous plants such as in potato, based on the results of genomic Southern hybridization of potato DNA with an isolated DNA fragment of the invention. Plants of the species *Solanum tuberosum* have few α-amylase genes and therefore yield a simple fragment pattern that make polymorphisms easy to evaluate. Thus, genomes may be analyzed for alleles encoding α-amylase as described above using the principles of restriction fragment length polymorphism, i.e. as described in (27). An example of the use of α-amylase genes in this technique is given in Example 28 and the accompanying Fig. 19 and 20.

In the present context, the abbreviations for the amino acids are those conventionally used.

DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the nucleotide sequence of a potato α-amylase messenger and the deduced amino acid sequence of potato α-amylase precursor. The figure shows the sequence of the messenger RNA-like strand of the combined inserts of potato α-amylase cDNA clones AmyZ3 and AmyZ4 without the terminal EcoRI sites. AmyZ3 and AmyZ4 have identical nucleotide sequences in the regions where they overlap (cf. Fig. 11), except that AmyZ3 has an intron sequence of 128 nucleotides which is underlined in the figure (nucleotides 296 to 423, inclusive). The consensus 5' and 3' splice junction nucleotides, GT and AG, respectively, are double-underlined in the figure. A consensus branch point, (nucleotides 390 to 396) is boxed. The nucleotide sequence of the 3' leader until nucleotide 540 contains four open reading frames, which are shown in detail in Fig. 17. The region encoding the α-amylase precursor starts at nucleotide 541 and terminates at nucleotide 1761, and the length of the α-amylase precursor is 407 amino acids. In the figure, the derived amino acid sequence is shown using the one letter code for the amino acids. The probable processing site of the precursor is shown by an arrow. The 3' untranslated region is at least 200 nucleotides in length, not including the polyA tail, but probably not much longer, since a putative polyA signal is found 30 nucleotides from the end. The polyA signal is underlined in the figure.

Fig. 2 shows the nucleotide sequence of potato α-amylase messenger and deduced amino acid sequence of potato α-amylase precursor. The figure shows the sequence of the messenger RNA-like strand of the insert of potato α-amylase cDNA clone AmyZ7 without the terminal EcoRI sites. The nucleotide sequence encodes an α-amylase precursor starting at amino acid number two and the sequence includes the G residue (No. 1 in the figure) of the initiation codon ATG. The α-amylase reading frame terminates at position 1219, and the length of the α-amylase.precursor is 407 amino acids, including the initiation codon. The probable processing site of the precursor is shown by an arrow. The sequence terminates with a 187 nucleotides long 3' untranslated region followed by a polyA tail nine residues long.

Fig. 3 shows the partial nucleotide sequence of potato α-amylase messenger and deduced amino acid sequence of a partial potato α-amylase precursor. The figure shows the sequence of the messenger RNA-like strand of the insert of potato α-amylase cDNA clone AmyZ1 without the terminal EcoRI sites. The partial α-amylase open reading frame starts at nucleotide 6 and terminates at nucleotide 1052 and the first amino acid aligns with amino acid 69 of the sequence shown in Fig. 1. The AmyZ1 clone thus lacks nucleotides encoding the signal peptide and approximately 50 codons from the N-terminus of mature α-amylase. The sequence terminates with a 163 nucleotides long 3' untranslated region.

Fig. 4 shows the partial nucleotide sequence of potato α-amylase messenger and deduced amino acid sequence of a partial potato α-amylase precursor. The figure shows the sequence of the messenger RNA-like strand of the insert of potato α-amylase cDNA clone AmyZ6 without terminal EcoRI sites. The partial α-amylase open reading frame starts at nucleotide 3 and terminates at nucleotide 647 and the first amino acid aligns with amino acid 133 of the sequence shown in Fig. 1. The sequence terminates with a 360 nucleotides long 3' untranslated region.

Fig. 5 shows the partial nucleotide sequence of a pseudo potato α-amylase messenger and deduced amino acid sequence of the α-amylase that the sequence encoded before the corresponding gene suffered two deletions. The figure shows the sequence of the messenger RNA-like strand of the insert of potato α-amylase cDNA clone AmyZ2 without the terminal EcoRI sites.

Fig. 6 is a schematic cross-sectional view of a potato showing the tissues from which samples were taken as described in Example 1; A: sprout in itself without any leaves or roots; B: connection between sprout and tuber; C: vascular tissue of the tuber; D: parenchym tissue.

Fig. 7 shows the structure and subcloning of barley α-amylase cDNA clone pM/C. Plasmid 036 was the clone received from J.C. Rogers with the information that it is a BamHI-HindIII subclone of pM/C (24).

However, the complete sequence was not published, and the short unknown sequence is indicated by a star. From plasmid 036, the indicated Hinf fragment was subcloned in pBS- to generate a plasmid yielding high copy numbers, pBS036, which was analyzed as described in Example 3 (the results of which are shown in Fig. 4), and used as a source of the hybridization probe which is the SacI fragment indicated at the bottom of the figure. Single lines indicate vector sequences and noncoding barley sequences; double lines indicate the barley α-amylase coding region.

Fig. 8 shows hybridization of barley α-amylase cDNA fragments with radioactive cDNA from polyA-rich RNA from potato (Dianella) sprouts. Restriction enzyme fragments from barley α-amylase cDNA clones and control plasmids were fractionated in duplicate on a 2% agarose gel and the ethidium bromide stained fragments are shown in panel b). The fragments were transferred to nitrocellulose filters and one filter was hybridized with 5 million cpm of radioactive cDNA made from Dianella sprout polyA-rich RNA (panel a). The other filter was hybridized with 4 million cpm of radioactive cDNA made from barley leaf polyA-rich RNA. Both filters were hybridized at low stringency (hybridization at 67 °C in 6 x SSC, wash at 67 °C in 4 x SSC). The panels a) and b) show autoradiograms of the filters. The plasmid DNA in the different lanes (2 µg/lane) was as follows: Lane 1, pBR327 digested with EcoRI and PstI (liberates a 723 bp fragment that serves as a negative control); lane 2, pKG3730 digested with PstI (liberates a 780 bp fragment encoding barley ubiquitin, ubiquitin genes are highly conserved and found in all eukaryotes (17) - the fragment serves as a positive control); lane 3, pBS036 digested with SacI (liberates the 800 bp fragment subsequently used as probe); lane 4, pBR327 digested with BstNI (size markers, 1855 bp, 928 bp, and 475 bp); lane 5, plasmid pBS050 digested with PstI (liberates a cDNA fragment encoding barley α-amylase type A); lane 6, plasmid 036 digested with BamHI and HindIII (liberates the fragment subcloned from pM/C, see Fig. 7). Strong bands in panel a) and b) are unexplained, false hybridizations to the unknown region of plasmids 036 and pBS036, indicated with stars in Fig. 7. The weaker bands indicated with triangles are the specific hybridizations of the barley α-amylase coding regions. Lane 2 in panel a) shows that potato messenger RNA, as expected, contains ubiquitin coding sequences.

Fig. 9 shows analyses of the putative potato α-amylase cDNA clones. Plasmid DNA from six of the eight purified plasmids from the potato cDNA library was digested with EcoRI, fractionated on a 2% agarose gel, and stained with ethidium bromide (black lanes). The fragments were transferred to a nitrocellulose filter and the filter was hybridized at low stringency (hybridization at 67°C in 6 x SSC, wash at 67°C in 4 x SSC) with a nick-translated barley α-amylase SacI probe. The white lanes show the resulting autoradiograms, and the hybridizing EcoRI fragments seen in the original autoradiogram are indicated with arrows. The full names given to the plasmids are AmyZ2, AmyZ3, AmyZ4, AmyZ5, AmyZ6 and AmyZ7. M1 and M2 are size markers, M1 is pBR327 digested with BstNI (1855 bp, 928 bp, and 475 bp), M2 is pBR327 digested with HinfI (1631 bp, 517 bp, 452 bp, 298 bp and 154 bp). Panel C shows the homologous hybridization of pBS036 digested with SacI to the SacI probe performed in the same experiment.

Fig. 10 shows EcoRI maps of potato α-amylase cDNA clones. All maps have been refined after DNA sequence determination. In the maps of the clones only the EcoRI sites are shown and the terminal EcoRI sites are in the EcoRI linkers by which the inserts were inserted into the EcoRI site in the vector, pBluescript SK-. The sequences of the inserts of AmyZ5, 7, and 8 are completely identical. The EcoRI fragments that hybridize to the barley α-amylase probe are indicated with an extra thin line. In Fig. 9 weak bands, 285 base pairs long, were seen to hybridize in lanes Z5 and Z7, and the cryptic EcoRI site that gave rise to these bands is shown in parentheses. The circled number above the figure indicates the EcoRI fragments used in Southern and Northern hybridizations (Fig. 19 and 21, respectively). The clone AmyZ2 includes a row of 76 unstable T residues that is the result of a common cloning artefact.

Fig. 11 shows the strategy for sequencing the inserts of plasmids AmyZ3 and AmyZ4 and a summary map of AmyZ3/4. The arrows show the direction and length of sequences obtained in individual sequence analyses. The abbreviations for the restriction enzyme sites are E = EcoRI, H = HindIII, Ha = HaeIII, X = XhoII. Above the maps, the location of the α-amylase precursor open reading frame is shown with boxes, the striped portions indicating the signal peptides and the white portions the mature α-amylase. In the combined map of AmyZ3 and 4 the location of the open reading frames in the 5' leader sequence is indicated; they are shown in more detail in Fig. 17.

Fig. 12 shows the strategy for sequencing the inserts of plasmids AmyZ1 and AmyZ6. The arrows show the direction and length of sequences obtained in individual sequence analyses. The abbreviations for the restriction enzyme sites are E = EcoRI, H = HindIII, B = BamHI, Bg = BglII, Ha = HaeIII, T = Taq. Above the maps, the location of the α-amylase open reading frame is shown with boxes.

Fig. 13 shows the strategy for sequencing the inserts of plasmids AmyZ2 and AmyZ7. The arrows show the direction and length of sequences obtained in individual sequence analyses. The insert in plasmid AmyZ2 has a string of 76 T residues at the left end that is the result of a common cloning artefact. In subclones used for sequence determination a portion of the Ts were deleted and this event is indicated by the arrow. The abbreviations for the restriction enzyme sites are A1 = AluI, E = EcoRI, H = HindIII, Bg = BglII. Above the maps, the location of the α-amylase open reading frame is shown with boxes.

Fig. 14 shows the homology between potato and barley α-amylase encoding nucleotide sequences. The top lines show the sequence of the EcoRI fragment from AmyZ4 that hybridizes with the barley SacI probe (Fig. 7) as shown in Fig. 9. The potato sequence is aligned with the corresponding region from the SacI probe (U = Uracil replaces T, when a sequence is written as RNA). The total homology between the sequences is 63.5% (unmatched nucleotides are included in the total length). A region of 146 nucleotides with a homology of 73% is boxed, and within this region is shown a smaller box of 46 nucleotides with 80% homology.

Fig. 15 shows the homology between potato AmyZ3/4 α-amylase and barley α-amylases. The figure shows the amino acid sequences of mature potato α-amylase from Fig. 1 (top lines) and mature barley amylase B, decoded from pM/C (bottom lines) (24). Identical amino acids are indicated by a dash and conserved amino acids by double dots. Gaps have been introduced to maximize the similarity. The percentage of identical amino acids is 45.6 (unmatched amino acids are included in the total length). The box indicates a peptide which is conserved in α-amylases found in mammals and insects.

Fig. 16 shows the homology between potato AmyZ1 α-amylase and barley α-amylase. The figure shows the partial amino acid sequence of mature potato α-amylase, bottom lines (from Fig. 3), and mature barley amylase B, decoded from pM/C, top lines (24). Identical amino acids are indicated by double dots and conserved amino acids by single dots. On gap has been introduced to maximize the similarity. The percentage of identical amino acids is 64.1 (the unmatched amino acid is included in the total length).

Fig. 17 shows open reading frames in the 5' end of the AmyZ3 sequence. The extent of the open reading frames and their frames are shown at the top, the amino acid sequences at the bottom. The position of the intron in AmyZ3 is also indicated.

Fig. 18 shows a hydrophilicity profile of potato α-amylase precursor with the amino acid sequence shown in Fig. 1. The analysis was performed by the method of Hopp and Woods (25). Part of the plot is expanded to illustrate the short hydrophobic region of the signal peptide. At the bottom the amino acids contributing to the hydrophobic stretch are indicated by small circles and the probable processing site by an arrow.

Fig. 19 shows Southern hybridizations of potato genomic DNA with potato α-amylase probes. Saturna DNA was digested with restriction enzymes and fractionated on an 8% agarose gel. The DNA fragments were transferred to nitrocellulose filters and the filters hybridized at medium high stringency with nick-translated EcoRI fragments from AmyZ3. The figure shows the resulting autoradiograms. Lanes 1 and 2 contain HindIII digested DNA, lane 3 contains BamHI digested DNA, and lanes 4 and 5 contain EcoRI digested DNA. Lane 6 is a weaker exposure of lane 3. The central lanes 2, 3, and 4 were hybridized with nick-translated EcoRI fragments 2, 3, and 4 from AmyZ3, and lanes 1 and 5 were hybridized with nick-translated EcoRI fragment 1 (Fig. 10). The numbers on the left are size markers in kbp from ethidium bromide stained HindIII fragments of phage λ DNA.

Fig. 20 shows Southern hybridizations of two varieties of potatoes with AmyZ3/4 and AmyZ1 types of α-amylase probes. The method used is described in Fig. 19. The left hand autoradiogram shows hybridization of the complete α-amylase insert from plasmid AmyZ3 to Saturna and Dianella potato variety genomic DNA. The right hand autoradiogram shows hybridization of the complete α-amylase insert from plasmid AmyZ6 to Saturna and Dianella potato variety genomic DNA. Abbreviations for restriction enzymes: H = HindIII, B = BamHI, E = EcoRI. Numbers in the middle are size markers in kbp from ethidium bromide stained HindIII fragments of phage λ DNA.

Fig. 21 shows northern hybridization of potato RNA. RNA from sprouts and tubers from varieties Saturna and Dianella was fractionated on agarose gels after denaturation and control lanes which also contained potato RNA were stained with methylene blue to visualize the ribosomal RNAs. Their migration is indicated by thin arrows; the top arrows show 26s RNA (approximately 4400 nucleotides) and the bottom arrows show 18s RNA (approximately 1850 nucleotides). The RNA in the remaining lanes were transferred to nitrocellulose filters which were hybridized with potato α-amylase specific probes and used to expose X-ray films. Saturna sprout RNA was hybridized to nick-translated EcoRI fragment 1 from AmyZ3 (Fig. 10); the three other RNAs were hybridized to nick-translated EcoRI fragments 2, 3 and 4 from AmyZ3. Dianella sprout RNA was the polyA-rich fraction used for construction of the cDNA library, whereas the three other lanes contained total RNA. Thick arrows mark the position of α-amylase specific bands seen in the original autoradiogram, and their approximate sizes are listed in the table in Example 17.

Fig. 22 shows reducing sugar levels in stored potatoes. The levels of glucose and fructose (including the phosphorylated forms) were determined in four varieties of potato at the time of harvest and during subsequent long term storage at 8 °C. The figure shows the sum of the glucose and fructose levels.

Fig. 23 shows reducing sugar levels at different storage temperatures. The levels of glucose and fructose (including the phosphorylated forms) were determined in four varieties of potato stored for 19 weeks at 8 °C or 6 °C, and 6 weeks at 4 °C. All potatoes were sampled on the same day. The figure shows the sum of the glucose and fructose levels.

Fig. 24 shows levels of α-amylase in stored potatoes. The activity of α-amylase was determined (method 2) in the juice of four varieties of potatoes stored for 19 weeks at 8 °C and at 6 °C. The figure shows the average values obtained at the two temperatures as a function of the level of the reducing sugars at 8 °C after 19 weeks of storage.

MATERIALS AND METHODS

Potato varieties

The following potato varieties were used: Dianella, Saturna, Bintje, Lady Rosetta.

These potato varieties were chosen in order to illustrate the relation between reducing sugar and α-amylase activity and to identify the variety and/or tissue, from which α-amylase clones could be isolated in the most advantageous manner.

Dianella is a variety used as a starting material for alcohol fermentation, due to its high content of starch. Dianella was chosen because it is a well-known variety and because it probably represents an extremity as regards sugar metabolism in potatoes generally.

Saturna is known for its low content of reducing sugars, making it particularly suitable for use in the production of e.g. potato chips. It is one of the few potato varieties with a sugar content low enough to make it suitable for producing potato chips after prolonged storage. However, it is a relatively sensitive variety with respect to cultivation, especially with regard to its demand for water. It is therefore desirable that new potato varieties with a low reducing sugar content equivalent to that of Saturna may be produced.

Bintje is a well-known established variety which is primarily used for potatoes intended for normal consumption. Its content of reducing sugars lies between that of Dianella and Saturna.

Lady Rosetta is a new variety for use in the production of e.g. potato chips. It is one of the few potato varieties with a reducing sugar content low enough for use in the production of chips, but it is still in the experimental stage.

Bacterial strains

HB101:	*hsm, hrs, recA, gal, pro* str^R
JM109:	*recA1, endA1, gyrA96, thi, hsd R17, supE44, relA1,* λ^-, Δ*(lac⁻proAB), [F', traD36, proAB,* $LacI^q Z\Delta MI5$*]*
XL1-Blue:	*endA1, hsdR17* (r_k^-, m_k^+), *supE44, thi-1,* λ^-, *recA1, gyrA96, relA1,* Δ*(lac)*, [F', *proAB,* $lacI^q$ $Z\Delta M15$*, Tn10 (*tet^R*)*]
BB4	$recA^+$, $lacI^q$, $Z\Delta M15$, tet^R
JM109:	see reference (48)
XL1:	see reference (20, 49)
BB4:	see reference (31)
HB101:	see reference (50)

Phages and plasmids

λZap:	see reference (31)
R408 Interference Resistant Helper Phage:	see reference (31)
pBR327:	see reference (51)
pBS+, pBS-:	see reference (31)

Medium and plates

L-Broth (LB) medium:

Per litre: 5 g of yeast extract, 5 g of NZ-amide, 5 g of NaCl, 5 g of bacto-peptone. Autoclave.

LB-plates:

LB medium plus 15 g of Bacto agar per litre. Autoclave. Pour into plastic petri dishes (25 ml/dish).

Tet-plates:

As LB-plates plus 17 mg tetracycline per litre after autoclaving.

Amp-plates:

As LB-plates plus 35 mg ampicillin per litre after autoclaving.

AXI-plates:

As LB-plates plus 35 mg ampicillin, 120 mg IPTG (isopropylthiogalactoside), 40 mg Xgal (dissolved in dimethylformamide) per litre after autoclaving.
Xgal: 5-bromo-4chloro-3indolyle-β-D-galactoside.

Minimal medium: 400 ml of H_2O, 1 ml of solution B, 100 ml of solution A, 5 ml (0.5 mg/ml) of thiamin, 5 ml of 20% glycerol. Solution A is 10 g of $(NH_4)_2SO_4$, 24 g of Na_2HPO_4, 15 g of KH_2PO_4, 15 g of NaCl per litre of H_2O. Solution B is 200 g of $MgCl_2.2H_2O$, 7.2 g $CaCl_2.2H_2O$ and 20 ml of micronutrient solution per litre. Micronutrient solution is 10 µM $FeCl_2$, 500 nM of $CaCl_2.2H_2O$, 400 nM of H_3BO_3, 80 nM of $MnCl_2$, 30 nM $CoCl_2$, 10 nM $CuCl_2$, 3 nM of $(NH_4)_6Mo_7O_{24}$. For plates add 15 g of agar per litre autoclave.

Freezing of potato samples

In order to be able to measure amylase activity, the potato samples were first frozen. A metal bucket was filled 2/3 full with liquid N_2, and a plastic bowl filled halfway up with the potato material was placed in the bucket with liquid N_2. A blender bowl which had been cooled with liquid N_2 was filled with the cooled potato material, and 2 teaspoons of ascorbic acid were added. The material was blended in a blender until the potato material had a flour-like consistency. An 800 ml beaker was cooled with liquid N_2 and a potato sample was poured into the beaker. Each sample was divided into 2 portions which were placed in 2 plastic bags, and the bags were placed in a freezer as quickly as possible. This frozen potato material is referred to in the following as "pretreated material".

Determination of reducing sugar levels in potatoes

25 g of the above pretreated material was weighed and ice-water was added to 125 ml. The mixture was blended for 1 min. and then centrifuged for 10 min. at a temperature of 0°C and 14,000 rpm. The supernatant was decanted from the precipitate and filtered through a sterile filter, heated in a water bath for 10 min., and filtered again through a sterile filter. Determination of levels of D-glucose before and after enzyme hydrolysis of sucrose, and determination of D-fructose after the determination of D-glucose, were carried out using a sucrose/D-glucose/D-fructose test kit from Boehringer Mannheim according to the manufacturer's instructions.

Qualitative measurement of amylase activity in potato

Potato tubers (varieties Saturna and Dianella) were placed in a dark cupboard for 14 days at 20°C.

Tissue extracts of these tubers and their white sprouts were prepared by grinding 2 g of fresh tissue (or in some cases 10 g of frozen tissue) with 5 ml of 0.1M Tris-HCl (pH 6.2), 1mM $CaCl_2 \cdot 6H_2O$. The homogenate was centrifuged for 5 min at 10.000 rpm and 4°C in 15 ml corex tubes. 5 µl of the supernatant was spotted on a glass plate covered with a thin film of 1% starch (w/v). The glass plate (with the thin film facing up) was placed in a petri dish on 4 pieces of pre-wetted filter paper. Care was taken to ensure that the starch plate was held moist under the incubation, so that the enzyme could function adequately. The petri dish was incubated at either 20°C or 37°C for 16 h. The starch plates were submerged in I_2/kI solution and rinsed under deionized water. The I_2/kI solution gives the starch a dark blue colour, which disappears if the starch has been digested to glucose and maltose (a "strong" reaction). In cases where there is a "weak" reaction, the spot is reddish, with a clear change in the surface compared to the surroundings (more smooth) (7).

Quantitative Determination of α-amylase activity (method 2)

The following procedure was used for each sample of pretreated potato material. All determinations were carried out in duplicate, with 4 samples and 8 determinations from each sample of pretreated potato material.

A 75 ml beaker was filled with the above pretreated material and sealed with parafilm. The material was allowed to thaw in the beaker, optionally in a water bath. After thawing, the liquid was poured into a centrifuge test tube and centrifuged at 14,000 rpm for 10 min. 10 ml of buffer with a pH of 5.5 was added to an Erlenmeyer flask with a 10 ml pipette, after which a tablet comprising Phadabas blue starch powder (Pharmacia Diagnostics Ltd.) was added to the buffer. The tablet was dissolved by gently shaking the flask. Finally, 5 ml of supernatant from the centrifuged liquid was added to the solution with a 5 ml pipette, being careful not to disturb the precipitate. Penicillin was added to the flask to achieve an activity of 10,000 I.U., and the flask was sealed with parafilm.

The flasks were placed in a 45°C water bath for 48 hours, after which they were cooled in a tub with cold water. The optical density of the samples were measured with a spectrophotometer at 620 nm using a buffer with a pH of 5.5 as a blind sample. The concentration of α-amylase in the samples was calculated automatically on the basis of these measurements.

The protein concentration was determined in the supernatants according to Lowry (52)

Harvest of potato tissue for RNA/DNA isolation

Tubers (Dianella, Saturna) were placed in a dark cupboard at 20 °C. The white sprouts which developed were harvested after 14 days and cut into smaller pieces. The tissue was frozen with dry ice immediately after cutting and stored at -80 °C until use. Tubers (Dianella, Saturna, Bintje, Lady Rosetta) were peeled, grated (with a grater) directly onto tin foil placed on dry ice. 10 g portions were stored at -80 °C until use.

Extraction of RNA from potato sprouts

Total RNA was extracted and purified from potato (*Solanum tuberosum*, Dianella and Saturna varieties) white sprouts after grinding under liquid N_2, using the following guanidine thiocyanate/N-sarcosine method of Kaplan et al. (8).

10 g of frozen sliced potato sprouts were homogenized in 6 vol. (w/v) of 5.0 M guanidine thiocyanate, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA and 5% 2-mercaptoethanol. The homogenate was made 4% (w/v) with respect to N-lauroylsarcosine and solid CsCl was added to 0.15 g/ml. The homogenate was centrifuged for 20 minutes at 10.000 rpm, 4 °C to remove debris. The supernatant was gently layered over a 2.5 ml cushion of 5.7 M CsCl, 0.1 M EDTA and centrifuged in a Beckman SW-41 rotor at 37,000 rpm for 18 hours at 20 °C. After centrifugation, the homogenate was carefully removed with a pasteur pipette and the polyallomer tubes were washed three times with water. After removal of the CsCl cushion, the RNA pellets were suspended in 10 mM Tris-HCl (pH 7.5) and precipitated by the addition of 2.5 vol. of cold ethanol.

The pellet was resuspended in 10 mM Tris-HCl, adjusted to 100 mM NaCl and precipitated with 2.5 vol. cold ethanol. The final RNA pellet was suspended in 10 mM Tris-HCl (pH 7.5) 2 mM EDTA, 100 mM NaCl and the RNA concentration was determined by measuring OD_{260} (OD = 1 for a solution with 40 µg RNA/ml).

The total RNA used for the isolation of polyA-rich RNA was suspended in 10 mM Tris-HCl (pH 7.5), 2 mM EDTA, 100 mM NaCl and 0.5% sodium dodecyl sulphate (SDS).

Extraction of RNA from tubers

It was not possible to isolate RNA from tuber tissue using the above explained conventional method, i.e. with the guanidine thiocyanate/N-sarcosine method. The guanidine thiocyanate reacted with the starch and the products had a jelly-like consistency. From this turbid liquid it was impossible to centrifuge the RNA through the CsCl cushion. Thus, the present inventors had to develop another method: the tuber tissue ground under liquid N_2 was suspended in 10 mM Tris-HCl (pH 7.5), 50 mM EDTA, 500 mM NaCl, 5% 2-mercaptoethanol, 1% SDS and placed at 65 °C for 20 min. The homogenate was made 3% (w/v) with respect to N-lauroylsarcosine and solid CsCl was added to 0,15 g/ml. The homogenate was centrifugated for 20 min at 10,000 rpm, 4 °C (International) to remove debris. The RNA was then isolated by centrifugation over a CsCl cushion as described above (9).

RNA electrophoresis

The RNA samples were denatured in 0.5 M glyoxal (deionized, stored at -20 °C), 10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.5), 50% DMSO (dimethylsulfoxide, stored at 4 °C) (10). They were then incubated at 50 °C for 1 hour and placed for 5 to 10 minutes on ice before adding tracking dye (35% ficoll, MW 400,000, 0.01 M Na_2HPO_4/NaH_2PO_4 (pH 6.5), 0.4% Bromophenol Blue. The samples were loaded onto a 1.5% agarose gel in 10 mM Na_2HPO_4/Na_2PO_4 (pH 6.5) and subjected to electrophoresis in the presence of 10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.5) with buffer recirculation, at 35 mA for approximately 2 1/2 hours (11). One or two lanes were cut from the gel and the RNA was stained overnight in Methylene Blue solution (33.5 ml 3M Na-acetate, 100 ml 1M acetic acid, H_2O up to 500 ml and 10 mg Methylene Blue).

Northern blotting

A glass plate was placed in a trough on a support that lifted the glass plate 5-6 cm. A piece of filter paper, wetted in 0.025 M Na_2HPO_4/NaH_2PO_4 (pH 6.5), was placed on the glass plate with the ends and sides touching the bottom of the trough.

0.025 M Na_2HPO_4/NaH_2PO_4 (pH 6.5) was poured into the trough so that the surface barely touched the glass plate, and the RNA gel was placed on top of the wet filter paper. A Gene Screen (New England Nuclear) membrane was cut to the exact size of the gel, soaked in 0.025 M Na_2HPO_4/NaH_2PO_4 (pH 6.5) and placed on top of the gel (avoiding trapping air bubbles). Four pieces of parafilm covered the edges of the membrane and a piece of filter paper wetted in 0.025 Na_2HPO_4/NaH_2PO_4 (pH 6.5) was placed on top of it. Several pieces (20-25) of filter paper were layered on top, and on top of these were layered several paper towels (6-8 cm). The filter paper and paper towels were cut to the size of the gel. A glass plate and a weight were placed on top of the paper towels. The transfer took place over a period of 16 hours. The paper towels, filter paper and parafilm were removed. The membrane was marked for size markers (18S rRNA and 28S rRNA) using the piece of gel stained in Methylene Blue overnight. The membrane was washed in 0.025 M Na_2HPO_4/NaH_2PO_4 (pH 6.5) air dried and baked for 2 h. at 80 • C (11).

Hybridization of RNA

The Gene Screen membrane was prewetted in 6 x SSC for 30 minutes at room temperature with constant agitation. Then the membrane was prehybridized in a solution of 50% formamide (deionized), 0.2% polyvinyl-pyrrolidone (MW 40,000), 0.2% bovine serum albumin, 0.2% ficoll (NW 400,000), 0.05 M Tris-HCl (pH 7.5), 1.0 M NaCl, 0.1% Na_4P_2O7, 1.0% SDS, 10% dextran sulphate (MW 500,000), denatured sonicated salmon sperm DNA (50 µg/ml) and 10 µg/ml polyA RNA. The volume of the prehybridization solution was 100 µl/cm² of the membrane placed in a sealable plastic bag. Prehybridization took place in a sealed plastic bag which was incubated for 6 hours at 42 • C with constant agitation. To the bag was added 1/5 of its liquid volume of the following solution: 50% formamide (deionized), 0.2% polyvinyl-pyrrolidone (MW 40,000), 0.2% bovine serum albumin, 0.2% ficoll (MW 400,000), 0.05 M Tris-HCl (pH 7.5), 0.1% $Na_4P_2O_7$, 1.0% SDS, denatured sonicated salmon sperm DNA (50 µg/ml), poly A (10 µg/ml) and the denatured radioactive probe (5 ng/ml prehybridization and hybridization solution). The bag was resealed and incubated with constant agitation for 16-20 hours at 42 • C. The hybridization solution was removed and the membrane was washed at room temperature in 2 x SSC, 1 mM EDTA, 10 mM Tris-HCl, pH 7.59 2 x 5 min., at 67 • C in 2 x SSC, 10% SDS, 1 x 10 min and 1 x 30 min., and finally at room temperature in 0.1 x SSC, 1 x 5 min. and 1 x 30 min., always with constant agitation (11). The membrane was air dried, covered with plastic film and autoradiographed with and without intensifying screen at -80 • C. The salmon sperm DNA and the radioactive probe were denatured by boiling for 10 min. in a water bath and placed on ice before being added to the hybridization solution.

Isolation of poly(A)–rich RNA

An oligo(dT) column was made as follows:
2 g oligo(dT)-cellulose/Type 2 (purchased from Collaborative Research, Inc., Research Product Division, 1365 Main Street, Waltham, Mass., USA) was washed once with 10 mM Tris-HCl (pH 7.5), washed once with 0.5 M KOH and neutralized by 8-10 washes with 10 mM Tris-HCl (pH 7.5). The column was poured and preserved at room temperature in 1% SDS. The column was run at 30 • C and the buffers were warmed to 30 • C before use. The 1% SDS was removed by passing 2 vol. 10 mM Tris-HCl (pH 7.5) over the column. Before adding the RNA sample the column was adjusted to high salt buffer by passing 2 vol. 10 mM Tris-HCl (pH 7.5), 500 mM NaCl over it. The total RNA dissolved in 2 mM EDTA, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.5% SDS was heated to 65 • C for 10 minutes (to remove aggregations), cooled to 30 • C and adjusted to 500 mM NaCl. The solution was then gently applied to the top of the column and the column was washed with 10 mM Tris-HCl (pH 7.5), 500 mM NaCl until the OD_{260} of the eluent was less than 0.01. The enriched poly(A)RNA was eluted with 10 mM Tris-HCl (pH 7.5), and 10 fractions of 1 ml were collected. The poly(A)-rich RNA was precipitated from the peak fractions (measuring OD_{260}) by adjusting to 100 mM NaCl and adding 2.5 vol. cold ethanol. The RNA was centrifuged at 10,000 rpm for 20 minutes at 4 • C (Sorvall cooling centrifuge) and dried under vacuum. The dry pellet was suspended in sterile water (1 µg poly(A)-rich RNA/µl) kept on ice and stored at -20 • C (12).

Isolation of genomic DNA

Genomic DNA was isolated from white potato sprouts. 10 g of frozen sliced potato sprouts was ground under liquid N_2 and homogenized in 4 vol. (w/v) proteinase K buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1% SDS and 0.2 mg/ml proteinase K). The mixture was incubated for 1 h at 30 • C with stirring from time to time and then centrifuged at 10,000 rpm for 15 min. at 4 • C. 1 vol. of chloroform/isobutanol (24:1) was added to the supernatant. This was followed by thorough mixing (6 to 7 times with standing in between periods of mixing, total about 20 min.) and centrifuging for 20 min. at 10,000 rpm and 4 °C. The organic phase (with chlorophyll, if present, and proteins in the interphase) was discarded and the mixture was extracted again with 1 vol. of chloroform/isobutanol (24:1) and centrifuged for 20 min. at 10,000 rpm and 4 °C. 2.5 vol. cold ethanol was added to the upper phase and the mixture was stored overnight at -20 °C. It was then centrifuged for 10 min. at 10,000 rpm and 4 °C, the pellet was thoroughly vacuum-dried and resuspended in 20 ml TE-buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). The solution was stored at 4 °C before CsCl banding. To test that the genomic DNA had a high molecular weight, 1 to 10 µl of the genomic DNA was run on a 1% agarose gel; DNA which has not been degraded will migrate close to the slot in a broad band. The genomic DNA was then banded on a CsCl gradient: 20 g of CsCl was poured into the 20 ml of genomic DNA and 1.25 ml ethidium bromide solution (stock: 5 µg/ml) was added. Another solution (A) was prepared: solid CsCl was mixed with TE-buffer (w/v) and 0.2 mg ethidium bromide/ml was added. The DNA solution was poured into quick-seal polyallomer tubes, which were then filled up with solution (A) and sealed. The tubes were centrifuged in a Beckman VTI 65 rotor at 15 °C and 45,000 rpm for 48 h. The centrifuge was stopped without using the brake. The genomic band was removed under UV-light with a syringe, and the ethidium bromide was extracted with CsCl-saturated isopropanol (7 to 8 times). The CsCl was then removed from the DNA by dialysis in TE-buffer for 72 h with 6 changes of buffer. It was generally not necessary to precipitate the DNA at this stage (precipitation was avoided, since high molecular weight DNA is very hard to resuspend) unless very little DNA was isolated. The DNA concentration was measured at $OD_{260\ nm}$ assuming 50 µg DNA/ml at OD_{260} = 1. The genomic DNA, which was used for restriction enzyme digestion, was stored at 4 °C (45).

Preparation of plasmid DNA

Small scale preparation of plasmid DNA was performed as follows: bacterial strains harboring the plasmids were grown overnight in 2 ml L-Broth (LB) medium with either 15 µg/ml tetracycline (tet) or 35 µg/ml ampicillin added. The operations were performed in 1.5 ml Eppendorf tubes and centrifugation was carried out in an Eppendorf centrifuge at 4 °C. The cells from the overnight culture were harvested by centrifugation for 2 min., washed with 1 ml 10 mM Tris-HCl (pH 8.5), 1 mM EDTA and centrifugated for 2 min. The pellet was suspended in 150 µl of 15% sucrose, 50 mM Tris-HCl (pH 8.5), 50 mM EDTA by vortexing. 50 µl of 4 mg/ml lysozyme was added and the mixture was incubated for 30 min. at room temperature and 30 min. on ice. 400 µl ice cold H_2O was added and the mixture was kept on ice for 5 min, incubated at 70-72 °C for 15 min. and centrifuged for 15 min. To the supernatant was added 75 µl 5.0 M Na-perchlorat and 200 µl isopropanol (the isopropanol was stored at room temperature), and the mixture was centrifuged for 15 min. at 5 °C. The pellet was suspended in 300 µl 0.3 M Na-acetate and 2-3 vol. cold ethanol was added. Precipitation was accomplished by storing at either 5 min. at -80 °C or overnight at -20 °C, centrifuging for 5 min., drying by vacuum for 2 min. and redissolving the pellet in 20 µl H_2O. The yield was 5-10 µg plasmid DNA (46).

Large scale preparation of plasmid DNA was accomplished by simply scaling up the small scale preparation ten times. Working in 15 ml corex tubes, all the ingredients were scaled up ten times. The centrifugation was carried out in a Sorvall cooling centrifuge at 4 °C. Only changes from the above will be mentioned in the following. After incubation at 70-72 °C, the centrifugation was for 30 min. at 17,000 rpm. After adding isopropanol and after adding cold ethanol, the centrifugation was for 15 min. at 17,000 rpm. The final plasmid DNA pellet was suspended in H_2O and transferred to an Eppendorf tube and then given a short spin to remove debris. The supernatant was adjusted to 0.3 M Na-acetate and 2-3 vol. cold ethanol were added. The pellet was resuspended in 40 µl H_2O. The yield was usually 20-80 µg plasmid DNA.

To obtain very pure plasmid DNA, 200-300 µg of isolated plasmid DNA from the upscaled method were banded on a CsCl gradient. Solid CsCl was mixed with H_2O (1:1 w/v) and 0.2 mg ethidium bromide/ml was added. The solution was poured into a quick-seal polyallomer tube and the plasmid DNA, mixed with solid CsCl (1:1 w/v), was added. The tube was filled, sealed and centrifuged in a Beckman VTI 65 rotor at 15 °C, 48,000 rpm for 16-18 hours. The centrifuge was stopped by setting the timer at zero without using the brake. The banded plasmid DNA was withdrawn from the tubes by using a syringe and the ethidium bromide was extracted with CsCl-saturated isopropanol 7-8 times. The CsCl was removed by dialysis in 10 mM Tris-HCl (pH 8.0), 1mM EDTA for 48 hours with three changes of buffer. The DNA was precipitated by adjusting to 0.3 M Na-acetate and adding 2-3 vol. cold ethanol (12).

Restriction Enzyme Digestions

All restriction endonucleases were from Biolabs or Boehringer Mannheim and were used according to the supplier's instructions. 1 unit of enzyme was used for 1 µg of DNA and incubation was for 2 hours. The buffer was changed in double digestions, either by changing the volume or by adding the necessary ingredient according to the enzyme instructions.

Electrophoretic fractionation of DNA fragments

Non-denaturing gels

Agarose gels were used for estimating the concentration of plasmid DNA, for separation of restriction enzyme digested genomic DNA (0,8 or 1% agarose gels), for mapping restriction enzyme digested plasmid DNA and for Southern blotting (2% agarose gels) and Northern blotting (1,5% agarose gels). The Northern blotting gels have been described above. The other gels were prepared in a horizontal slab gel apparatus, and electrophoresis was either carried out in 2 x McArdle buffer (2 x McArdle:80mM Tris, 36 mM NaCl, 24 mM Na-acetat, 4.4mM EDTA, adjusted to pH 8.0 with glacial acetic acid) at 45 mA for 18h, or carried out in 1xTBE buffer (1xTBE:89mM Tris-borate, 89mM boric acid, 2mM EDTA) at 70mA for 2-3h. The gels contained 5 µg ethidium bromide/ml gel. Tracking dye (35% ficoll (M.W 40,000), 5 mM EDTA, 0.04% Bromophenol Blue) was added to the DNA samples before loading. The gels were photographed using long wave UV light with an orange filter and Polaroid film No. 665 (13, 14).

5% acrylamide gels were used for mapping restriction enzyme digested plasmid DNA and for isolation of specific DNA fragments. The gel (0.1x12x15cm) was formed between two glass plates held apart by spacers. One gel contains 37 ml H_2O, 17.5 ml 19% acrylamide, 1% bis-acrylamide, 4.4 ml TEMED (0,5% N,N,NN-tetraethylethylenediamine) 7 ml 10xTBE, 4.4 ml amper (1.6% ammoniumperoxodisulphate). Electrophoresis was carried out for 2 h at 180-200 V in a vertical slab gel apparatus. The gel was stained in 5µg/ml ethidium bromide for 30 min., after which the bands were visualized using long wave UV light and photographed as described above (13).

DNA sequencing gels

8% acrylamide - 8 M urea gels (0.035 x 20 x 47cm) were used for separation of DNA fragments after sequence reactions. The gel contained the following: 15.75 ml 38% acrylamide, 2% bis-acrylamide, 36 g urea and H_2O up to 70 ml. The solution was deionized (one spoonful ion exchanger, with stirring for 30 min and removal by filtration) and 7.5 ml 10 x TBE, 1 ml H_2O, 2.7 ml amper (1.6%) were added. The mixture was degassed, cooled on ice and 40 µl concentrated TEMED was added. The gel was immediately poured between two glass plates held apart by spacers. The gel was run at 40 W for 1 1/2 - 4 hours, then dried by a gel drier and autoradiographed for 3-48h at room temperature (12).

Recovery of DNA from gels

DNA fragments were eluted from acrylamide gels to circumvent problems with enzyme-toxic compounds found in agarose. A DNA fragment cut from a 5% acrylamide gel was placed in a dialysis bag with 200 µl 1/2 x TBE. The bag was placed parallel to the electrodes in a horizontal gel apparatus and soaked with 1/2 x TBE. Electrophoresis was at 150 V for 2-4 hours. The current was inverted 30 sec. and the bag was controlled under UV light. The DNA solution was adjusted to 0.3 M Na-acetate and precipitated with 2-3 vol. of cold ethanol (12).

Southern Transfer

A 2% agarose gel was soaked in denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 2 x 15 min. The liquid was constantly stirred with a magnetic stirrer. The gel was then soaked in neutralization buffer (0.5 M Tris-HCl (pH 7.0), 3.0 M NaCl) for 3 x 10 min. The neutralized gel was placed on a solid support covered with a piece of filter paper prewetted in 20 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7.0), the ends of the filter paper having been dipped into a tray with 20 x SSC to form a wick. A nitrocellulose filter, cut to the size of the gel, was wetted in H_2O and in 20 x SSC and laid on top of the gel, avoiding air bubbles. Four pieces of parafilm (an "umbrella") were placed on the edges of the nitrocellulose filter. A piece of filter paper prewetted in 20 x SSC was placed on top of this and over the filter paper was placed a stack of dry filter paper. On this stack were placed several layers of paper towels cut to the size of the gel. Finally, a glass plate and a weight were placed on top. The blotting took place for a period of 16-18 hours, after which the nitrocellulose filter was marked, washed for 10 minutes in 3 x SSC, air dried and baked in a vacuum oven for 2 hours at 80 °C. The procedure was identical for 0.8% agarose gels with large DNA fragments, except that the gels were pretreated by soaking 2 x 15 minutes in 0.25 M HCl (15).

Nick translation

The following was mixed in an Eppendorf tube:
3 µl of 10 x nick buffer (500 mM Tris-HCl (pH 8.0), 50 mM $MgCl_2$, 100 mM β-mercaptoethanol)
2 µl of a mix containing 1 mM dCTP, 1 mM dGTP and 1 mM dTTP in H_2O
0.4-2 µg of an isolated DNA fragment (from 5% acrylamide gel)
4 µl of DNase (1mg/ml) diluted to 10^{-4}
25 µ Ci of α^{32}P-dATP
1 µl DNA polymerase I (Kornberg)
The total volume was adjusted to 30 µl with H_2O.

The mixture was incubated at 14 °C for 2-3 h (16).

A column was made from a pasteur pipette, plugged with ordinary glass wool, and filled to a height of 7 to 8 cm with Sephadex G-100 (the Sephadex G-100 is equilibrated in TE-buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA).

The nick-translated DNA was passed over the column using TE-buffer, the first peak was collected and the incorporated radioactivity was estimated by liquid scintillation counting. Probes having a specific activity of 2 x 10^7 to 8 x 10^8 cpm/µg DNA were used. They were heat denatured before being added to the hybridization solutions.

For hybridization to λ-ZAP phage filters, the amounts of the ingredients in the nick were doubled, except for the α^{32}p-dATP, which was used in an amount of 100 µCi (double nick translation). This usually gave 1.7 x 10^8 cpm/5 µl of probe used (1.6 µg), which was enough for one plastic bag with filters with a size of 22 x 22 cm.

Labelling of cDNA

Radioactive cDNA for hybridization experiments was prepared by first strand synthesis of polyA-mRNA in the presence of a radioactive nucleotide.

The following was mixed in an autoclaved Eppendorf tube:
1 µl of 20 x cDNA stock (1 x cDNA stock: 50 mM Tris-HCl (pH 8.3), 100 mM KCl, 10 mM $MgCl_2$, 5 mM dithriothreitol),
1 µl of each of dCTP (10 mM), dGTP (10 mM) and dTTP (10 mM).
1 µl of dATP (100 µM plus 50 µCi α^{32}-PdATP,
2 µl of oligo (dT) (P.L. Biochemical),
2-3 µg of polyA-rich RNA,
20 units of RNasin (an RNase inhibitor, Biotec Inc.)
40 units of avian myeloblastosis virus reverse transcriptase (J.W. Beard, Life Science Inc.) and sterile H_2O up to 20 µl.

The mixture was incubated for 45 min. at 37 °C, after which 1 µl of dATP (10 mM) was added and the mixture was kept at 37 °C for 15 min. (chase 15). The RNA-DNA hybrids were purified on a Sephadex G-100 column (7 to 8 cm in a pasteur pipette plugged with ordinary glass wool and equilibrated in TE-buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA)). The first peak was collected and the hybrids were precipitated with 5 µl tRNA (1 mg/ml), 80 mM NaCl (final concentration) and 2.5 vol. cold ethanol. The reaction was checked by running 5 x 10^5 cpm of the RNA-DNA hybrids on a 5% acrylamid gel. The gel was exposed to an X-ray film overnight at room temperature and it showed a smear which was strongest near the slots. The RNA was hydrolysed with 0.4 M NaOH (final concentration) for 1 h at 50 °C and neutralized with HCl. The cDNA was then ready to add to the hybridization solution (17).

Employing another method of preparing radioactive cDNA, a small portion of polyA-rich RNA was used for constructing the λ-ZAP library. The radioactive cDNA was prepared to ensure that the RNA had not been degraded during its isolation. Both the first and the second strand were synthesized from 2 µg polyA-rich RNA and 20 µCi α^{32}P-dATP by using a Boehringer Mannheim cDNA synthesis kit. 1/20 of the double-stranded cDNA was run on a 5% acrylamid gel. The gel was dried, and exposed to X-ray film at room temperature. The resultant band and smear showed that the cDNA had a high molecular weight (12).

Hybridization of DNA

The nitrocellulose filters from Southern transfer or λ-ZAP plaque-filters were wetted in 2 x SSC [1 x SSC: 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7.0] and placed in a heat-sealed plastic bag with pre-warmed 67 • C) prehybridization solution. Prehybridization took place for 2 h at 67 • C, the bag being gently shaken. The solution was exchanged with prewarmed (67 • C) hybridization solution, the radioactive probe was added and hybridization was carried out at 67 • C for 18 h. The bag was gently shaken to ensure constant movement of the liquid over the nitrocellulose filters. After hybridization, a washing procedure was carried out. Different stringency conditions were used:

Low stringency conditions:

Prehybridization and hybridization solutions: 10 x Denhardt (0.2% polyvinylpyrrolidone (MW 40,000), 0.2% ficoll (MW 400,000), 0.2% bovine serum albumin), 6 x SSC, 0.1% SDS, 10 µg/ml polyA, 50 µg/ml of denatured (heat) sonicated *E. coli* DNA (not salmon DNA), and the denatured (heat) radioactive probe. The filters were washed in pre-warmed (67 • C) solutions: 2 x 15 min in 10 x Denhardt, 4 x SSC, 0.1% SDS; 4 x 15 min in 4 x SSC, 0.1% SDS. The filters were air-dried and covered with plastic film. Autoradiography was carried out for 3 h to 24 h at -80 • C with and without intensifying screens.

Medium high stringency conditions:

Prehybridization and hybridization solutions were the same as above, except that 6 x SSC was replaced with 4 x SSC. The following wash solutions were prewarmed to 67 • C:

10 x Denhardt, 2 x SSC, 0.1% SDS for 2 x 15 min. and 1 x SSC, 0.1% SDS for 4 x 15 min. The filters were air-dried and covered with plastic wrap, and X-ray film was exposed to the filters for 3 h to 3 weeks with and without intensifying screens.

The procedure for genomic filters was as follows (medium high stringency):
Prehybridization and hybridization solution: 10 x Denhardt, 3 x SSC, 0.1% SDS, 10% (w/v) Dextransulphate, 10 µl/ml polyA and 50 µg/ml denatured sonicated *E. coli* DNA. The filters were washed in prewarmed (67 • C) hybridization solution without Dextransulphate for 5 x 10 min at 67 • C, and for 4 x 15 min. in 10 x Dehnhardt, 1 x SSC, 0.1% SDS. The filters were rinsed in 3 x SSC and air-dried, and X-ray film exposed to the filters as described above (12 and 18).

Filling of recessed 3′ ends (for subcloning Hinf fragment from pO36)

The following was combined in an Eppendorf tube: 10 µl containing up to 1 µg of DNA fragment, 1 µl of a 2 mM solution of the four dNTP's, 2 µl of a 10 x nick translation buffer (0.5 M Tris-HCl (pH 7.2), 0.1 M $MgSO_4$, 1 mM dithiotreitol, 500 µg/ml bovine serum albumin) and H_2O up to 20 µl. 2 units of Klenow polymerase were added and the mixture was mixed and incubated for 30 min at 22 • C. The mix was heated for 5 min at 70 • C to inactivate the polymerase, extracted twice with saturated phenol (the phenol was first mixed with 0.1 M Tris-HCl, then mixed twice with TE-buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and once with chloroform, precipitated with 0.3 M Na-acetate and 2.5 vol. cold ethanol and rinsed twice with 70% cold ethanol. The blunt-ended DNA fragment was then ligated to a blunt-ended vector in T4-DNA ligase buffer (12).

Subcloning

Preparation of vectors

Vectors (pBS- or pBS+) were digested with one or two restriction enzymes, extracted twice with saturated phenol (the phenol was first mixed with 0.1 M Tris-HCl, then mixed twice with TE-buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and once with chloroform and precipitated with 0.1 M NaCl and 2.5 vol. cold ethanol. The pellet was rinsed in 80% cold ethanol and dissolved in H_2O, giving a concentration of 25-50 ng/µl. The vectors were always tested for background before use (self-ligation with and without T4-DNA-ligase).

Ligation

The plasmid comprising the fragment to be subcloned was digested with one or more appropriate restriction enzymes and run on a 5% acrylamid gel, after which the fragment was isolated as described under "Recovery of DNA from gels". 1 µl (25 ng/µl) of a solution containing the vector was combined with the fragment (ratio of vector: fragment 1:2 based on the number of molecules),. 2 µl of T4-ligation buffer (5 x (50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 5% (w/v) polyethylene glycol-8000)) 0.5 µl of T4-DNA ligase (BRL) and H_2O up to 10 µl. The mixture was incubated for 20 h at 14 °C if the ligated DNA fragments had sticky ends. If the DNA had blunt ends, then 1 µl of T4-DNA ligase was added and the incubation was for 1 h at room temperature. The ligation mix was stored at -20 °C if not used immediately. Usually only 5 µl of the ligation mix was used for transformation.

Preparation of competent cells

JM109 cells (or other cells, for example HB101) were inoculated in 4 ml L-Broth made to 10 mM $MgSO_4$ and 10 mM $MgCl_2$ (from 1 M $MgSO_4$, 1 M $MgCl_2$; autoclaved). The cells were grown overnight at 37 °C. 1 ml of the overnight culture was added to 40 ml of prewarmed (37 °C) LB medium (10 mM $MgSO_4$, 10 mM $MgCl_2$). The culture was shaken at 250-275 rpm. The cells were harvested from 30 ml of culture when the OD_{450} had reached 0.8 to 0.9 by centrifugation at 5000 rpm for 10 min at 4 °C. It was important that the OD was below 1 to ensure that the cells were aerated as much as possible. The pellet was very gently suspended in 30 ml of cold 0.1 M $CaCl_2$ (autoclaved) in a centrifuge tube, the tube being cooled by an ice-bath during the process, followed by centrifuging for 10 min at 5000 rpm and 4 °C. The pellet was suspended very gently in 15 ml of cold 0.1 M $CaCl_2$, kept on ice for 20 min and centrifuged for 10 min at 5000 rpm and 4 °C. The cells were gently suspended in 3 ml of cold 0.1 M $CaCl_2$ and kept for at least 1 h on ice before being ready for use (19).

Transformation

5 µl of ligation mix was combined with 95 µl of cold sterile TCM (10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 10 mM $MgCl_2$), and 200 µl of competent JM109 cells (or another type of cells). The mixture was allowed to stand for at least 40 min on ice, then 5 min at 37 °C (or 2 min at 42 °C). The mixture was then transferred to a sterile glass tube containing 0.8 ml of L-Broth, 10 mM $MgSO_4$, 10 mM $MgCl_2$ and incubated for 45 min at 37 °C with gentle shaking, then plated out (sterile technique) on 5 AXI plates (or other suitable plates, for example amp or tet) at 0.2 ml/plate. The plates were allowed to stand for 10 min before being inverted and incubated overnight at 37 °C. They were stored in plastic bags upside down at 4 °C.

Isolation and testing of subclones

1 to 6 recombinant clones (white on AXI plates when the vector is pBlueScript) from each plate were isolated and plasmid prepared from the clones were digested with the appropriate restriction enzyme(s) and run on a 2% agarose gel to ensure that the inserted fragment had the right size. If this was the case, then the clone was cultivated overnight in 4 ml of L-Broth, mixed with 25% sterile glycerol and stored at -80 °C. For sequencing of an inserted fragment, see "plasmid sequencing". For fragment isolation (probes) and nick translation, see the relevant sections (12, 20).

Screening of the λ-ZAP library

The litre of the amplified λ-ZAP library's titer was determined in duplicate prior to the screening. Infection competent BB4 cells were prepared by inoculating the cells in 30 ml fresh L-Broth containing 300 µl of 20% maltose and cultivating them overnight at 37 °C. The cells were harvested at 10,000 rpm for 10 min and 4 °C and resuspended gently in cold sterile 10 mM $MgSO_4$ (30 ml), and kept on ice until use. 100 µl of λ-ZAP phages, diluted in ϕ-buffer (22 mM KH_2PO_4, 49 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.001% of gelatine; autoclaved) were mixed with 0.2 ml of freshly made BB4 cells, incubated for 30 min. at room temperature, mixed with 2.5 ml warm (42 °C) 0.6% top agar containing 10 mM $MgCl_2$ and plated out on LB plates.

For screening of the library, 22 x 22 cm LB plates (dried for 3-4 h at 37 °C) were used. Each plate can carry about 1 x 10^5 λ-ZAP plaques, and they were mixed with 1 ml of BB4 cells (prepared as above) and allowed to stand for 30 min at room temperature. This mixture was then added to 25 ml of warm (42 °C)

0.3% top agarose + 10 mM $MgCl_2$ and the solution was poured onto a fresh dry LB plate. The large LB plates were incubated (not upside down) overnight at 37 °C. Phages from the plaques were transferred to nitrocellulose filters in duplicate. The plates were placed at 4 °C for 1 to 2 h to prevent the agarose layer from sticking to the nitrocellulose filters. Just before use, they were placed on ice and they remained on ice when working with the nitrocellulose. Two nitrocellulose filters (A and B) plus the plate were marked at three sites for orientation of the filters. Filter A was laid on the plaques for 45 sec; then floated on denaturation buffer (0.5 M NaOH, 1.5 M NaCl), with the phages facing up, for 45 sec, then floated on neutralization buffer (0.5 M Tris-HCl (pH 7.5), 3.0 M NaCl) for 5 min and finally floated on 2 x SSPE (1 x SSPE: 180 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4) for at least 2 min. The filter was air-dried and baked in a vacuum oven for 2 h at 80 °C. Filter B was laid on the same plate, after filter A, for 120 sec and then treated as filter A (12). These filters were used in the hybridization. X-ray film from both filters was orientated so that the signals from filter A fit the signals from filter B. The positive plaques were cut out with a scalpel (1 x 1 cm blocks) and submerged in 1 ml of ϕ-buffer (shaking the tube before use). All tubes with phages in ϕ-buffer were stored airtight (parafilm) at 4 °C after 2-3 drops of chloroform had been added. The plaque plates (22 x 22 cm) were stored by placing a piece of soaked (chloroform) Wattman filterpaper in the lid, then making the plates airtight (parafilm) and letting them stand with the plaques facing up at 4 °C. To isolate a positive plaque from the 1 x 1 cm block (containing about 2000 plaques), dilutions in ϕ-buffer were made and plated with BB4 cells on round fresh LB plates (8.5 cm diameter) with 2.5 ml of 1% warm (42 °C) top-agar + 10 mM $MgCl_2$. The dilution which gave 1500-3000 plaques was used to make the nitrocellulose filter prints. The method was exactly as described for the large (22 x 22 cm) LB plates, except that the filters (A and B) were marked by sticking a syringe needle through the filter down into the plate. After hybridization to the round filters and autoradiography, the positive signals form A and B were matched. The plaques were isolated by sticking the tip of a pasteur pipette through the plate and releasing the plaques (by blowing the other end) in 1 ml of ϕ-buffer. Again, dilutions were made, and a dilution with 150-300 plaques was used to make the nitrocellulose prints. The procedure was the same as described for the dilution with the 1500-3000 plaques. The positive plaques were isolated and diluted, and the dilution giving 15-30 plaques was used to make nitrocellulose prints. At this point, all plaques on the filters (A and B) generally hybridized, showing that the isolated phages were pure. If this was not the case, another round was necessary, after which the phages were pure. The phages were then ready for the "Excision Protocol for λ-ZAP phages".

Excision Protocol for λ-ZAP phages

XL1-Blue cells were inoculated in 4 ml of fresh LB medium for an overnight culture (gently shaken at 37 °C).

0.5 ml of the overnight culture was added to 25 ml of fresh LB medium and placed, with continuous shaking, at 37 °C until OD_{450} = 0.5. To a prewarmed sterile glass tube was added:
100 µl of XL1-Blue cells OD = 0.5
100 µl of a clean λ-ZAP phage stock (containing > 1 x 10^5 phage particles.
5 µl of R408 helper phage (1 x 10^6 pfu/ml)
2 µl of 1 M sterile $MgSO_4$.

The tube was held for 15 min at 37 °C. 2.5 ml of fresh LB medium was added, and the tube was shaken vigorously for 4 h at 37 °C. The tube was then heated for 20 min. at 70 °C before transferring 1.5 ml to an autoclaved Eppendorf tube. The tube was given a short spin, after which the supernatant was transferred to a new autoclaved Eppendorf tube. The rescued phages can be stored at this point for 1 to 2 months at 4 °C. To transfer the packaged, rescued plasmid to cells, the following procedure was followed: XL1-Blue cells were inoculated in 4 ml of fresh LB medium for an overnight culture and cultivated as described above. Two sterile glass tubes containing 100 µl of XL1-Blue cells OD = 0.5 were prepared. To the first glass tube, 200 µl of the packaged plasmid was added and to the second glass tube 2 µl were added. The tubes were allowed to stand at 37 °C for 15 min. 100 µl from each glass tube was transferred to a new sterile glass tube containing 100 µl of fresh LB medium. The 200 µl was plated out on a dry (dried at 37 °C for 20 min) LB-ampicillin plate and incubated upside down overnight at 37 °C (31).

The colonies appearing on the plates contained the pBlue Script plasmid with the inserted cDNA. The colonies from these plates were grown in LB medium containing 35 mg of ampicillin per litre.

DNA sequencing

The plasmid (double stranded template) to be sequenced was purified by the plasmid small scale preparation method. The DNA was denatured in 0.2 M NaOH (5 min at room temperature), the mixture was neutralized by adding 0.4 vol of 5 M ammonium acetate (pH 7.5) and then precipitated with 4 vol. of cold ethanol (5 min at -80 °C). The pellet was rinsed with 70% cold ethanol and resuspended in 10 µl H_2O. Two reactions were set up for each plasmid, one for each primer that was used. A 17-mer sequencing primer #1212 (Biolabs) and reverse sequencing primer #1201 (Biolabs) were used.

Sequencing was accomplished with a Sequenase™ DNA Sequencing Kit from United States Biochemical Corp., following the sequencing Protocol enclosed in the kit (22).

EXAMPLE 1

Amylase activity in potato tissue

It is desirable to know in which tissue amylase is active, since presumably amylase mRNA is present in such tissue. Growing tuber sprouts demand nutrition (such as sucrose) from the tuber, and starch is broken down to accommodate this demand. Different tissues from sprouting potatoes were therefore tested for amylase activity. Thin slices of sprouting tubers (Dianella, Saturna) were placed in I_2/kl solution. The periderm (cells making up the tuber skin), the vascular tissue and the sprouts were not coloured, while the rest of the tuber tissue (parenchyma, storage cells) was coloured. While this test indicates where starch is present (dark blue colour) and where starch is absent (the colourless areas), it does not show where amylase is present. There was no visible difference in the colour pattern of Saturna and Dianella potatoes.

Thin slices of the sprouts were placed on a 1% starch plate, and incubated for 1 h at 37 °C with no visible effect. For comparison, mouse urine spotted on a 1% starch plate gives a clear spot after incubation for 20 min at 37 °C (23). Although the slices were very thin, the distance the enzyme has to travel to come into contact with the substrate may still be too long. On the basis of the colouring pattern seen for the sprouting tubers, it was decided to make extracts of the different regions indicated in Fig. 6.

The extracts were prepared as described above ("Materials and Methods") from both Dianella and Saturna. After incubation at 20 °C for 16 h, only Dianella sprouts showed a weak reaction (orange spot). Another set of extracts was incubated at 37 °C and here Dianella sprouts showed a strong reaction (clear spot), demonstrating the presence of amylase in these sprouts. Saturna sprouts showed a weak reaction after incubation at 37 °C, weaker than the reaction seen for Dianella sprouts at 20 °C. The clear difference observed in the amylase level of Dianella and Saturna sprouts is correlated with the sugar level seen in these varieties (cf. Example 20 and Fig. 22). In order to obtain a stronger reaction from Saturna sprouts, 10 g were frozen (-70 °C for 5 min, -20 °C for 1h) before grinding in 5 ml extraction buffer (see Materials and Methods). After incubation at 37 °C, the spot was pink-red, but the reaction was still not as evident as the reaction seen with the 2 g of fresh Dianella sprouts.

The rest of the tissue extracts (B, C and D from both Dianella and Saturna) showed a darker blue colour than the surroundings (on the 1% starch plate) after staining with I_2/kl solution. There is thus more starch in these spots than in the surroundings. The parenchym tissue (D on the drawing) from both varieties was also tested after having been frozen, and the spots were still darker than the surroundings. The frozen parenchym tissue was then ground without extraction buffer and the resulting potato juice (after centrifugation to remove debris) was tested. A very weak reaction was seen after incubation at 37 °C for juice from both Saturna and Dianella. This reaction may be due to there being so much substrate (i.e. starch) for the amylase in the parenchym tissue that the enzyme needs much more time to degrade the starch at the plate. A maximum incubation time of about 16 h was used, since the plates curl up if they are incubated much longer than 16 h at 37 °C.

On the basis of the above, if may be concluded that amylase activity is present in both sprouts and tuber parenchym tissue of Saturna and Dianella potatoes. The highest activity is seen with Dianella sprouts.

EXAMPLE 2

Preparation of barley α-amylase probes

Two plasmids (050 and 036), each of which codes for a barley α-amylase, were provided by John C. Rogers of Washington University Medical Center, St. Louis, USA. The two plasmids were originally constructed from cDNA derived from polyA-rich RNA isolated, after hormone induction, from the aleurone cell layer of barley grains. Plasmid 050 codes for a type A (= clone E) (27) α-amylase, and plasmid 036 codes for a type B (= pM/C) (24) α-amylase. According to information received with the plasmids p050 contains the barley PstI insert from clone E inserted into the PstI site in the polylinker of the vector pSP64 (26). To increase plasmid DNA yields the same PstI fragment was inserted into the vector pBS- (31). This new clone pBS050 thus contains the identical barley insert cloned in clone E. According to information received, plasmid 036 contains a BamHI-HindIII fragment from clone pM/C inserted into the polylinker of the vector pSP64 (Fig. 7). The complete sequence of the fragment has not been published, and as shown in Example 3, the fragment includes sequences that give rise to a strong false hybridization signal. In heterologous hybridizations at low stringency ("Materials and Methods") the signal to noise ratio can be increased if the heterologous probe only includes sequences that will hybridize in control experiments (Example 3). The barley insert in p036 contains two SacI sites that divide the coding region and cut near the termination codon (positions 599 and 1388 in Fig. 2 of reference 24). The 5' leader sequence and the start of the coding region encoding the signal peptide of the barley α-amylase was not expected to be conserved between barley and potato, and a HinfI site at position 152 in the above-mentioned figure was used to remove the first 152 nucleotides from the barley probe as follows. The HinfI fragment shown in Fig. 7, in which the righthand site is in the vector pSP64, was subcloned in the vector pBS- and from the recombinants one was chosen in which the fragment cloned has the orientation shown in Fig. 7. This plasmid, pBS036, liberates after digestion with SacI two fragments containing essentially only regions coding for mature barley α-amylase. In addition, pBS036 gives a better yield of plasmid DNA than 036.

EXAMPLE 3

Detection of hybridization between barley α-amylase gene sequences and potato nucleotide sequences

PolyA-rich DNA prepared from Dianella sprouts as described under "Materials and Methods" was selected since this tissue was found to have the highest α-amylase activity (Example 1). In order to elucidate whether the complex mixture of messengers in the polyA RNA preparation contains sequences that will hybridize with the barley α-amylase sequences, plasmids pBS050 and pBS036 were hybridized with radioactive copy DNA synthesized from the polyA-rich RNA, using a method similar to first strand synthesis for cDNA cloning as described in "Materials and Methods". The hybridization conditions (hybridization at 67°C in 6 x SSC, wash at 67°C in 4 x SSC) were selected based on previous experience with heterologous hybridizations in complex mixtures of molecules (28, 29).

The result of the experiment is shown in Fig. 8. It shows that the potato copy DNA does hybridize specifically with α-amylase coding regions from barley. In particular, the 800 bp SacI fragment from pBS036 that encodes the C-terminal part of barley α-amylase type B hybridizes well, whereas the 350 bp SacI fragment encoding the N-terminal part does not hybridize. That the hybridization is specific is indicated by the lack of hybridization of a similar size DNA fragment from a control plasmid (lane 1, Fig. 8). The barley insert from plasmid pBS050 encoding α-amylase type A also hybridizes, albeit weakly, with the potato copy DNA. As a positive control for the hybridization a plasmid encoding a highly conserved, ubiquitous protein, ubiquitin, was used, and as expected it hybridizes with the potato copy DNA. The intensity of the hybridization to the ubiquitin coding fragment, the 800 bp SacI fragment from pBS036, and the Pst insert from pBS050 is weak compared with the hybridization of two strong bands seen in lanes containing pBS036, and it was concluded that the strong bands are due to hybridization of the copy DNA to the small region indicated by a star in Fig. 7. The sequence of the region is not known but it is proposed that the strong hybridization is the result of a chance homology with ribosomal RNA sequences (found in even the most purified polyA-rich RNA). This suggestion is supported by the finding that the barley copy DNA gives a similar strong hybridization.

The results of the experiment show that 1) Dianella sprout polyA-rich RNA contains sequences that hybridize specifically with barley α-amylase coding sequences, 2) the 800 bp SacI fragment from pBS036 is the most suitable probe, and 3) the hybridization conditions are sufficiently permissive to detect hybridization without creating an excessive background hybridization. It was concluded that the 800 bp SacI fragment (the probe in Fig. 7) could be used to screen a cDNA library made from polyA-rich RNA from Dianella sprouts with hybridization at 67°C in 6 x SSC and washing at 67°C in 4 x SSC.

EXAMPLE 4

Construction of a potato sprout cDNA library

PolyA-mRNA was prepared from Dianella sprouts as described under "Materials and Methods". The quality of the polyA-mRNA was tested by synthesizing a small portion into cDNA as described above.

The polyA-mRNA was cloned into the vector λ-ZAP with the aid of EcoRI linkers (30).

λ-ZAP is a hybrid vector consisting of a λ vector with the pBlue Script SK(-) plasmid inserted. λ-ZAP has six unique cloning sites that can accommodate inserts from 0-10 Kb in length. It has the ability to automatically excise the inserted region with a helper phage and to circularize it, forming the pBlueScript SK(-) plasmid. The inserted cDNA is situated in the polylinker of the pBlueScript SK(-)plasmid (31). The estimated titer of the primary library was approximately 7.0 x 10^6 pfu (plaque forming units), of which approximately 9% were non-recombinants. The stock of the unamplified library was 8.0 x 10^5 pfu/ml and the stock of the amplified library (amplified 10^6 pfu from the primary library) was 4.0 x 10^{10} pfu/ml.

Preparation of the Sac 800 probe

3 x 10 µg of very pure (CsCl-banded) pBS 036 (Fig. 7) plasmid was digested with SacI and run on a 5% acrylamide gel. pBR327 digested with BstNI was size marker giving 3 bands: 1855 bp, 928 bp and 475 bp. The Sac 800 band was isolated, suspended in 30 µl H_2O and stored at -20 °C. Each time a double nick was made (i.e. using double the amount of the ingredients), 5 µl of the store probe was used.

EXAMPLE 5

Isolation of α-amylase cDNA clones from potato

One set of nitrocellulose filters (3A and 3B, with 1 x 10^5 plaques) was hybridized at low stringency (hybridized at 6 x SSC and washed at 4 x SSC as described above) with approximately 1.7 x 10^8 cpm of the radioactive Sac800 probe. Another set of nitrocellulose filters (2A and 2B, with 1 x 10^5 plaques) was hybridized at a somewhat higher stringency (hybridization and washing at 2 x SSC), again with approximately 1.7 x 10^8 cpm of the radioactive Sac 800 probe. After 3 hours of autoradiography with two intensifying screens, the X-ray film from filters 3A and 3B (the low stringency filter) showed positive signals, while the X-ray film from filters 2A and 2B (the higher stringency filters) showed no signals. Even after 18 hours of autoradiography of 2A and 2B, they still did not show positive signals.

Among about 100,000 clones, eight positive signals from filters 3A and 3B were found and an additional positive signal was found on filter 3A in a region where the two filters did not overlap. The frequency of α-amylase cDNA clones in the library gives an indication of the prevalence of α-amylase messenger RNA in total Dianella sprout messenger RNA. The frequency was 8 x 10^{-5}, whereby the α-amylase mRNA was fairly rare, constituting approximately 0.008% of the total mRNA. All nine signals were located on the original L-Broth plate (22 x 22 cm) and isolated therefrom (Step 1) (see "Materials and Methods"). They were given the names ZAP1 and ZAP9. They were plated out, after serial dilutions, and two duplicate replicas (A, B) were made from each plate (with 1500-3000 plaques per plate). The nitrocellulose filters were hybridized at low stringency with the radioactive sac800 fragment (1.5 x 10^7 cpm to 3.0 x 10^7 cpm added to each bag with 4 or 6 round filters, the filters always being in pairs in one bag, i.e. filters A and B from the same plate). ZAP2, 3, 4, 5, 6 and 7 showed positive signals (one to many candidates) and they were serially diluted from this step (Step 2). Zap1, 8 and 9 had been too much diluted too much and showed no signals; they were therefore serially diluted again from the original isolate (step 1). An appropriate dilution, 200-500 plaques for ZAP2, 3, 4, 5, 6, and 7 and 1500-3000 plaques for ZAP 1, 8 and 9, was used to make replicas of A and B. The resulting nitrocellulose filters were hybridized with the radioactive Sac800 fragment as described above.

ZAP1 and ZAP8 showed positive signals at this point (step 2), but ZAP 9 showed no positive signals and was not further investigated. It was possible to isolate a single positive plaque from the plates with ZAP2, 3, 4, 5, 6 and 7, and they were at this point ready for excision from the plasmid.

ZAP1 and ZAP8 were serially diluted (step 2) and an appropriate dilution from each was used to make replicas. ZAP2, 3, 4, 5, 6 and 7 were also diluted, plates with 20-50 plaques being used for replicas. This step (step 3) was performed as an extra control of these phages. All filters were hybridized with the radioactive Sac800 fragment at low stringency. ZAP1 and ZAP8 showed positive signals and it was possible to isolate a single positive plaque from each. ZAP1 gave a weaker signal throughout the isolation process than the others; Nevertheless, it was present throughout the entire process.

ZAP1 and ZAP8 were also plated for control hybridization of purity. All eight phages ZAP1-8 were pure, since all plaques on the plates hybridized. The plasmids from each of ZAP1 to ZAP8 were excised following the excision protocol under "Materials and Method".

A large number of ampicillin-resistant colonies were obtained and two colonies from each of ZAP1-8 were grown overnight for plasmid preparation. The plasmids were named AmyZ1 to AmyZ8. The colonies all contained plasmids. Plasmids AmyZ2-7 were digested with EcoRI and run on a 2% agarose gel with pBR327 digested with either BstNI or HinfI as size markers (Fig. 9). The gel also contained SacI digested pBS036 (Fig. 7). The gel was used for southern transfer as described in "Materials and Methods" and the nitrocellulose filter was hybridized at low stringency with the radioactive Sac800 fragment (1 x 10^6 cpm added). All plasmids showed at least one positive fragment (Fig. 9), confirming that the correct cDNA-clones had been isolated. AmyZ2, 3, 4, 5, 6 and 7, and subsequently AmyZ1 and 8, have all been frozen in 25% sterile glycerol for prolonged storage (-80 °C).

EXAMPLE 6

Mapping of the α-amylase cDNA clones and DNA sequence analysis

AmyZ2, 3, 4, 5, 6 and 7 were digested with EcoRI and run on a 5% acrylamide gel, yielding the same fragments seen in Fig. 9. AmyZ1 and 7 were treated the same way and the resulting EcoRI fragments from all eight clones are illustrated in Fig. 10.

The EcoRI fragments as well as fragments obtained by digestion with other restriction enzymes were isolated and subcloned in the pBS-vector (see "Materials and Methods"). The fragments were cloned into the polylinker region situated between the sequence priming sites. The subclones were tested for correct insert size and frozen in 25% sterile glycerol for prolonged storage.

The strategy for sequencing the potato inserts of AmyZ3 and Z4 is shown in Fig. 11, the strategy for the inserts of AmyZ1 and AmyZ6 is shown in Fig. 12, and the strategy for sequencing AmyZ2 and AmyZ7 is shown in Fig. 13. EcoRI fragments from AmyZ5 and AmyZ8 were also sequenced and the results showed that these two clones are identical to AmyZ7.

The nucleotide sequences were analyzed with the aid of Beckman Micro-Genie Sequence Software, and the sequences of the potato inserts are shown in Figs. 1-5 as follows: Fig. 1 = AmyZ3/4, Fig. 2 = AmyZ7, Fig. 3 = AmyZ1, Fig. 4 = AmyZ6 and Fig. 5 = AmyZ2.

EXAMPLE 7

At least five α-amylase genes of two dissimilar types are active in potato sprout tissue

The cDNA in AmyZ1-8 contains copies of α-amylase messenger RNAs from potato sprout tissue and are thus copies of products of potato α-amylase genes. The sequences shown in Fig. 1-5 are all different and are therefore products of different genes. This shows that at least five different genes are active in potato sprouts from the variety Dianella. Pairwise comparisons of the five sequences from Fig. 1-5 show that they fall into two groups as illustrated in the following table. The sequences were aligned and the table shows the percentage homology between pairs of sequences to the extent that they overlap. Gaps were introduced to optimize the alignments and the gaps were included in the total length for the calculation of the homoglogy.

% Nucleotide Sequence Homology				
	AmyZ1	AmyZ6	AmyZ3/4	AmyZ7
AmyZ6	91,1			
AmyZ3/4	55,5	55,4		
AmyZ7	55,5	55,4	98,9	
AmyZ2P	60,1	59,2	98,5	99,2

It is seen that the sequences of AmyZ3/4, AmyZ7 and AmyZ2, *including the 3' untranslated regions*, are very homologous indeed. This finding suggests that the three corresponding genes are alleles of the same gene, which is possible because the potato variety used (as well as other commercial potato varieties) are tetraploid. In the following, the clones/sequences AmyZ3/4, AmyZ7 and AmyZ2 will be referred to as the AmyZ3/4 type. AmyZ1 and AmyZ6 are likewise very homologous, but in this case the homology is slightly lower and the differences between the genes are concentrated in 3' untranslated regions. AmyZ1 and AmyZ6 may be the products of alleles of one gene but they may also be different genes belonging to a sub-gene family. In the following, the clones/sequences AmyZ1 and AmyZ6 will be referred to as the AmyZ1 *type*. From the above table it can be seen that the homology between the nucleotide sequences of any of the AmyZ3/4 type and either of the AmyZ1 type is low, 55-60%, and the result shows that two distinctly different types of potato α-amylase cDNA clones have been isolated.

Nucleotide sequences are characterized by an additional parameter, the nucleotide composition. This parameter, usually expressed as the CG content, is particularly useful for discriminating between coding regions and non-coding regions. It has been found that coding regions have CG contents 15-20% higher than non-coding regions (Salinas, J., Matassi, G., Montero, L.M., and Bernardi, G. (1988), *Compositional compartmentalization and compositional patterns in the nuclear genomes of plants, Nucleic Acids Res. 16*, pp. 4269-4285) and this information is helpful in locating introns and exons in genes. Moreover, the coding regions (and genomes) of different plant species have different CG contents and there is a relatively sharp division between monocotyledon plants and dicotyledon plants. The CG content of the coding regions of the potato α-amylase cDNAs is in all cases below 50% whereas the CG content of cereal α-amylase coding regions is above 50%. The examples indicate that α-amylase coding regions from other plants may be allocated to the monocotyledon or dicotyledon group of the angiosperms on the basis of the CG content.

EXAMPLE 8

Nucleotide sequence of potato α-amylase AmyZ3/4 messenger including leader, main coding region, and 3' untranslated region

Fig. 1 shows the sequence of the messenger RNA-like strand of the combined inserts of potato α-amylase cDNA clones AmyZ3 and AmyZ4, and Fig. 11 gives a summary of the structure of the two clones AmyZ3 and AmyZ4 have identical nucleotide sequences in the regions where they overlap (compare Fig. 11), except that AmyZ3 has an intron sequence of 128 nucleotides in the 5' leader sequence (sometimes called the 5' untranslated region). The intron terminates in consensus 5' and 3' splice junction nucleotides, GT and AG, respectively, and the intron contains a consensus branch point. Consensus splice junctions and branch points for plant introns have been compiled by Brown (32). The nucleotide sequence of the 3' leader up to nucleotide 540 contains four open reading frames shown in detail in Fig. 17. The region encoding the α-amylase precursor starts at nucleotide 541 and terminates at nucleotide 1761, and the derived length of the α-amylase precursor is 407 amino acids. The 3' untranslated region is at least 200 nucleotides in length, not including the polyA tail, but probably not much longer since a putative polyA signal is found 30 nucleotides from the end. PolyA signals are extremely well conserved in animals in which practically all genes have AATAAA about 20 base pairs from the polyadenylation site (33), but are quite degenerate in plants, although AT-rich (34).

EXAMPLE 9

Special features of the nucleotide sequences of the potato α-amylase AmyZ3/4 messenger RNA

Fig. 11 illustrates the unusual structure of the potato α-amylase AmyZ3/4 messengers: they have very long 5' leader regions containing open reading frames (Fig. 17). In addition, two different types of transcripts from the same gene have been isolated in the AmyZ3 and AmyZ4 clones, one containing an intron in the leader region. Normally, unspliced transcripts constitute a very minor fraction of PolyA RNA, and the fact that an unspliced transcript has been isolated (in a cDNA clone) indicates that they are fairly abundant and may have a specific function. In other systems such introns have been found to have a stabilizing effect on the messenger (Callis, J., Fromm, M. and Walbot, V. (1987), *Genes and Development 1*, pp. 1183-1200; Huang, M.T.F. and Gorman, C.M. (1990); *Nucleic Acids Res. 18*, pp. 937-94). The amount of protein produced from the messenger may thus be regulated by the degree to which the leader intron is spliced out. Long leaders with open reading frames have also recently been found in Pea phytochrome transcripts (47). It is not known whether the reading frames in the potato α-amylase or the pea phytochrome 5' leaders are actually translated in the plants. This might be tested by raising antibodies against artificial peptides having the sequences shown in Fig. 17 and reacting such antibodies with plant cell extracts.

EXAMPLE 10

The nucleotide sequences of potato α-amylase AmyZ7 and AmyZ2 messengers

Fig. 2 shows the nucleotide sequence of the messenger RNA-like strand of the insert of potato α-amylase cDNA clone AmyZ7. The sequence is very homologous to the sequence of AmyZ3/4, but the clone is shorter in the 5' end and the first nucleotide in Fig. 2 aligns with nucleotide 543 in Fig. 1, which is the third nucleotide of the initiation codon. The alignment continues without gaps beyond the translation stop codon, and the α-amylase precursor encoded by AmyZ7 is 407 amino acids long as for AmyZ3/4. The 3' untranslated region is 187 nucleotides long followed by a polyA tail nine residues long.

Fig. 5 shows the nucleotide sequence of the messenger RNA-like strand of the insert of potato α-amylase cDNA clone AmyZ2. The sequence is denoted AmyZ2P to indicate that a string of 76 T residues erroneously inserted during the cloning in the 5' end is not included in Fig. 5. The sequence is very homologous to the sequence of AmyZ3/4, but the clone is shorter both in the 5' and 3' end. The first nucleotide in Fig. 5 aligns with nucleotide 1023 in Fig. 1, and the last nucleotide in Fig. 5 aligns with nucleotide 1756 in Fig. 1. unexpectedly, the alignment requires that two gaps are introduced into the AmyZ2 sequence (shown as blank spaces in Fig. 5). It follows that the α-amylase open reading frame is interrupted and AmyZ2 does not encode a partial potato α-amylase. AmyZ2 has two deletions relative to AmyZ3/4; one deletion could have been ascribed to a mistake made by the enzymes during the synthesis of the double-stranded cDNA in the cloning process, but two such mistakes in one clone is very unlikely and it may be concluded that the gene corresponding to AmyZ2 has suffered at least two deletions in the coding region. In other words, the AmyZ2 has undergone fatal mutations and has become a pseudo-gene.

. During further evolution such genes suffer more mutations, against which there is no selection, and in time, the gene will also lose the ability to be transcribed. Thus, it is relatively rare that pseudo-genes are active. Although AmyZ2 no longer encodes a partial potato α-amylase, its isolation contributes to the elucidation of the structure of potato α-amylase genes and gene families and it can be employed in hybridization studies in the same way as AmyZ3/4 and AmyZ7 or fragments thereof.

EXAMPLE 11

Nucleotide sequences of potato α-amylase AmyZ1 and AmyZ6 messengers

Fig. 3 shows the sequence of the messenger RNA-like strand of potato α-amylase cDNA clone AmyZ1. The sequence is significantly different from the AmyZ3/4 type (Example 7), but the first nucleotide in Fig. 3 aligns approximately with nucleotide 740 in Fig. 1. The first codon in frame with the α-amylase reading frame is a stop codon, but this stop codon is so close to the cloning site that it is ascribed to a cloning error and it is not likely that AmyZ1 is the product of a pseudo-gene as described for AmyZ2 in Example 10. The α-amylase open reading frame is 350 codons long and the sequence terminates with a 163 nucleotides long 3' untranslated region that does not have a polyA tail.

Fig. 4 shows the sequence of the messenger RNA-like strand of potato α-amylase cDNA clone AmyZ6. It is 91% homologous to the sequence of AmyZ1, but half of the nucleotide differences between AmyZ1 and AmyZ6 are concentrated in the region downstream from the stop docon in AmyZ1. AmyZ6 is shorter than AmyZ1 in the 5' end; nucleotide 1 in Fig. 4 aligns with nucleotide 201 in Fig. 3. AmyZ6 has a one nucleotide deletion corresponding to position 816 in Fig. 3 with the result that the AmyZ6 α-amylase open reading frame is 66 codons shorter than the AmyZ1 open reading frame in the 3' end. This finding is further discussed in Example 14. The AmyZ6 sequence terminates with a 360 nucleotides long 3' untranslated region and since AmyZ6 and AmyZ1 are very homologous, the first part of the 3' untranslated region in AmyZ6 corresponds to the region in AmyZ1 that encodes the terminal 66 amino acids.

EXAMPLE 12

Borderline homology between potato and barley α-amylase sequences

Having determined the nucleotide sequence of potato α-amylase cDNA clones, the actual homology was determined between the SacI fragment of the barley α-amylase probe (Fig. 7) with which the isolation of corresponding potato cDNA clones was accomplished. In Fig. 10, the EcoRI fragments from AmyZ clones that hybridize with the barley probe are indicated. In Fig. 14, the nucleotide sequence of the hybridizing EcoRI fragment from AmyZ4 is aligned with the corresponding sequence of the barley probe. It is seen that the homology is only 63.5%. However, within these sequences there is a shorter region, 146 nucleotides long with a homology of 73% that includes a core of 46 nucleotides with 80% homology. The same type of comparison was carried out between the ExoRI fragment from AmyZ6 that hybridizes to the barley probe (Fig. 9 and Fig. 10). The overall homology is 66%, a shorter region of 110 nucleotides has 77% homology and a core of 62 nucleotides has 84% homology. Levels of homology of this magnitude have previously been determined to be just sufficient to allow detection of sequences in heterologous hybridizations at low stringency of hybridization (29 and 35). This analysis demonstrates the importance of the careful optimization of 1) the source of messenger used for the potato cDNA library, 2) the conditions of hybridization, and 3) the limitation of the length of the barley probe to the subfragment that includes the regions of sufficient homology for the isolation of the potato α-amylase clones.

EXAMPLE 13

Properties of the α-amylase precursor encoded by AmyZ3, AmyZ4 and AmyZ7

The nucleotide sequence shown in Fig. 1 contains one long open reading frame of 407 codons and the derived amino acid sequence is shown below the nucleotide sequence. To confirm the identity of the potato α-amylase clone, the derived amino acid sequence was compared with the sequence of the barley α-amylase derived from the sequence of pM/C (24) and the comparison is shown in Fig. 15. The percentage of identical amino acid is 45.6 and at 72 positions similar amino acids are found. This degree of similarity is significant, in spite of a number of gaps introduced to maximize the similarity, and indicates that the two sequences have a common evolutionary origin (36). The plasmids AmyZ3 and AmyZ4 are therefore potato α-amylase cDNA clones. Furthermore, the peptide indicated by a box in Fig. 15 is even conserved in α-amylases found in mammals and insects (see 55).

The nucleotide sequence shown in Fig. 2, which is 99.2% homologous to the sequence shown in Fig. 1 in the region where they overlap, like AmyZ3/4 encodes a 407 amino acids long peptide (except that the first two nucleotides of the initiation codon are lacking in AmyZ7). The sequences of the α-amylase precursors encoded by AmyZ3/4 and AmyZ7 differ at three residues and in each case similar amino acids are found: position 37 has leucine to isoleucine, position 333 has phenylalanine to tyrosine and position 385 has valine to methionine.

α-Amylase is synthesized as a precursor in barley and wheat with a so-called signal peptide in the N-terminal of the peptide. Signal peptides mediate the transport across membranes and have common properties which are partly conserved between prokaryotes and eukaryotes (37). They are 15 to 30 amino acids long and include a hydrophobic region near the middle. The residues before and just after the processing site have a distinct pattern used to predict the processing site in precursors in which it has not been determined experimentally (38). To illustrate the structure of the complete α-amylase precursor as well as of the signal peptide, a hydrophilicity profile was calculated for the peptide (Fig. 18). The figure shows the short hydrophobic region near the N-terminal of the precursor and the residues are indicated. The most likely processing site is indicated, the site being found using the rules proposed by von Heijne: the glycine at position -1 is most significant, but the arginine at position -3 is unusual. The hydrophobic region is short compared with other eukaryotic signal peptides, and short hydrophobic regions are more often found in prokaryotic signal peptides. It is concluded that the potato α-amylase precursor starts with a signal peptide and that the probable processing site is after glycine 18. However, the structure of the signal peptide is atypical and may signify a special transport mechanism. The final location of the mature enzyme is not known precisely in potato sprouts or in cereals.

The hydrophilicity profile of the mature α-amylase (Fig. 18) does not show pronounced hydrophobic or hydrophilic regions and the analysis predicts that the enzyme is soluble.

The amino acid composition of the mature α-amylase encoded by AmyZ3 and AmyZ4 is compared with the composition of the two types of barley α-amylase in the following table:

α-AMYLASES: AMINO ACID COMPOSITION (%)			
Amino Acid	Potato	Barley A (low pI) (Amy2)	Barley B (high pI) (Amy1)
Ala	6.7	11.4	8.7
Arg	4.9	3.6	4.5
Asn	4.6	4.1	4.0
Asp	6.9	8.9	9.2
Cys	0.8	1.0	0.7
Gln	4.1	2.9	3.2
Glu	4.1	3.1	4.0
Gly	8.2	10.6	10.9
His	3.9	2.9	4.0
Ile	6.4	5.6	6.2
Leu	6.4	6.0	7.2
Lys	6.2	5.3	5.9
Met	1.8	2.9	1.5
Phe	4.4	3.6	4.0
Pro	4.4	4.1	4.7
Ser	7.5	5.1	3.2
Thr	5.1	4.1	4.2
Trp	4.1	3.9	4.0
Tyr	4.4	3.9	3.7
Val	5.1	6.8	6.2
Mw	44397	45288	45083
Acidic (D,E)	11.1	12.1	13.1
Basic (R,K)	11.1	8.9	10.4
Aromatic (F,W,Y)	12.9	11.4	11.6
Hydrophobic (F,W,Y,I,L,M,V)	32.6	32.6	32.7
Net charge at pH 7.0	0	-13	-11

The barley (and wheat) amylases are acidic (pIs 4.9-6.0) and acidic to neutral (pIs 6.3-7.5). In the newer nomenclature, the higher pI types are preferably called Amy1 and the lower pI types are called Amy2. The potato α-amylase type AmyZ3/4 is neutral, and as described in Example 15, the sequence is equally different from the cereal type 1 and type 2 sequences.

The amino acid sequences are in all cases deduced from nucleotide sequences. The N-terminal amino acid of the mature peptides have been determined in the case of barley amylase A, and is deduced from the structure of the precursor peptides in the two other cases.

EXAMPLE 14

Properties of the partial α-amylase encoded by AmyZ1 and AmyZ6

The low nucleotide sequence homology between the potato AmyZ3/4 type and the AmyZ1 type (55-60%, Example 7) suggests that they encode distinctly different α-amylases. This is indeed the case: the amino acid sequence homology between the AmyZ3/4 α-amylase and the partial AmyZ1 α-amylase is only 45.9%. α-Amylase has been purified from potato (Fan, M.L., *Taiwania 1975, 20*, pp. 71-76), but it is not possible to deduce if the preparation was a mixture of the AmyZ3/4 types and the AmyZ1 types or only contained one type (which again presumably would be a mixture of the closely related α-amylases found for each type and represented in Figs. 1-2 and Figs. 3-4, respectively).

As shown in Figs. 3 and 4 and described in Example 11, the AmyZ6 α-amylase is 66 amino acids shorter in the C-terminal end than the AmyZ1 α-amylase. Variable lengths of the C-terminal region have also been found for barley α-amylases (Huang, J.-K., Swegle, M., Dandekar, A.M., and Muthukrishnan, S. (1984), *Plant Mol. Biol. 9*, pp. 3-17). This indicates that the C-terminal portion is not important for the catalytic function of the enzyme, but the variations in length may well influence other properties of the enzymes, such as specific activity, temperature dependency and pH optimum.

Fig. 16 shows a comparison of the amino acid sequences decoded from barley α-amylase clone pM/C and potato α-amylase clone Amy21. The numbering of the amino acids refers to the barley α-amylase precursor. Despite the finding that the local *nucleotide* sequence homology between the pM/C sequence and the AmyZ3/4 and Amy Z1 type sequences, respectively, are not significantly different (Example 12), the amino acid sequence homology between the barley pM/C α-amylase and the potato AmyZ1 α-amylase is 64.1% and thus significantly greater than between the barley α-amylase and the potato AmyZ3/4 type α-amylases (45.6%, Fig. 15). However, like AmyZ3/4 α-amylase, the AmyZ1 α-amylase is preferably not homologous with one of the types of cereal α-amylases (Example 15).

About 87% of the complete mature amino acid sequence of potato α-amylase type AmyZ1 is shown in Fig. 3, and a comparison of this peptide with the corresponding peptide in AmyZ3/4 α-amylase indicates that the AmyZ1 type α-amylase is more acidic than the AmyZ3/4 α-amylase but less acidic than the two barley α-amylases listed in the table in Example 13. Thus, the charge of the partial α-amylase shown in Fig. 3 is approximately -6 at pH 7.

EXAMPLE 15

A survey of cereal α-amylase gene sequences and comparisons of the deduced amino acid sequences and their relationship to the potato α-amylases

In the following table, the plant α-amylase gene/cDNA sequences found in the GenBank and EMBL sequence databases are listed with their types as far as deduced, the database identifications, the clone names, and the literature references.

Type	Name	Clone	Reference
Amy1	BLYAMY1 (HVAMY1)	amy1	Knox, C.A.P., Sonthayanon, B., Chandra, G. R. and Muthukrishnan, S. (1987) Plant Mol. Biol. 9, 3-17.
	BLYAMY1A (HVAMY1A)	p141.117	Knox, C.A.P., Sonthayanon, B., Chandra, G. R. and Muthukrishnan, S. (1987) Plant Mol. Biol. 9, 3-17. Muthukrishnan, S. (1988) Unpublished.
	BLYAMYABE (HVAMYABE)	pHV19	Chandler, P. M., Zwar, J. A., Jacobsen, J. V., Higgins, T. J. V. and Inglis, A. S. (1984) Plant Mol. Biol. 3, 407-418.
	BLYAMYABD (HVAMYABD)	pM/C	Rogers, J. C. (1985) J. Biol. Chem. 260, 3731-3738
	BLYAMYABA (HVAMYABA)	103	Huang,J.-K., Swegle, M., Dandekar, A. M. and Muthukrishnan, S. (1984) J. Mol. Appl. Genet 2, 579-588.
	BLYAMYABB (HVAMYABA)	168	
	BLYAMYABC (HVAMYABC)	96	
Amy2	BLYAMY2 (HVAMY2)	amy2	Knox, C.A.P., Sonthayanon, B., Chandra, G. R. and Muthukrishnan, S. (1987) Plant Mol. Biol. 9, 3-17.
	BLYAMYG (HVAMYG)	Lambda-amy32b	Rogers, J. C. and Milliman, C. (1984) J. Biol. Chem. 259, 12234-12240. Whittier, R. F., Dean, D. A. and Rogers, J. C. Nucl. Acid Res (1987).
	BLYAMYAA (HVAMYA)	E	Rogers, J. C. and Milliman, C. (1983) J. Biol. Chem. 258, 8169-8174.
	BLYAMY2A (HVAMY2A)	p155.3	Knox, C.A.P., Sonthayanon, B., Chandra, G. R. and Muthukrishnan, S. (1987) Plant Mol. Biol. 9, 3-17.
Amy1	M24286	M24286	O'Neill, S. D., Kumagai, M. H., Majumdar, A., Huang, N., Sutliff, T. D. Rodriguez, R. L. (1989) un published.
Amy3?	(OSAMYA)	M24941	Sutliff, T. D., Huang, N., Rodriguez, R. L. (1989) unpublished.
?	(OSAAMYB)	pOS137	O'Neill, S. D., Kumagai, M. H., Majumdar, A., Huang, N., Sutliff, T. D. Rodriguez, R. L. (1989) un published.
Amy1		Amy1/13	Baulcombe, D. C., Huttly, A. K., Martienssen, R. A., Barker, R. F. and Jarvis, M. G. (1987) Mol. Gen. Genet 209, 33-40.
Amy2		Amy2/54	
Amy3	WHTAMYA (TAMY3 or TAAMYA)	lambda-amy3/33	

The name is the one given the clone in the database: Gen Bank , compiled by the National Instituеs of Health. The The name in parentheses is the one given the clone in the database: EMBL, compiled by the European Molecular Biology laboratory. The first 11 clones has been isolated from barley (Hordeum vulgare), the next 3 are isolated from rice (Oryza sativa) and the last 3 has been isolated from wheat (Triticum aestivum).

The table shows that a number of α-amylase gene sequences have been determined in barley, wheat and rice. The cereals are, like all grasses, monocotyledon plants, whereas potato is a dictyledon plant - no α-amylase gene sequences from dicotyledon plants were in the databases.

All nucleotide sequences listed in the table were decoded and paired homologies of all α-amylase amino acid sequences were calculated. The amino acid sequences of the potato α-amylase shown in Fig. 1-4 were similarly compared to each other and to all the cereal amino acid sequences. The result is shown in the following table.

% AMINO ACID HOMOLOGY

BARLEY→

	BLYAMY1	BLYAMY1A	BLYAMYABE	BLYAMYABD	BLYAMY2	BLYAMYG	BLYAMYAA	BLYAMY2A
BLYAMY1A	**91.6**							
BLYAMYABE	**98.4**	**92.7**						
BLYAMYABD	**99.8**	**91.4**	**98.6**					
BLYAMY2	72.8	70.8	75.6	73.0				
BLYAMYG	72.8	70.1	76.1	73.0	**96.4**			
BLYAMYAA	73.0	71.0	75.9	73.2	**99.8**	**96.7**		
BLYAMY2A	69.7	62.2	73.4	69.9	**87.9**	**87.6**	**88.1**	
M24286	**81.8**	**80.5**	**85.0**	**82.1**	74.6	66.9	74.8	70.6
OSAMYA	70.0	68.6	72.5	70.3	67.8	66.9	67.8	63.3
OSAMYB	69.1	68.1	72.2	69.4	67.2	64.2	67.4	63.6
Amy1/13	**93.0**	**92.8**	**94.5**	**93.2**	73.4	73.3	73.6	69.3
Amy2/54	72.2	70.3	75.3	72.5	**94.3**	**93.3**	**94.5**	**84.9**
WHTAMYA	62.3	63.3	67.8	66.0	62.6	63.0	64.0	60.4
AmyZ1	64.1	62.2	68.1	64.1	63.3	62.7	63.3	59.9
AmyZ6	67.1	65.1	68.6	67.1	67.8	67.3	67.8	61.5
AmyZ3/4	45.9	45.1	47.2	46.2	44.3	45.7	44.3	41.5
AmyZ7	45.9	45.1	47.5	46.2	44.3	45.7	44.3	41.5

	RICE →								
	M24286								
OSAMYA	**70.3**	OSAMYA							
OSAMYB	**67.8**	**75.8**	OSAAMYB	**WHEAT→**					
Amy1/13	82.7	69.8	69.7	Amy1/13					
Amy2/54	73.5	67.5	66.4	72.5	Amy2/54				
WHTAMYA	69.5	72.1	66.8	64.8	64.2	WHTAMYA		**POTATO →**	
AmyZ1	61.6	62.7	61.0	64.4	62.8	56.0	AmyZ1		
AmyZ6	64.1	68.3	68.3	67.1	67.8	59.5	**91.7**	AmyZ6	
AmyZ3/4	45.0	43.3	42.7	46.2	44.6	43.6	45.9	56.6	AmyZ3/4
AmyZ7	45.0	43.3	42.7	46.2	44.6	43.9	45.9	57.1	**99.3**

Among the many observations that can be made from the homology matrix, the following are particularly relevant for the potato α-amylases. Barley and wheat are closely related and in the table the figures in bold face show that barley and wheat have closely related sub-gene families that code for Amylase type 1 and type 2, respectively. In wheat a third type of α-amylase, Amylase type 3, has been found that has not yet been found in barley. Rice is more distantly related to barley and wheat than they are to each other and the assignment of the rice α-amylase to the three types of wheat α-amylases is less clearcut. Potato is very distantly related to wither the cereal plants and it is observed that the homology of each of the potato sequences is approximately the same to *all* the cereal sequences, and the potato α-amylases cannot on the basis of amino acid comparisons be categorized as belonging to any of the three cereal types of amylases. On the other hand, it is striking that the potato AmyZ1 type α-amylases are more homologous to the cereal α-amylases than to the potato AmyZ3/4 type α-amylases.

EXAMPLE 16

Arrangement of α-amylase genes in potato

DNA from potato was analyzed by Southern hybridizations (15) and two sets of experiments were carried out. In the first experiment (Fig. 19), separate EcoRI fragments from AmyZ3 were hybridized to DNA from potato variety Saturna and in this experiment the alleles/genes belonging to the AmyZ3/4 type of sequences were investigated. In the second experiment (Fig. 20), DNA from two potato varieties were hybridized separately with complete inserts from AmyZ3/4 and AmyZ1 type sequences. This experiment demonstrates that the two types of sequences do not cross hybridize at normal hybridization stringency due to the low sequence homology between the two types of sequences. In addition, the second experiment demonstrates DNA fragment polymorphisms of the α-amylase genes in different potato varieties.

In Fig. 19, DNA from potato variety Saturna was digested with EcoRI, HindIII, and BamHI, fractionated on agarose gels and the fragments transferred to nitrocellulose filters. One filter was hybridized to labelled EcoRI fragments No. 2, 3, and 4 from AmyZ3 (compare Fig. 10) and another, identical filter was hybridized to EcoRI fragment No. 1. The EcoRI patterns show four strong bands (approximately 6.0, 4.0, 2.2 and 1.5 kb) and six weaker bands (approximately 7.7, 6.8, 4.5, 3.1, 2.8, 2.5, and 1.0 kb) with probes 2, 3, and 4. Some of the bands also hybridize with probe 1, and two additional bands can be seen (1.7 and 1.2 kb). The HindIII patterns show three strong bands (approximately 15, 7.0 and 1.4 kb) and three weaker bands (5.4, 4.4, 1.8 kb) with probes 2, 3 and 4. The 1.8 kb and especially the 1.4 kb bands are very prominent with probe 1 and these fragments contain the part of the AmyZ3/4 type genes that harbors the start of the α-amylase open reading frame. In Fig. 20, DNA from potato varieties Saturna and Dianella was digested with ExoRI, HindIII, or BamHI, and two identidal filters were prepared as described above. One filter was hybridized to a labelled complete insert from AmyZ3 and the other filter was hybridized to a labelled complete insert from AmyZ6. The fragments hybridizing to Saturna DNA with AmyZ3 sequences are seen to be the same in Fig. 19 and Fig. 20, except that the weaker bands in Fig. 19 are not clearly seen in Fig.

20. When the DNA fragment pattern obtained with the AmyZ3 probe in Fig. 20 is compared in Saturna and Dianella DNA, som bands are seen to be the same, but sometimes with altered relative intensity, whereas other bands are not seen in one of the DNAs. One example is the absence of the 15 kb HindIII fragment in Dianella DNA, which instead has a stronger, clearly double 7.0 kb fragment. This type of DNA fragment polymorphism can be explored in RFLP mapping as described in Example 27.

For the AmyZ3/4 type amylase sequences in variety Dianella, the isolation of three different, but highly homologous cDNA clones suggested that these three sequences were derived from three alleles of the same α-amylase gene. The very simple fragment pattern seen in Fig. 19 and 20 combined with the knowledge that the AmyZ3/4 type sequences contain both EcoRI and HindIII sites suggests that potato only has one α-amylase gene of the AmyZ3/4 type, but up to four different alleles may be present in one variety. However, the limitations of Southern analyses must be taken into account - both very large and small DNA fragments may escape detection.

In Fig. 20, Saturna and Dianella DNA was hybridized with complete inserts from AmyZ3 and AmyZ6. It is seen that the DNA fragment patterns are completely different for the two different types of α-amylase sequences, showing that they do not cross hybridize, as is expected from the low sequence homology between the two types of genes. As described in Example 7, AmyZ1 and AmyZ6 may be the products of alleles of one gene, but they may also be different genes belonging to a sub-gene family. The fragment pattern seen with the AmyZ6 probe is more complex than the pattern seen with the AmyZ3 probe, which may indicate that there is more than one AmyZ1 type gene in potato, and the Southern analysis therefore does not rule out that AmyZ1 and AmyZ6 represent two genes (with the possibility of up to eight different alleles). As with the AmyZ3 probe, the fragment patterns are seen to be different in the two varieties of potato also with the AmyZ6 probe. Thus, both types of α-amylase sequences can be employed in RFLP mapping.

EXAMPLE 17

α-amylase messenger RNA in potato sprouts and tubers

Saturna Sprout RNA was hybridized in two different experiments with AmyZ3 EcoRI fragment No. 1 and EcoRI fragments Nos. 2+3+4 (see Fig. 10). In both experiments a band of 1500 nucleotides was detected. The other RNA samples were hybridized with AmyZ3 EcoRI fragments Nos. 2+3+4. The result of the hybridizations is shown in Fig. 21 and the sizes of the transcripts detected are given in the following table.

Potato variety	Tissue	Transcript size, nucleotides ±100
Dianella	Sprout	1950
Saturna	Sprout	1500
Dianella	Tuber	(1950)
Saturna	Tuber	1700 and 2400

The transcript size estimates are approximate, but the *differences* in the transcript sizes are clear. In addition, the one band seen in Dianella sprout RNA is known to contain at least three types of α-amylase transcripts: mature messenger and a precursor with an intron 128 nucleotides long, corresponding to AmyZ3 and AmyZ4 (Fig. 11), and a messenger corresponding to AmyZ2 (Fig. 12).

The tuber RNAs were isolated from tubers stored at 8°C for 19 weeks. α-Amylase transcripts were detected in both Saturna and Dianella, but the Dianella RNA quality was sub-optimal, and it is possible that Dianella tubers also contain large transcripts in addition to mature size α-amylase messenger.

The hybridization was performed under stringent conditions and therefore only α-amylase sequences of the AmyZ3/4 type were detected, but from the cloning results it is known that at least two transcripts of the AmyZ1 type are present in Dianella sprouts. The large transcript in Saturna tuber RNA is likely to be an abundant unspliced α-amylase transcript precursor and the presence of this transcript suggests that control of α-amylase gene expression in part may be controlled at the level of RNA maturation.

EXAMPLE 18

Isolation of additional potato α-amylase cDNA clones and potato α-amylase genes

Two very different types of α-amylase cDNA clones have been isolated from potato with a barley α-amylase probe using particular low stringency hybridization conditions. The AmyZ3/4 type clones (AmyZ2,3,4,5, and 7) or parts thereof on the other hand and the AmyZ1 type clones (AmyZ1 and 6) or parts thereof on the other hand now allow the isolation of all of the two types of potato α-amylase sequences, either genes, pseudo-genes, or messengers (including cDNAs) using standard methods (12). Standard conditions implies reasonable high stringency of hybridization (e.g. hybridization at 67° in 2xSSC, final wash at 67°C in 1xSSC), which lowers the hybridization background and occurence of false signals and therefore greatly facilitates the isolation of additional α-amylase sequences.

EXAMPLE 19

Isolation of α-amylase genes and cDNA clones from other dicotyledon plants

The nucleotide sequence homology between the potato α-amylase cDNA clones type AmyZ3/4 and type AmyZ1 and any other α-amylase genes or messengers from a dicotyledon plant, in particular from the *Solanaceae* family, is better than between such dicotyledon α-amylase coding sequences and the barley (monocotyledon) α-amylase probe.

α-Amylase genes and cDNA clones from dicotyledon plants other than potato are therefore isolated with AmyZ3/4 type or AmyZ1 type sequences, or parts thereof as a probe under hybridization conditions identical to or more stringent than those described above for the isolation of the AmyZ series of clones with the barley α-amylase probe.

EXAMPLE 20

Levels of reducing sugars in four potato varieties stored at 8°C

A series of biochemical studies was carried out to characterize potato varieties with respect to reducing sugar levels in stored potatoes (this example), the effect of cold induction (Example 21), and activities of α-amylase (Example 22). The results constitute the basis for methods for increasing or decreasing reducing sugar levels in potatoes, employing α-amylase gene constructs in transgenic potato varieties. Reducing sugar levels were determined in potatoes from the time they are taken from the field and during the winter storage period in the four potato varieties described in "Materials and Methods". The results for potatoes stored at 8°C are shown in Fig. 22. The curves illustrate the substantial differences in reducing sugar levels found in the different varieties. The varieties represent approximately the known spectrum of levels of reducing sugars in cultivated potato.

EXAMPLE 21

Reducing sugar levels at different storage temperatures

Potatoes stored for 19 weeks at 8°C or at 6°C or for 6 weeks at 4°C were sampled on the same day and the levels of glucose and fructose were measured. The result (Fig. 23) shows the so-called cold-induced sweetening of potatoes, a response which is a natural reaction to low temperatures. The comparison of the reducing sugar levels in four different varieties of potatoes shows that the relative increase in sugar level is highest in low-sugar varieties and lowest in the high-sugar variety, but the relationship between the sugar levels in the four varieties stays the same. This indicates that a method that decreases the intrinsic level of reducing sugars also will improve the storage characteristics at low temperatures.

EXAMPLE 22

Correlation between reducing sugar levels and α-amylase activity in stored potatoes

In the experiment described in the preceding Example the α-amylase activity was determined (method 2) in four potato varieties after 19 weeks of storage at 6 °C or 8 °C. As far as the present inventors are aware, these are the first successful measurements of α-amylase activity in stored potatoes (3, 62). The activities were similar at 6 °C and 8 °C, and in Fig. 24 the average activity at the two temperatures is correlated with the reducing sugar levels at 8 °C. The figure shows a good correlation between reducing sugar levels and α-amylase activity. This observation is the basis for methods of reducing and increasing the level of reducing sugars in stored potatoes by controlling the production of α-amylase.

EXAMPLE 23

Correlation between reducing sugar levels and α-amylase gene activity

To further substantiate the notion that α-amylase activity is genetically determined such that α-amylase activity is determined by the level of expression of the α-amylase genes, RNA samples prepared from the four potato varieties mentioned above are assayed by semiquantitative Northern hybridizations using inserts from the isolated potato α-amylase cDNA clones as probes. Relative transcript levels are determined by scanning of autoradiograms resulting from the Northern hybridizations (40).

EXAMPLE 24

A method for decreasing the α-amylase (reducing sugar) level in potatoes

A plasmid is constructed in *E. coli*, the plasmid containing the following elements in said order 1) a plant promoter, 2) the α-amylase insert from e.g. AmyZ4 in orientation opposite to the orientation shown from left to right in Fig. 7, 3) a plant transcription termination sequence, e.g. from the nopaline synthetase (NOS) gene as provided between multiple cloning sites on pCaMVCN (Pharmacia LKB Biotechnology). The promoter may be the CaMV promoter or the NOS promoter (gene cartridges, Pharmacia LKB Biotechnology) for strong and medium strong constitutive expression of the antisense strand of the α-amylase sequence. The promoter may also be from a potato polyubiquitin gene isolated using pKG3730 as a heterologous probe (see Example 3) for low constitutive expression of the antisense strand of the α-amylase sequence, or a potato α-amylase promoter which is specifically active in the same plant cells as the α-amylase gene from which the promoter is taken. These examples of promoters and terminators are illustrative, and other sequences can fulfil the same roles.

An antisense construction including the entire sequence of AmyZ4 was made using the pBSK vector sites SacI and EcoRV of the AmyZ4 clone. The potato fragment was cloned into the plasmid pEnhanced-Peter-Linker (pEPL, (63)), which was first digested with SacI and then with SmaI. The resultant plasmid is called p(anti-AmyZ4).

pEPL was constructed from pCaMVCN (64,65) in which the CAT gene was removed by a PstI digestion. A small linker (linker: PstI-BamHI-BalI-PstI) has been inserted into this plasmid PstI site, giving the plasmid called pLise (pL). pL was digested with HincII and BglII and the resultant fragment containing the 35S promoter and the NOS terminator was cloned into another pL plasmid digested with EcoRV and BglII. Both EcoRV and HincII are blunt ended sites. The resulting construct is called pEnhanced-Lise (pEL). pEL differs essentially from pCaMVCN in that it contains a variant 35S promoter with a tandem duplication of the 250 base pairs of the upstream sequence of the promoter. The variant 35S promoter has a transcriptional activity approximately ten times higher than the natural 35S promoter (66). pEL was digested with PstI and BglII, thereby removing the NOS terminator, and a CaMV terminator (DW2t) was inserted instead. The plant virus terminator functions more efficiently than the plant gene terminator (67). Finally, a linker (PstI-BamHI-SmaI-SacI-SalI-SphI) was inserted into the PstI site situated between the enhanced 35S promoter and the CaMV terminator. This plasmid is called pEPL.

An antisense construction was also made with AmyZ6 in which the potato clone was digested with SacI and EcoRV (as AmyZ4). The potato fragment was cloned into pEPL, first digested with SacI and then with SmaI. The resultant plasmid is called p(anti-AmyZ6). Both p(anti-AmyZ4) and p(anti-AmyZ6) were digested with HindIII in order to isolate the fragments containing the entire enhanced 35S promoter, the inserted AmyZ4 or AmyZ6 in the antisense direction and the CaMV terminator. It was necessary to make a partial digestion with p(antiAmyZ4) since the AmyZ4 potato insert contains a HindIII site. The isolated fragments were cloned into the HindIII site of the binary vector pBI121 (68, 69, 70). The HindIII site in pBI121 is situated between a kanamycin resistance gene and the β-Glucuronidase (GUS) gene. The antisense construction (anti-AmyZ4 or anti-AmyZ6), the kanamycin resistance gene and the GUS gene all have their own promoter and terminator. The constructed pBI121-anti-AmyZ4 (or-anti-AmyZ6) is isolated from *E.coli* and transformed into the *Agrobacterium tumefaciens* strain LBA4404 which contains the disarmed helper plasmid pAL4404 (71,72) by the triparental mating method (73). A Solanum tuberosum transformation model system has been made with the potato varieties Dianella and Saturna. A pBI121 plasmid containing a herbicide resistance marker gene was used to create the model system in these potato varieties. The pBI121 with the marker gene was mated with LBA4404 as described above, and the resultant *A. tumefaciens* strain was grown overnight in LB medium with 50 µg/ml kanamycin. The cells were centrifuged and resuspended in Murashige and Skoog medium (MS medium) before transformation (Murashige, T. and Skoog, F. (1962), *Physiol. Plant 15*, pp. 473-497). Potato tubers were peeled, washed briefly in distilled water and surface sterilized for 15 min. in a 10% solution of Na-hypochlorite with a few drops of "tween®20". The potatoes were then washed extensively in sterile distilled water, and immersed in MS medium. Cylindrical samples were taken from the tubers using a sterile cork borer, and these samples were sliced into 1-2 mm thin discs with a scalpel. The tuber discs were floated in 20 ml of MS medium containing *Agrobacterium* and completely wetted by gentle shaking. 20 min. later, the tuber dics were transferred to supplemented MS plates (supplemented MS medium contains in addition to the MS salts 1 mg/l thiamine HCL, 0.5 mg/l nicotinic acid and 0.5 mg/l pyridoxine HCL (the vitamins) and 3% sucrose, 5 µM zeatin riboside and 3 µM IAA aspartic acid, pH 5.9. The medium was solidified with 0.8% Difco agar) and after 48 hours, they were transferred to new plates supplemented with 500 µg/ml carbencillin (to eliminate *Agrobacterium*) and 100 µg/ml kanamycin for selection of transformed potato issue. The sealed plates were incubated at 25°C under a day-night light cycle. The tuber discs were subcultured every 3 weeks onto fresh supplemented MS medium with 200 µg/ml carbencillin. Developing shoots were removed and planted in large test tubes with MS medium containing vitamins, 200 µg/ml carbencillin and 100 µg/ml kanamycin for induction of roots. The shoots were investigated for GUS expression (70) and a blue color indicated that the shoots have been transformed with the marker gene. This model system is used for carrying the anti-AmyZ4 construct or the anti-AmyZ6 construct into the genome of e.g. both Dianella and Saturna, by co-cultivation with the pB121-anti-AmyZ4 or pBI121-anti-AmyZ6 in pAL4404 and following the method already described for the marker gene.

Successfully transformed potato plantlets express α-amylase anti-messenger from the inserted tripartite gene construction which inhibits the translation of α-amylase by base pairing with α-amylase messenger. The lower level of α-amylase will in turn limit the degradation of starch, thereby limiting the formation of reducing sugars. An even greater inhibition of translation of α-amylase is obtained using constructs containing *both* anti-AmyZ4 and anti-AmyZ6.

EXAMPLE 25

A method for increasing the α-amylase (reducing sugar) level in potatoes

A plasmid is constructed in *E. coli*, the plasmid containing the following elements in said order 1) a plant promoter, 2) a potato α-amylase gene isolated as described in Example 18 from which the promoter is removed up to the transcription initiation site ± approximately 20 bp. The plant promoters used are the same as specified in Example 24. In addition, a patatin promoter can be used. Patatin genes are highly expressed in the parenchym tissue of tubers (44) (region D in Fig. 6).

AmyZ4 (the cDNA clone containing the full length copy of the α-amylase messenger-RNA) was digested with BamHI and SalI (these sites are situated in the pBSK polylinker) and the resultant potato fragment was cloned into the pEPL (described in Example 24) plasmid digested with the same enzymes. This construct, called p(senseAmyZ4) has the enhanced 35S promoter (described in Example 24) followed by the AmyZ4 insert in the sense direction and after this, the CaMV terminator (described in Example 24). The tripartite fragment just described was isolated by a partial HindIII digestion of p(sense-AmyZ4) and was then cloned into the unique HindIII site of pBI121 and then transferred into e.g. the Dianella potato genome by the method described in Example 24.

Successfully transformed plantlets express α-amylase messenger at a higher level than untransformed plants, thus producing more α-amylase than the parent plant. The increased level of α-amylase will in turn enhance the degradation of starch, thereby increasing the level of reducing sugars. Using a construction with a patatin promoter, the excess α-amylase will accumulate in the middle of the tubers, converting some of the starch to sugars, and will be available for starch degradation during the first heating step prior to fermenation.

EXAMPLE 26

Production of potato α-amylase in microorganisms

The complete open reading frame for potato α-amylase precursor is excised from AmyZ4 with HaeII and DraI. HaeII cuts between the initiation codon ATG and codon number 2, and the HaeII site is filled in and ligated to a filled-in NcoI site (yielding *CCATG*), restoring the initiation codon. NcoI single sites are commonly found in initiation codon of *E. coli* expression vectors (e.g. pKK233-2, Pharmacia LKB Biotechnology). DraI cuts 51 bp after the termination codon, directly yielding a blunt end. The NcoI site is also restored in the expression plasmid for easy transfer of the complete potato α-amylase precursor open reading frame to other expression vectors for *E. coli*, other prokaryotes or eukaryotes such as yeast. Since signal peptides have similar features in prokaryotes and eukaryotes (Example 13), the potato α-amylase precursor may be processed correctly in the new host to yield mature α-amylase. Alternatively, coding regions for other signal peptides are used to replace the region coding for the potato α-amylase signal peptide.

To produce the AmyZ1 type α-amylase, a full length cDNA clone is isolated as described in Example 18 and appropriate restriction enzymes are used to excise the open reading frame precisely for insertion into an expression vector as described above.

EXAMPLE 27

Dot blot quick screening of potato varieties in breeding programmes

Based on the observations that the reducing sugar content in stored potatoes is correlated with α-amylase activity (Example 22) and that the correlation extend to sprouts (Example 17) and therefore probably to leaves, potato varieties can be screened for their tendency to accumulated sugar in stored potatoes already at a stage when young plantlets have formed a few leaves. RNA extracted from 0.1 to 0.5 grams of leaf material is spotted on filters suitable for hybridization and hybridized with radioactively or biotin labeled α-amylase cDNA sequences. As a reference, hybridization with similarly labeled ubiquitin coding regions from any organism, e.g. barley, can be used, since ubiquitin sequences are extremely well-conserved and the ubiquitin genes are constitutively expressed in different plant tissues (17). The results are compared with similar dot blots of potato varieties with known sugar characteristics (e.g. the four varieties described in "Materials and Methods"). Such a dot blot assay can be carried out on a large amount of breeding material and can lead to an early assessment of quality as regards sugar characteristics.

EXAMPLE 28

RFLP mapping with α-amylase gene sequences

Restriction fragment length polymorphisms are increasingly used to follow specific alleles of genes, so far primarily in humans, but also in plants (58). The results of genomic Southern hybridization of potato DNA with the isolated α-amylase sequences showed (Figs. 19 and 20) that potato has few α-amylase genes and therefore yields a simple fragment pattern that makes polymorphisms easy to evaluate. Examples of polymorphisms are shown in Fig. 20. Polymorphisms in α-amylase sequence hybridizing fragment length as in corresponding studies with other probes, is used either to follow the α-amylase alleles themselves or as (linked or unlinked) markers in crosses involving other characteristics, e.g. pathogen resistance and morphological characteristics such as tuber colour.

EXAMPLE 29

Controlled expression of other enzymes

α-Amylase genes from potato are expressed in a developmental and tissue (cell) specific fashion as is the case with most other plant genes. α-Amylase gene expression is characteristic for genes encoding enzymes (as opposed to structural proteins or storage proteins). The level of expression is relatively low, on the order of 0.01% of the total messenger in sprouts being α-amylase messenger (Example 5). Using an α-amylase promoter fused to genes without promoters encoding enzymes not found in potato or enzymes made in potatoes in small, but suboptimal amounts or in cells other than the cells that produce α-amylase, the intermediate metabolism and the metabolism of e.g. phytohormones may be fine-tuned. The fusion construct may be inserted into an *A. tumefaciens* vector for the production of transgenic plants as described in Example 24.

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Fig 1

AmyZ3/4

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1    CGA CAG TCG CCG TCG TCA CCG CCA CCG CAC TAT TCT CGA CGC GTC GTC   48
49   TAT CTC CTC CAC CCC ACA GCC GTC AAT TCC AAG CTT CCA ATG AAC CGT   96
97   TGC CAT GTG TCA CTG CCT ATT CAC CGC GAA ACA TGA ATA TCA CTG ACG  144
145  AAC GAT TTC GGA GCG GAA CGA ATC CAG AAA ATG GAT TAC TTT CTA TAA  192
193  ATT CCT CGA ATC TCA ACT CCA TTT CGT AAA AAT AAA ATT AAA AAT ATT  240
241  GTT TCT TTT TTG ATT TCT GGT TTA TGT GGT GAT CGA ATT TTC AAT TTT  288
289  TTT ACT GGT AGT GAT TCC TAC TTT TCT TCA ATT CGA TTT CTC CTT TTT  336
337  CCA TTT CAC GGT TGA GAA TTC ATG ATT CCT TAT CAG AGG AAT CGA TCC  384
385  GAT TTG ACT AAT TTC ACT TTT CGT CTG TAT AAA TAC CAG AGT ATC TAG  432
433  GTT GAG GAA CGT AAT TTC AAG CTG CGA TCG GCT TTT TCC CCT GAA CGA  480
481  GCA AAC ACA GGT TGT GGG TTC GAG TTA GCA AGG GAC GTA TAA TCT CAA  528
529  CTA CAA TCC ATT ATG GCG CTT GAT GAA AGT CAG CAG TCT GAT CCA TTG  576
1                     M   A   L   D   E   S   Q   Q   S   D   P   L    12
577  GTT GTG ATA CGC AAT GGA AAG GAG ATC ATA TTG CAG GCA TTC GAC TGG  624
13    V   V   I   R   N   G   K   E   I   I   L   Q   A   F   D   W    28
625  GAA TCT CAT AAA CAT GAT TGG TGG CTA AAT TTA GAT ACG AAA GTT CCT  672
29    E   S   H   K   H   D   W   W   L   N   L   D   T   K   V   P    44
673  GAT ATT GCA AAG TCT GGT TTC ACA ACT GCT TGG CTG CCT CCG GTG TGT  720
45    D   I   A   K   S   G   F   T   T   A   W   L   P   P   V   C    60
721  CAG TCA TTG GCT CCT GAA GGT TAC CTT CCA CAG AAC CTT TAT TCT CTC  768
61    Q   S   L   A   P   E   G   Y   L   P   Q   N   L   Y   S   L    76
769  AAT TCT AAA TAT GGT TCT GAG GAT CTC TTA AAA GCT TTA CTT AAT AAG  816
77    N   S   K   Y   G   S   E   D   L   L   K   A   L   L   N   K    92
817  ATG AAG CAG TAC AAA GTT AGA GCG ATG GCG GAC ATA GTC ATT AAC CAC  864
93    M   K   Q   Y   K   V   R   A   M   A   D   I   V   I   N   H   108
865  CGT GTT GGG ACT ACT CAA GGG CAT GGT GGA ATG TAC AAC CGC TAT GAT  912
109   R   V   G   T   T   Q   G   H   G   G   M   Y   N   R   Y   D   124
913  GGA ATT CCT ATG TCT TGG GAT GAA CAT GCT ATT ACA TCT TGC ACT GGT  960
125   G   I   P   M   S   W   D   E   H   A   I   T   S   C   T   G   140
961  GGA AGG GGT AAC AAA AGC ACT GGA GAC AAC TTT AAT GGA GTT CCA AAT 1008
141   G   R   G   N   K   S   T   G   D   N   F   N   G   V   P   N   156
1009 ATA GAT CAT ACA CAA TCC TTT GTT CGG AAA GAT CTC ATT GAC TGG ATG 1056
157   I   D   H   T   Q   S   F   V   R   K   D   L   I   D   W   M   172
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Fig.1 continued

Amy23/4

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1057  CGG TGG CTA AGA TCC TCT GTT GGC TTC CAA GAT TTT CGT TTT GAT TTT 1104
 173   R   W   L   R   S   S   V   G   F   Q   D   F   R   F   D   F   188

1105  GCC AAA GGT TAT GCT TCA AAG TAT GTA AAG GAA TAT ATC GAG GGA GCT 1152
 189   A   K   G   Y   A   S   K   Y   V   K   E   Y   I   E   G   A   204

1153  GAG CCA ATA TTT GCA GTT GGA GAA TAC TGG GAC ACT TGC AAT TAC AAG 1200
 205   E   P   I   F   A   V   G   E   Y   W   D   T   C   N   Y   K   220

1201  GGC AGC AAT TTG GAT TAC AAC CAA GAT AGT CAC AGG CAA AGA ATC ATC 1248
 221   G   S   N   L   D   Y   N   Q   D   S   H   R   Q   R   I   I   236

1249  AAT TGG ATT GAT GGC GCG GGA CAA CTT TCA ACT GCA TTC GAT TTT ACA 1296
 237   N   W   I   D   G   A   G   Q   L   S   T   A   F   D   F   T   252

1297  ACA AAA GCA GTC CTT CAG GAA GCA GTC AAA GGA GAA TTC TGG CGT TTG 1344
 253   T   K   A   V   L   Q   E   A   V   K   G   E   F   W   R   L   268

1345  CGT GAC TCT AAG GGG AAG CCC CCA GGA GTT TTA GGA TTG TGG CCT TCA 1392
 269   R   D   S   K   G   K   P   P   G   V   L   G   L   W   P   S   284

1393  AGG GCT GTC ACT TTT ATT GAT AAT CAC GAC ACT GGA TCA ACT CAG GCG 1440
 285   R   A   V   T   F   I   D   N   H   D   T   G   S   T   Q   A   300

1441  CAT TGG CCT TTC CCT TCA CGT CAT GTT ATG GAG GGC TAT GCA TAC ATT 1488
 301   H   W   P   F   P   S   R   H   V   M   E   G   Y   A   Y   I   316

1489  CTT ACA CAC CCA GGG ATA CCA TCA GTT TTC TTT GAC CAT TTC TAC GAA 1536
 317   L   T   H   P   G   I   P   S   V   F   F   D   H   F   Y   E   332

1537  TGG GAT AAT TCC ATG CAT GAC CAA ATT GTA AAG CTG ATT GCT ATT CGG 1584
 333   W   D   N   S   M   H   D   Q   I   V   K   L   I   A   I   R   348

1585  AGG AAT CAA GGC ATA CAC AGC CGT TCA TCT ATA AGA ATT CTT GAG GCA 1632
 349   R   N   Q   G   I   H   S   R   S   S   I   R   I   L   E   A   364

1633  CAG CCA AAC TTA TAC GCT GCA ACC ATT GAT GAA AAG GTT AGC GTG AAG 1680
 365   Q   P   N   L   Y   A   A   T   I   D   E   K   V   S   V   K   380

1681  ATT GGG GAC GGA TCA TGG AGC CCT GCT GGG AAA GAG TGG ACT CTC GCG 1728
 381   I   G   D   G   S   W   S   P   A   G   K   E   W   T   L   A   396

1729  ACC AGT GGC CAT CGC TAT GCA GTC TGG CAG AAG TAA TCT TAC AGC TAT 1776
 397   T   S   G   H   R   Y   A   V   W   Q   K                       407

1777  TCC GTT ACT TAA TAT ATT AGT AGA AAT ATA TAT GTT TTA AAC CCG AGC 1824

1825  ACC TAC TTC TAA CAC TAG ATC CGC CTC TAC AGG CTT GGA TGG AGT GAT 1872

1873  GAG TTT TTT TTT CCT GTT CAT TAG ACA TTG CAA CAT GGG ATG TAT GTT 1920

1921  TTG TTA ATA AAA GTG TTC TTG ATC AAT GCA ATG TAA TAA GGG         1962
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Fig. 2

Amy27

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  1    G GCG CTT GAT GAA AGT CAG CAG TCT GAT CCA TTG GTT GTG ATA CGC    46
  1       A   L   D   E   S   Q   Q   S   D   P   L   V   V   I   R    15

 47   AAT GGA AAG GAG ATC ATA TTG CAG GCA TTC GAC TGG GAA TCT CAT AAA    94
 16    N   G   K   E   I   I   L   Q   A   F   D   W   E   S   H   K    31

 95   CAT GAT TGG TGG ATA AAT TTA GAT ACG AAA GTT CCT GAT ATT GCA AAG   142
 32    H   D   W   W   I   N   L   D   T   K   V   P   D   I   A   K    47

143   TCT GGT TTC ACA ACT GCT TGG CTG CCT CCG GTG TGT CAG TCA TTG GCT   190
 48    S   G   F   T   T   A   W   L   P   P   V   C   Q   S   L   A    63

191   CCT GAA GGT TAC CTT CCA CAG AAC CTT TAT TCT CTC AAT TCT AAA TAT   238
 64    P   E   G   Y   L   P   Q   N   L   Y   S   L   N   S   K   Y    79

239   GGT TCT GAG GAT CTC TTA AAA GCT TTA CTT AAT AAG ATG AAG CAG TAC   286
 80    G   S   E   D   L   L   K   A   L   L   N   K   M   K   Q   Y    95

287   AAA GTT AGA GCG ATG GCG GAC ATA GTC ATT AAC CAT CGT GTT GGG ACT   334
 96    K   V   R   A   M   A   D   I   V   I   N   H   R   V   G   T   111

335   ACT CAA GGG CAT GGT GGA ATG TAC AAC CGC TAT GAT GGA ATT CCT ATG   382
112    T   Q   G   H   G   G   M   Y   N   R   Y   D   G   I   P   M   127

383   TCT TGG GAT GAA CAT GCT ATT ACA TCT TGC ACT GGT GGA AGG GGT AAC   430
128    S   W   D   E   H   A   I   T   S   C   T   G   G   R   G   N   143

431   AAA AGC ACT GGA GAC AAC TTT AAT GGA GTT CCA AAT ATA GAT CAT ACA   478
144    K   S   T   G   D   N   F   N   G   V   P   N   I   D   H   T   159

479   CAA TCC TTT GTC CGG AAA GAT CTC ATT GAC TGG ATG CGG TGG CTA AGA   526
160    Q   S   F   V   R   K   D   L   I   D   W   M   R   W   L   R   175

527   TCC TCT GTT GGC TTC CAA GAT TTT CGT TTT GAT TTT GCC AAA GGT TAT   574
176    S   S   V   G   F   Q   D   F   R   F   D   F   A   K   G   Y   191

575   GCT TCA AAG TAT GTA AAG GAA TAT ATC GAG GGA GCT GAG CCA ATA TTT   622
192    A   S   K   Y   V   K   E   Y   I   E   G   A   E   P   I   F   207

623   GCA GTT GGA GAA TAC TGG GAC ACT TGC AAT TAC AAG GGC AGC AAT TTG   670
208    A   V   G   E   Y   W   D   T   C   N   Y   K   G   S   N   L   223

671   GAT TAC AAC CAA GAT AGT CAC AGG CAA AGA ATC ATC AAT TGG ATT GAT   718
224    D   Y   N   Q   D   S   H   R   Q   R   I   I   N   W   I   D   239

719   GGC GCG GGA CAA CTT TCA ACT GCA TTC GAT TTT ACA ACA AAA GCA GTC   766
240    G   A   G   Q   L   S   T   A   F   D   F   T   T   K   A   V   255

767   CTT CAG GAA GCA GTC AAA GGA GAA TTC TGG CGT TTG CGT GAC TCT AAG   814
256    L   Q   E   A   V   K   G   E   F   W   R   L   R   D   S   K   271

815   GGG AAG CCA CCA GGA GTT TTA GGA TTG TGG CCT TCA AGG GCT GTC ACT   862
272    G   K   P   P   G   V   L   G   L   W   P   S   R   A   V   T   287
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Fig. 2 continued

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 863  TTT ATT GAT AAT CAC GAC ACT GGA TCA ACT CAG GCG CAT TGG CCT TTC   910
 288   F   I   D   N   H   D   T   G   S   T   Q   A   H   W   P   F   303

 911  CCT TCA CGT CAT GTT ATG GAG GGC TAT GCA TAC ATT CTT ACA CAC CCA   958
 304   P   S   R   H   V   M   E   G   Y   A   Y   I   L   T   H   P   319

 959  GGG ATA CCA TCA GTT TTC TAT GAC CAT TTC TAC GAA TGG GAT AAT TCC  1006
 320   G   I   P   S   V   F   Y   D   H   F   Y   E   W   D   N   S   335

1007  ATG CAT GAC CAA ATT GTA AAG CTG ATT GCT ATT CGG AGG AAT CAA GGC  1054
 336   M   H   D   Q   I   V   K   L   I   A   I   R   R   N   Q   G   351

1055  ATA CAC AGC CGT TCA TCT ATA AGG ATT CTT GAG GCA CAG CCA AAC TTA  1102
 352   I   H   S   R   S   S   I   R   I   L   E   A   Q   P   N   L   367

1103  TAC GCT GCA ACC ATT GAT GAA AAG GTT AGC ATG AAG ATT GGG GAC GGA  1150
 368   Y   A   A   T   I   D   E   K   V   S   M   K   I   G   D   G   383

1151  TCA TGG AGC CCT GCT GGG AAA GAG TGG ACT CTC GCG ACC AGT GGC CAT  1198
 384   S   W   S   P   A   G   K   E   W   T   L   A   T   S   G   H   399

1199  CGC TAT GCA GTC TGG CAG AAG TGA TCT TAC AGC TAT TCC GTT ACT TAA  1246
 400   R   Y   A   V   W   Q   K                                       406

1247  TAT ATT AGT AGA AAT ATA TAT GTT TTA AAC CCG AGC ACC TAC TTC TAA  1294

1295  TAC TAG ATC CGC CTC TAC AGG CTT GGA TGG AGT GAT GAG TTT TTT TTT  1342

1343  TCT GTT CAT TAG ACA TTG CAA CAT GGG ATG TAG TTT TGG TAA TAA AAG  1390

1391  TGT TCT TGA TCA ATG CAA AAA AAA A                                 1415
```

Fig. 3

AmyZ1

1	TC	TGA	TCA	GTT	TTC	AGT	TCC	AGG	AAC	GAT	TCC	AAG	TTT	GGG	AAT	CAG	47
1			S	V	F	S	S	R	N	D	S	K	F	G	N	Q	15
48	CAA	CAA	CTG	AAA	ACT	CTT	ATT	AAG	GCT	TTA	CAT	GAC	CAC	GGG	ATC	AAA	95
16	Q	Q	L	K	T	L	I	K	A	L	H	D	H	G	I	K	31
96	TCG	GTT	GCT	GAT	ATA	GTG	ATA	AAT	CAT	AGA	ACT	GCT	GAT	AAC	AAA	GAT	143
32	S	V	A	D	I	V	I	N	H	R	T	A	D	N	K	D	47
144	AGC	AGG	GGA	ATA	TAC	AGC	ATC	TTT	GAA	GGA	GGA	ACG	TCT	GAT	GAC	CGG	191
48	S	R	G	I	Y	S	I	F	E	G	G	T	S	D	D	R	63
192	CTT	GAT	TGG	GGT	CCA	TCT	TTC	ATT	TGC	AAG	AAC	GAC	ACA	CAA	TAT	TCT	239
64	L	D	W	G	P	S	F	I	C	K	N	D	T	Q	Y	S	79
240	GAT	GGC	ACA	GGG	AAT	CCA	GAC	ACG	GGT	TTG	GAC	TTT	GAA	CCT	GCC	CCT	287
80	D	G	T	G	N	P	D	T	G	L	D	F	E	P	A	P	95
288	GAT	ATC	GAT	CAT	CTT	AAT	ACA	AGA	GTG	CAG	AAA	GAG	TTA	TCA	GAC	TGG	335
96	D	I	D	H	L	N	T	R	V	Q	K	E	L	S	D	W	111
336	ATG	AAT	TGG	CTG	AAA	TCT	GAA	ATT	GGA	TTT	GAT	GGT	TGG	CGT	TTC	GAT	383
112	M	N	W	L	K	S	E	I	G	F	D	G	W	R	F	D	127
384	TTT	GTT	AGG	GGA	TAT	GCA	CCT	TGC	ATT	ACC	AAG	ATT	TAT	ATG	AGA	AAC	431
128	F	V	R	G	Y	A	P	C	I	T	K	I	Y	M	R	N	143
432	ACA	TCC	CCG	GAT	TTT	GCA	GTT	GGT	GAA	TTT	TGG	AAC	TCT	CTT	GCT	TAT	479
144	T	S	P	D	F	A	V	G	E	F	W	N	S	L	A	Y	159
480	GGC	CAG	GAT	GGG	AAA	CCG	GAA	TAT	AAC	CAG	GAC	AAT	CAT	AGG	AAT	GAG	527
160	G	Q	D	G	K	P	E	Y	N	Q	D	N	H	R	N	E	175
528	CTA	GTT	GGT	TGG	GTT	AAA	AAT	GCA	GGG	CGG	GCT	GTA	ACA	GCC	TTT	GAT	575
176	L	V	G	W	V	K	N	A	G	R	A	V	T	A	F	D	191
576	TTT	ACA	ACT	AAG	GGA	ATT	CTT	CAA	GCA	GCA	GTT	CAA	GGA	GAG	TTA	TGG	623
192	F	T	T	K	G	I	L	Q	A	A	V	Q	G	E	L	W	207
624	AGA	TTG	AAG	GAT	CCC	AAT	GGA	AAA	CCT	CCT	GGG	ATG	ATA	GGT	GTT	TTG	671
208	R	L	K	D	P	N	G	K	P	P	G	M	I	G	V	L	223
672	CCT	CGA	AAA	GCT	GTG	ACT	TTT	ATC	GAT	AAT	CAT	GAT	ACT	GGA	TCG	ACA	719
224	P	R	K	A	V	T	F	I	D	N	H	D	T	G	S	T	239
720	CAA	AAT	ATG	TGG	CCT	TTC	CCT	TCA	GAC	AAA	GTT	ATG	CAA	GGA	TAT	GCA	767
240	Q	N	M	W	P	F	P	S	D	K	V	M	Q	G	Y	A	255
768	TAC	ATT	CTA	ACT	CAT	CCA	GGA	ATC	CCA	TCA	GTG	TTT	TAT	GAC	CAT	TTC	815
256	Y	I	L	T	H	P	G	I	P	S	V	F	Y	D	H	F	271
816	TTT	GAT	TGG	GGC	TTC	ATG	GAT	GGA	ATT	TCA	GCA	CTA	ATC	TCT	ATT	AGG	863
272	F	D	W	G	F	M	D	G	I	S	A	L	I	S	I	R	287

Fig. 3 continued

AmyZ1

864 AAG AGG AAT AGG ATT TGT GCA ACA AGC AAT GTG CAA ATA ATG GCT TCT 911
288 K R N R I C A T S N V Q I M A S 303

912 GAT TCA GAT CTT TAT ATA GCA ATG ATT CAT CAC AAA ATT ATT GTC AAG 959
304 D S D L Y I A M I H H K I I V K 319

960 ATT GGG CCA AAA CTT GAT CTT GGA AAT CTT ATT CCA CCT AAT TAT GAG 1007
320 I G P K L D L G N L I P P N Y E 335

1008 GTG GCA ACT TCT GGA CAA GAC TAT GCT GTA TGG GAG CAA AAG GCA TAA 1055
336 V A T S G Q D Y A V W E Q K A 350

1056 TCA TAT TGT AGT ACC ATT TAT TTA CCA CAC TAA AAG TGA CCA TGG ACA 1103

1104 AAA TGG TTC TTA GTG TTA ATG TTA TAT GAT TGA AAA TGT AAT TTA TAT 1151

1152 CGG CAT AAT GAA GGC CAA AAA TTC AAG AAA TTA TAT TCA ATT CGA TAG 1199

1200 ACC TTG CTC AAT TCA C 1215

Amy26

Fig. 4

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1    GT CCG TCT TTA ATT TGC AAG GAT GAC ACA CAA TAT TCT GAT GGC ACG    47
1        P   S   L   I   C   K   D   D   T   Q   Y   S   D   G   T     15

48   GGG AAT CTA GAC ACG GGT TTG GAC TTT GAA CCT GCC CCT GAT ATT GAT   95
16    G   N   L   D   T   G   L   D   F   E   P   A   P   D   I   D    31

96   CAT CTT AAT ATC AGA GTG CAG AAA GAG TTA TCA GAT TGG ATG AAT TGG   143
32    H   L   N   I   R   V   Q   K   E   L   S   D   W   M   N   W    47

144  CTC AAG TCT GAA ATT GGA TTT GAT GGC TGG CGT TTC GAT TTT GTT AGG   191
48    L   K   S   E   I   G   F   D   G   W   R   F   D   F   V   R    63

192  GGA TAT GCA CCT AGC ATC ACC AAG ATT TAT ATG GGA AAC ACG TCC CCG   239
64    G   Y   A   P   S   I   T   K   I   Y   M   G   N   T   S   P    79

240  GAT TTT GCA GTT GGT GAA TTT TGG AAC TCT CTT GCG TAT GGC CAG GAC   287
80    D   F   A   V   G   E   F   W   N   S   L   A   Y   G   Q   D    95

288  GGG AAA CCG GAC TAT AAC CAG GAC AAT CAT AGG AAT GAG CTA GTT GGT   335
96    G   K   P   D   Y   N   Q   D   N   H   R   N   E   L   V   G    111

336  TGG GTT CAA AAT GGG GGT GGG GCT GTA AAA GCC TTT GAT TTC ACA ACT   383
112   W   V   Q   N   G   G   G   A   V   K   A   F   D   F   T   T    127

384  AAG GGA ATT CTT CAA GCT GCA GTT GAA GGA GAG TTA TGG AGA TTG AAG   431
128   K   G   I   L   Q   A   A   V   E   G   E   L   W   R   L   K    143

432  GAT TCC AAT GGA AAA CCT CCT GGG ATG ATA GGT GTT TTG CCT CAA AAT   479
144   D   S   N   G   K   P   P   G   M   I   G   V   L   P   Q   N    159

480  GCT GTG ACT TTT ATC GAT AAT CAC GAT ACT GGA TCG ACA CAA AAT ATG   527
160   A   V   T   F   I   D   N   H   D   T   G   S   T   Q   N   M    175

528  TGG CCT TTC CCT TCA GAC AAA GTT ATG CAA GGA TAT GCA TAC ATT CTC   575
176   W   P   F   P   S   D   K   V   M   Q   G   Y   A   Y   I   L    191

576  ACT CAT CCA GGA ATT CCA TCA CTG TTT TAT GAC CAT TTC TTG ATT GGG   623
192   T   H   P   G   I   P   S   L   F   Y   D   H   F   L   I   G    207

624  GCT GGA AGG ATG GAA TTT CAG CAC TAA TCT CTA TTA GGA AGA GGA ATG   671
208   A   G   R   M   E   F   Q   H                                    215

672  GGA TTT GTG CAA CAA GCA ATG TGC AGA TAA TGG CTT CTG ATT CTG ATC   719

720  TTT ATA TAG CAA TGA TTG ATC AGA AAA TTA TTG TCA AGA TTG GGC CAA   767

768  AAC TTG ATC TTG GAA ATC TTA TCC CAC CTA GTT ATC AGG TGG CAA CTT   815

816  CTG GAC AAG ACT ATG CTG TAT GGG AGC AAA AGG CAT TCA GTT ACC ATA   863

864  CAA AAT GTC ATC ATG GAC CAA ATG TCA GCA TGG ACA AAA TGG TTC TCA   911

912  GTG TTT AAT GTC ATT TGA CTG AAA ATG TAG TGT ATA TCA GAA ACA CAT   959

960  AAT GAA GGC AAA AAA TTC AAG AAA TTA TAT GCA ATT TGC GAT CAT GGT  1007

1008  TG                                                              1009
```

Fig. 5

AmyZ2P

```
1     A TCC TTT GTC CGG AAA GAT CTC ATT GAC TGG ATG CGG TGG CTA AGA   46
1        S   F   V   R   K   D   L   I   D   W   M   R   W   L   R    15

47   TCC TCT GTT GGC TTC CAA GAT TTT CGT TTT GAT TTT GCC AAA GGT TAT  94
16    S   S   V   G   F   Q   D   F   R   F   D   F   A   K   G   Y   31

95   GCT TCG AAG TAT GTA AAG GAA TAT ATC GAG GGG GCT GAG CCA ATA TTT  142
32    A   S   K   Y   V   K   E   Y   I   E   G   A   E   P   I   F   47

143  GCA GTT GGA GAA TAC TGG GAC ACT TGC AAT TAC AAG GGC  GC AAT TTG  190
48    A   V   G   E   Y   W   D   T   C   N   Y   K   G       N   L   63

191  GAT TAC AAC CAA GAT AGT CAC AGG CAA AGA ATC ATC AAT TGG ATT GAT  238
64    D   Y   N   Q   D   S   H   R   Q   R   I   I   N   W   I   D   79

239  GGC GCG GGA CAA CTT TCA ACT GCA TTC GAT TTT ACA ACA AAA GCA GTC  286
80    G   A   G   Q   L   S   T   A   F   D   F   T   T   K   A   V   95

287  CTT CAG GAA GCA GTC AAA GGA GAA TTC TGG CGT TTG CGT GAC TCT AAG  334
96    L   Q   E   A   V   K   G   E   F   W   R   L   R   D   S   K   111

335  GGG AAG CCA CCA GGA GTT TTA GGA TTG TGG CCT TCA AGG GCA GTC ACT  382
112   G   K   P   P   G   V   L   G   L   W   P   S   R   A   V   T   127

383  TTT ATT GAT AAT CAC GAC ACT GGA TCA ACT CAG GCG CAT TGG CCT TTC  430
128   F   I   D   N   H   D   T   G   S   T   Q   A   H   W   P   F   143

431  CCT TCA CGT CAT GTT ATG GAG GGC TAT GCA TAC ATT CTT ACA CAC CCA  478
144   P   S   R   H   V   M   E   G   Y   A   Y   I   L   T   H   P   159

479  GGG ATA CCA TCA GTT TTC TAT GAC CAT TTC TAC GAA TGG GAT AAT TCC  526
160   G   I   P   S   V   F   Y   D   H   F   Y   E   W   D   N   S   175

527  ATG CAT GAC CAA ATT GTA AAG CTG ATT GCT ATT CGG AGG AAT CAA GGC  574
176   M   H   D   Q   I   V   K   L   I   A   I   R   R   N   Q   G   191

575  ATA CAC AGC CGT TCA TCT ATA AGG ATT CTT GAG  CA CAG CCA AAC TTA  622
192   I   H   S   R   S   S   I   R   I   L   E       Q   P   N   L   207

623  TAC GCT GCA ACC ATT GAT GAA AAG GTT AGC ATG AAG ATT GGG GAC GGA  670
208   Y   A   A   T   I   D   E   K   V   S   M   K   I   G   D   G   223

671  TCA TGG TGC CCT GCT GGG AAA GAG TGG ACT CTC GCG ACC AGT GGC CAT  718
224   S   W   C   P   A   G   K   E   W   T   L   A   T   S   G   H   239

719  CGC TAT GCA GTC TGG C                                            734
240   R   Y   A   V   W                                               244
```

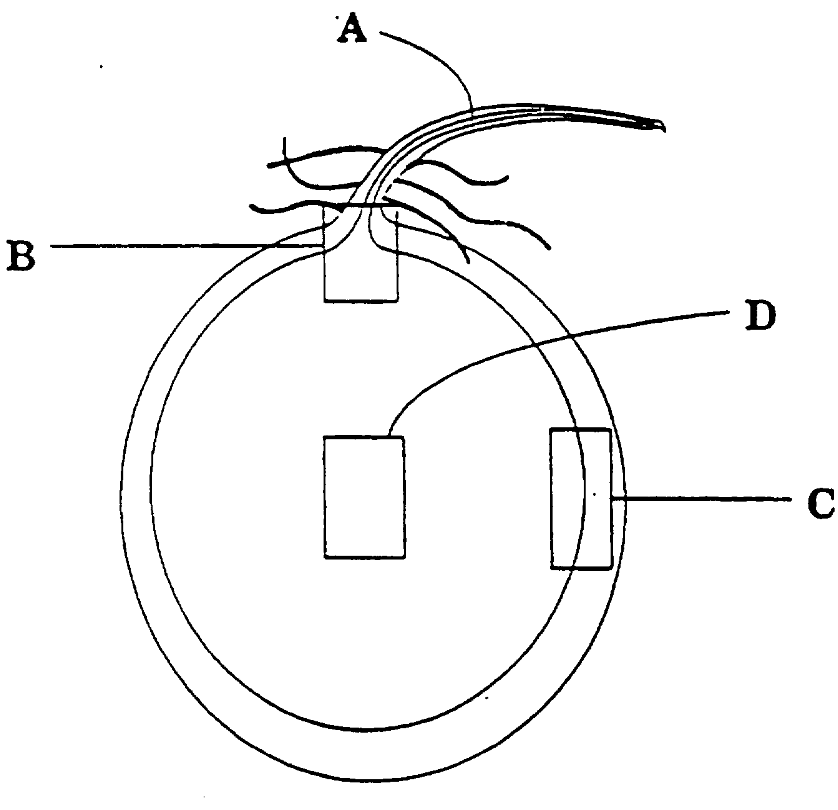


Fig. 6

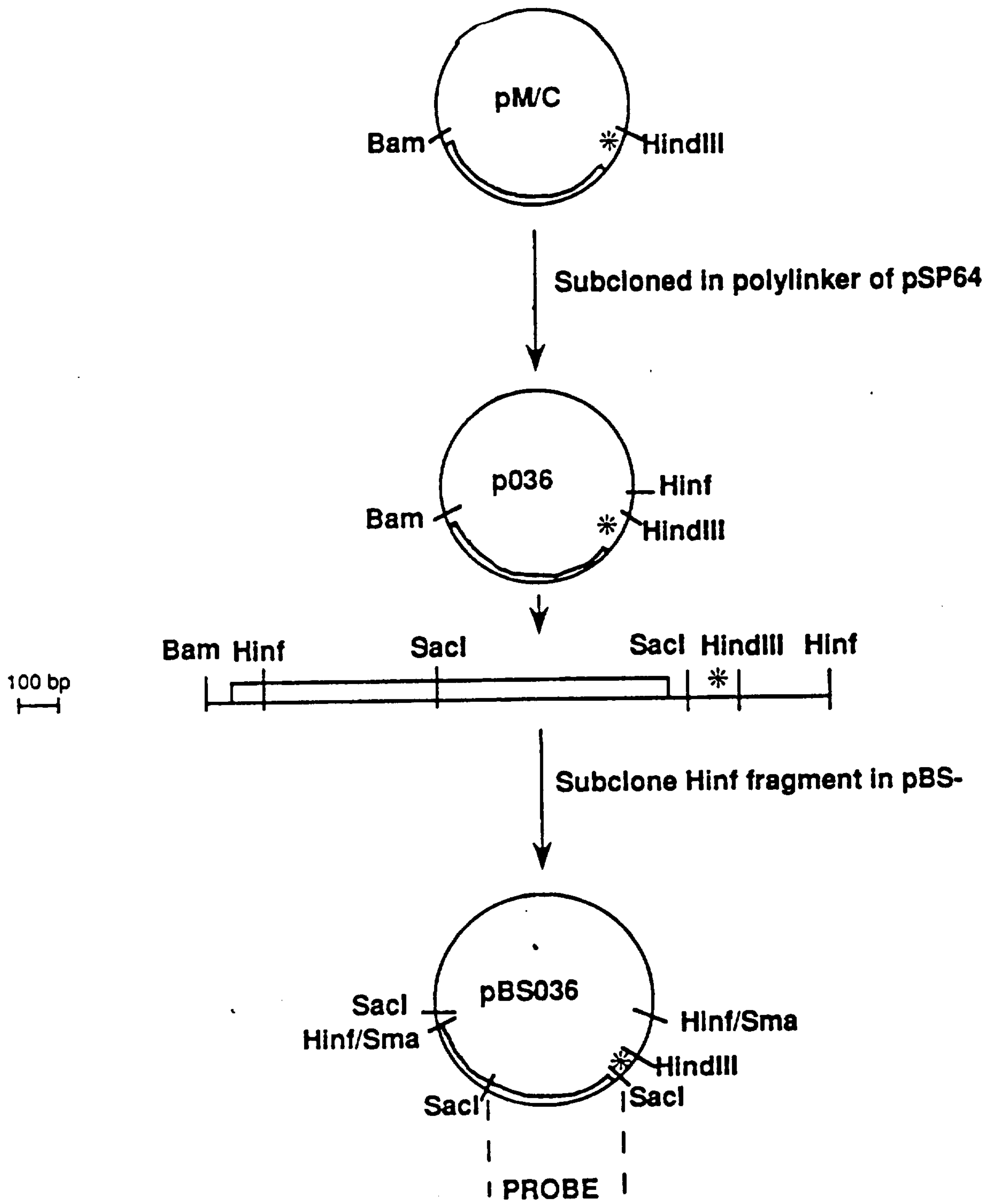

pM/C
Bam
HindIII
Subcloned in polylinker of pSP64
p036
Hinf
Bam
HindIII
Bam Hinf
SacI
SacI HindIII Hinf
100 bp
Subclone Hinf fragment in pBS-
pBS036
SacI
Hinf/Sma
Hinf/Sma
HindIII
SacI
SacI
PROBE

Fig. 7

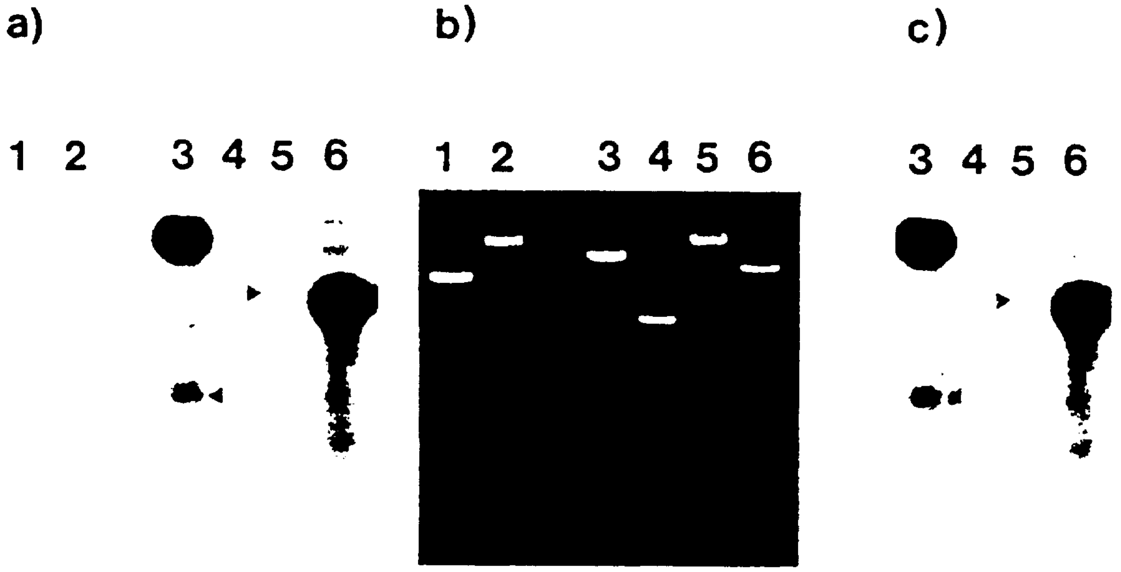


Fig. 8

Fig. 9

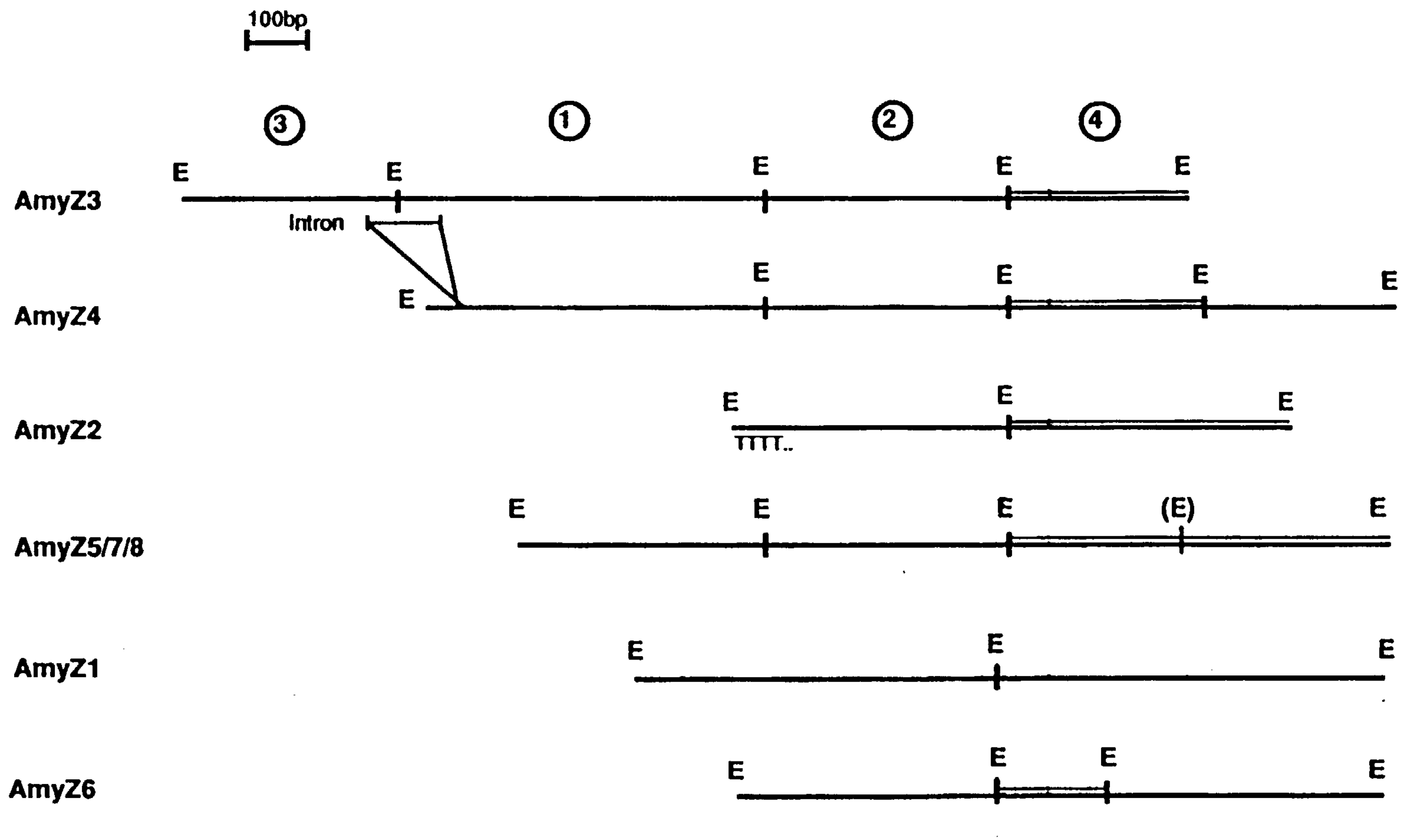

100bp
3
1
2
4
AmyZ3
AmyZ4
AmyZ2
AmyZ5/7/8
AmyZ1
AmyZ6
Intron
E
(E)
TTTT..

Fig.10

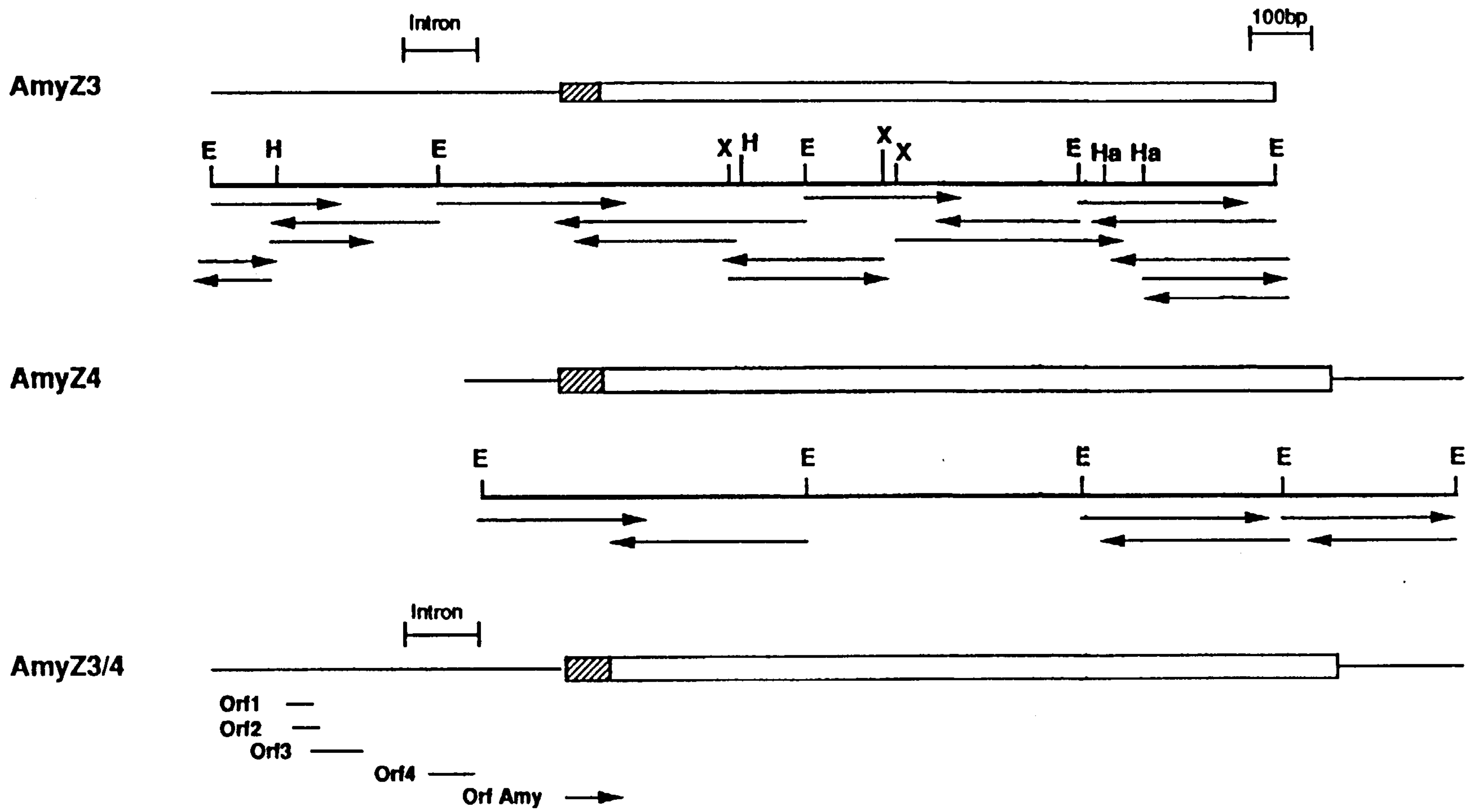

Intron
100bp
AmyZ3
E
H
E
X
H
E
X
X
E
Ha
Ha
E
AmyZ4
E
E
E
E
E
Intron
AmyZ3/4
Orf1
Orf2
Orf3
Orf4
Orf Amy

Fig. 11

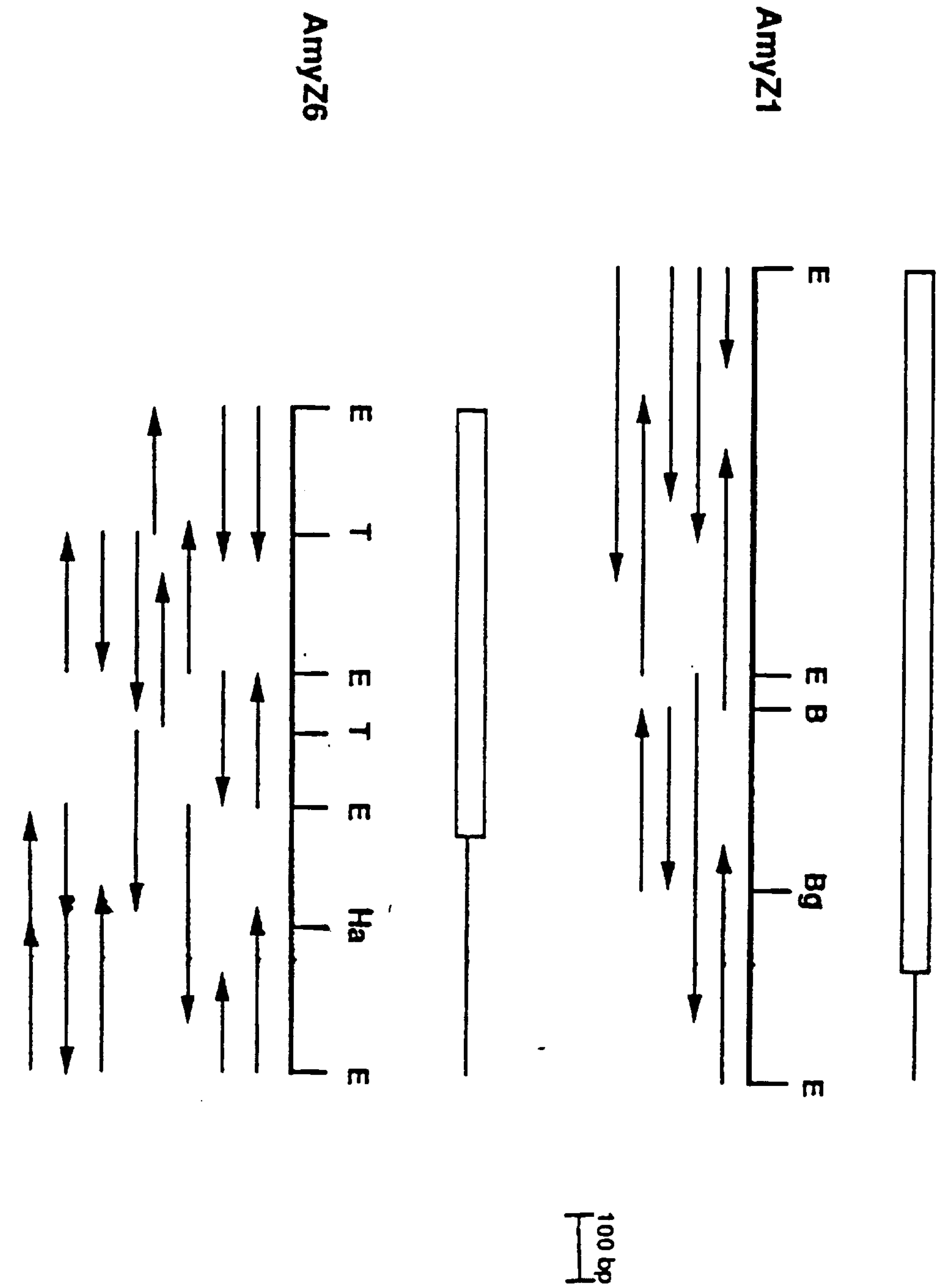


Fig. 12

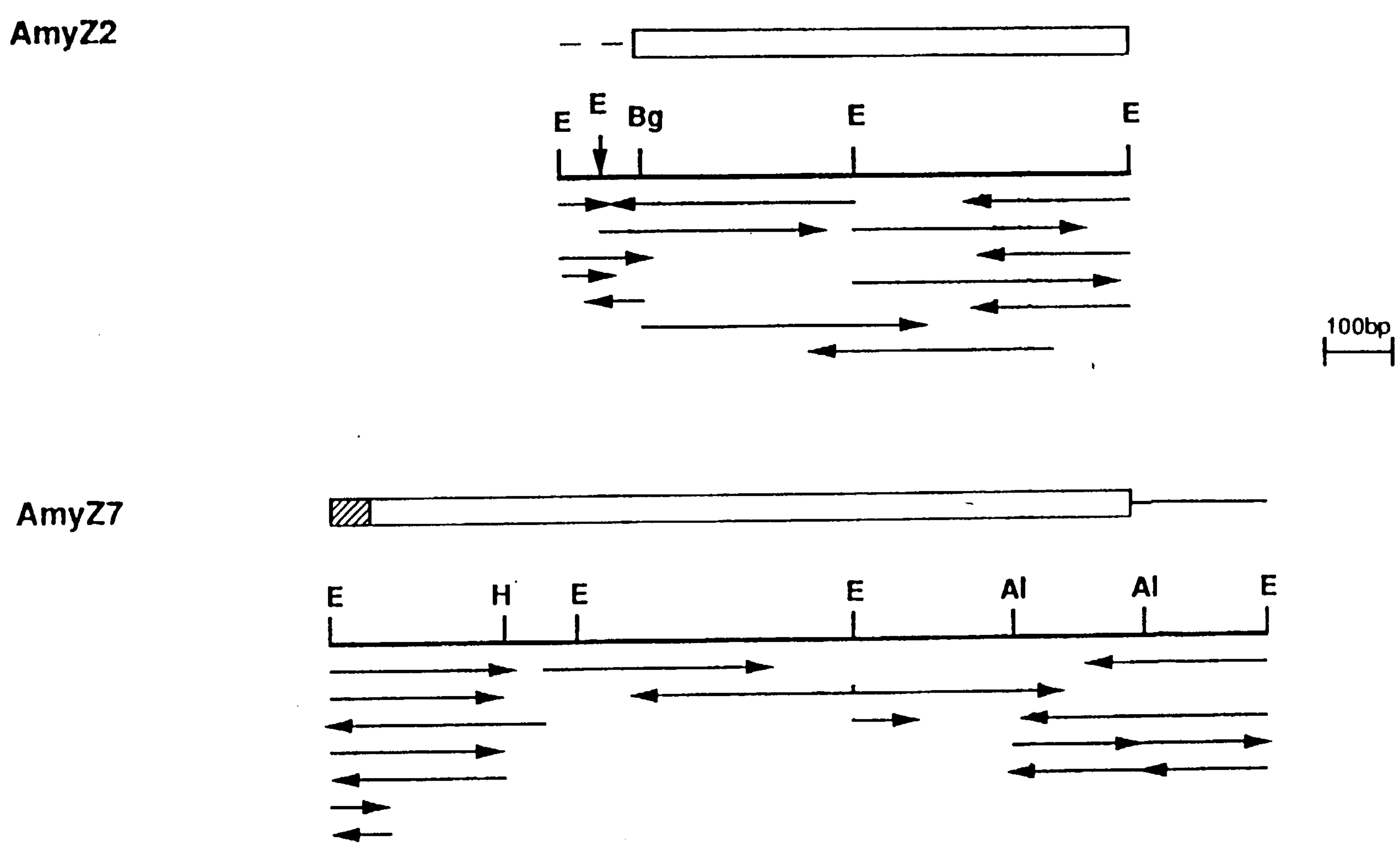


Fig. 13

```
         EcoRI
1330     GAATTCTGGCGTTTGCGTGACTCTAAGGGGAAGCCCCCAGGAGTTTTAGGATTGTGGCCT
308      GAGCUGUGGCGGCUGCGCGGCACAGACGGUAAGGCGCCAGGCAUGAUCGGGUGGUGGCCG

1390     TCAAGGGCTGTCACTTTTATTGATAATCACGACACTGGATCAACTCAGGCGCAT TGGCC
368      GCCAAGGCGGUGACCUUUGUGGACAACCACGACACCGGCUCCACGCA GCACAUGUGGCC

1449     TTTCCCTTCACGTCATGTTATGGAGGGCTATGCATACATTCTTACACACCCAGGGATACC
427      CUUCCCUUCUGACAGGGUCAUGCAGGGAUAUGCCUACAUCCUCACGCACCCAGGGACGCC

1509     ATCAGTTTTCTTTGACCATTTCTACGAATGGGATAATTCCATGCATGACCAAATTGTAAA
487      AUGCAUCUUCUACGAUCAUUUCUUCGACUGGG      GCC UGAAGGAGGAGAUCG AUC
                                                          EcoRI
1569     GCTGATTGCTATTC GGAGGAATCAAGGCATACACAGC CGTTC ATCTATAAGAATTC
540      GCUUGGUGUCAGUCAGGACCCGGCACGGGAUACACAACGAGAGCAAGCUGCAAAUCAUA
```

Matches = 190   Mismatches = 97   Unmatched = 12
Length = 299   Matches/length = 63.5 percent

Fig. 14

Potato amylase AmyZ3/4
Barley amylase B (pM/C)

```
19    K E I I L Q A F D W E S H K H D     W W
          : :   |   |   | | |   | |       | :
25      Q V L F Q G F N W E S W K H N G G W Y

37    L N L D T K V P D I A K S G F T T A W L
          |     | |   | | |   : |   |     | |
44    N F L M G K V D D I A A A G I T E V W L

57    P P V C Q S L A P E G Y L P Q N L Y S L
      | |     | | : |     | | : |     | |   |
64    P P A S Q S V A E Q G Y M P G R L Y D L

77    N S   K Y G S E D L L K A L L N K M K Q
        :   | | |         | | : | :     : :
84    D A S K Y G N K A Q L K S L I G A L E G

96    Y K V R A M A D I V I N E R V G T   T Q
          | : | : | | | | | | | |
104   K G V K A I A D I V I N E R T A E E K D

115   G E G G M Y N R Y D G   I P       M S W
      | : |   : |     : : |     |       :   |
124   G R G   I Y C I F E G D T P D A R L D W

131   D E E A I T   S C   T   G   G R G N K S
          |   |                   |   | |
143   G P E M I C R D D R P Y A D G T G N P D

147   T G D N F N G V P N I D E T Q S F V R K
      | |     |       |   | | |   :     |   |
163   T G A D F G A A P D I D E L N L R V Q K

167   D L I D W M R W L R S S V G F Q D F R F
      : | : : | :   | | : :               : | |
183   E L V E W L N W L K A D E R L D G W R F

187   D F A K G Y A S K Y V K E Y I E G A E P
      | | | | | | :         |   | | :   : | |
203   D F A K G Y S A D V A K I Y I D R S E P

207   I F A V G E Y W D T C N Y K G S N L   D
        | | |   |   |   :     |   |
223   S F A V A E I W T S L A Y G G D G K P N

226   Y N Q D S E R Q R I I N W I D G A G Q L
        | | |   | | |   : : | | : |     |
243   L N Q D Q E R Q E L V N W V D K V G G K

246   S     T A F D F T T K A V L Q E A V K G
            | : | | | | |   : | :   | |   |
263   G P A T T F D F T T K G I L N V A V E G

264   E F W R L R D S K G K P P G V L G L W P
      |   | | | |   :   | |   | | : : |   | |
283   E L W R L R G T D G K A P G M I G W W P

284   S R A V T F I D N H D T G S T Q A E W P
      : : | | | | : | | | | | | | | |     | |
303   A K A V T F V D N H D T G S T Q E M W P

304   F P S R E V M E G Y A Y I L T E P G I P
      | | |   : | |   | | | | | | | | | |   |
323   F P S D R V M Q G Y A Y I L T E P G T P

324   S V F F D E F Y E W D N S M E D Q I V K
        : | : | | | : : |       : : :   |   :
343   C I F Y D E F F D W     G L K E E I D R

344   L I A I R R N Q G I E S R S S I R I L E
      | : : : |       | | |     |   :   | : |
361   L V S V R T R E G I E N E S K L Q I I E

364   A Q P N L Y A A T I D E K V S V K I G D
      |       | |   |   | |   | |   | | : |
381   A D A D L Y L A E I D G K V I V K L G

384   G S W S   P A     G K E W T L A T S G E
          :                 :   : | :   |
400   P R Y D V G N L I P G G F K V A A E G N

401   R Y A V W Q K
        | | | | | |
420   D Y A V W Q K I
```

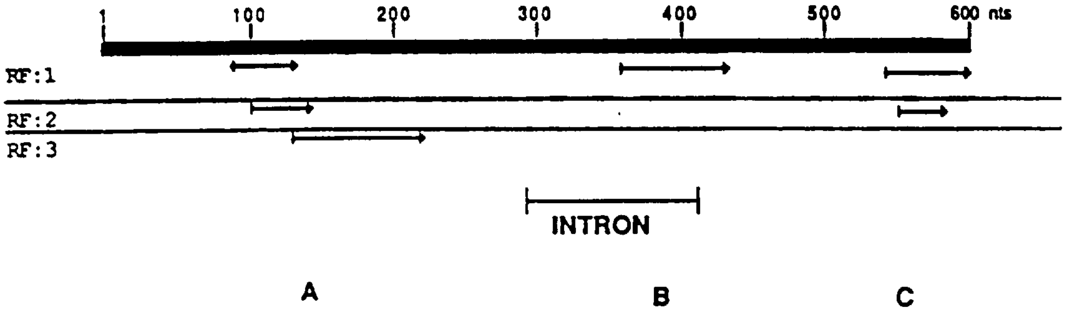
Fig. 15

```
         80        90       100       110       120       130
pM/C    GRLYDLDASKYGNKAQLKSLIGALHGKGVKAIADIVINHRTAEHKDGRGIYCIFEGDTPD
              ..::.::..:::.:: :::..:.:..::::::::::..::.::::.::::.:.:
AmyZ1   SVFSSRNDSKFGNQQQLKTLIKALHDHGIKSVADIVINHRTADNKDSRGIYSIFEGGTSD
                10        20        30        40        50        60

        140       150       160       170       180       190
pM/C    ARLDWGPHMICRDDRPYADGTGNPDTGADFGAAPDIDHLNLRVQKELVEWLNWLKADHRL
        .:::::: .::..: .:.::::::::: ::..:::::::: :::::: .:.::::..  .
AmyZ1   DRLDWGPSFICKNDTQYSDGTGNPDTGLDFEPAPDIDHLNTRVQKELSDWMNWLKSEIGF
                70        80        90       100       110       120

        200       210       220       230       240       250
pM/C    DGWRFDFAKGYSADVAKIYIDRSEPSFAVAEIWTSLAYGGDGKPNLNQDQHRQELVNWVD
        :::::::..::.. ..:::. ...:.:::.:.:.::::: ::::. :::.::.:::.::
AmyZ1   DGWRFDFVRGYAPCITKIYMRNTSPDFAVGEFWNSLAYGQDGKPEYNQDNHRNELVGWV-
               130       140       150       160       170

        260       270       280       290       300       310
pM/C    KVGGKGPATTFDFTTKGILNVAVEGELWRLRGTDGKAPGMIGWWPAKAVTFVDNHDTGST
        : .:. ..:.:::::::::..::.::::::....::.:::::  : :::::.::::::::
AmyZ1   KNAGR-AVTAFDFTTKGILQAAVQGELWRLKDPNGKPPGMIGVLPRKAVTFIDNHDTGST
        180        190       200       210       220       230

        320       330       340       350       360       370
pM/C    QHMWPFPSDRVMQGYAYILTHPGTPCIFYDHFFDWGLKEEIDRLVSVRTRHGIHNESKLQ
        :.:::::::.:::::::::::::.:..:::::::::....:. :.:.:.:. : ..:..:
AmyZ1   QNMWPFPSDKVMQGYAYILTHPGIPSVFYDHFFDWGFMDGISALISIRKRNRICATSNVQ
        240       250       260       270       280       290

        380       390       400       410       420
pM/C    IIEADADLYLAEIDGKVIVKLGPRYDVGNLIPGGFKVAAHGNDYAVWQKI
        :...:.:::.: :. :.:::.::. :.::::: ...::. :.:::::..
AmyZ1   IMASDSDLYIAMIHHKIIVKIGPKLDLGNLIPPNYEVATSGQDYAVWEQK
        300       310       320       330       340
```

64.1% identical amino acids in 343 amino acid overlap between barley clone pM/C and AmyZ1

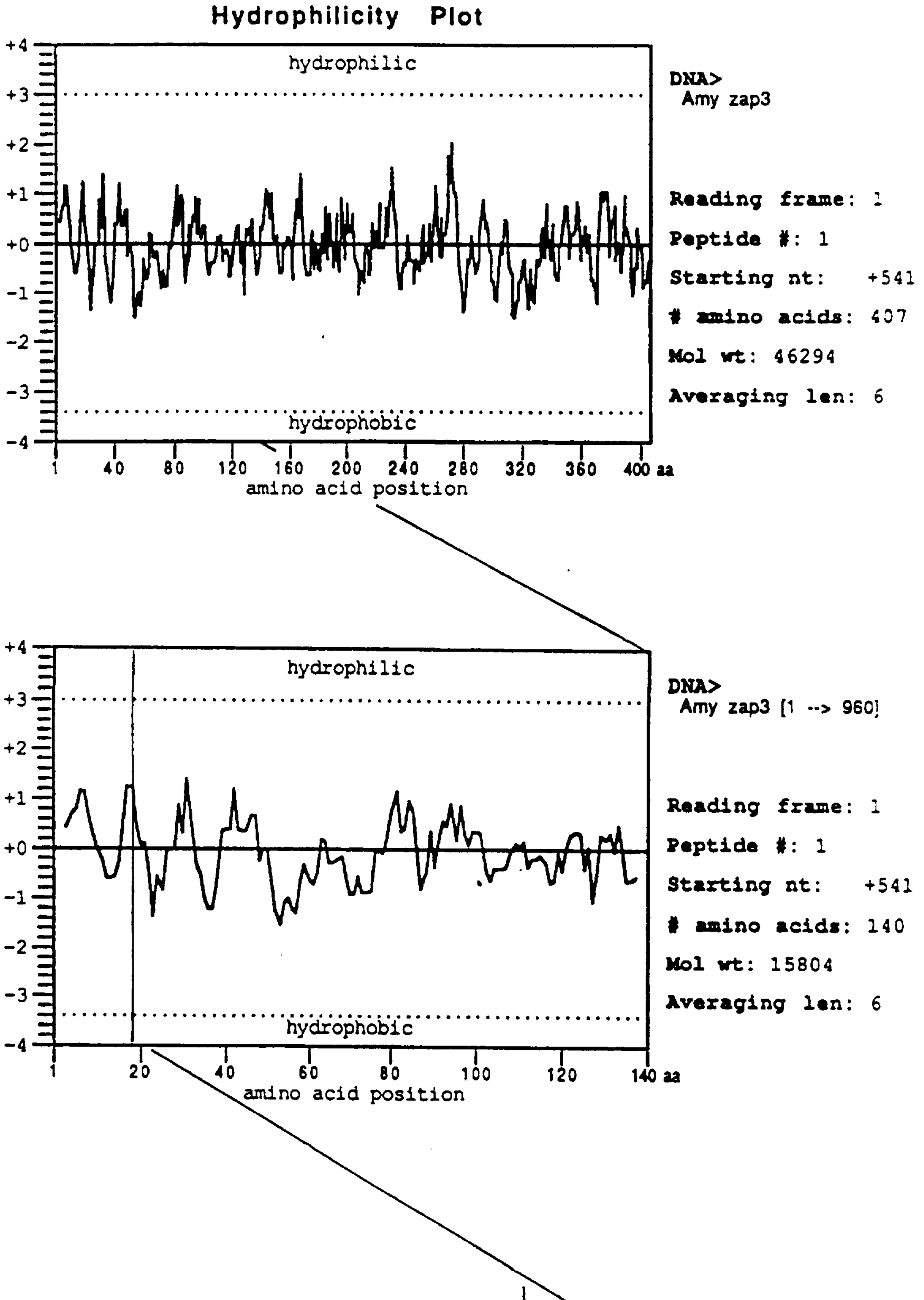
Fig. 16

OPEN READING FRAMES IN THE 5´END OF Amy Z3



A, RF1 MNRCHVSLPIHRET
A, RF2 MCHCLFTAKHEYH
A, RF3 MNITDERFRSGTNPENGLLSINSSNLNSIS
B, RF1 MIPYQRNRSDLTNFTFRLYKYQSI
C, RF1 MALDESQQS...... α-amylase precursor

Fig. 17



M A L D E S Q Q S D P L V V I R N G K E I

Fig. 18

Fig. 19

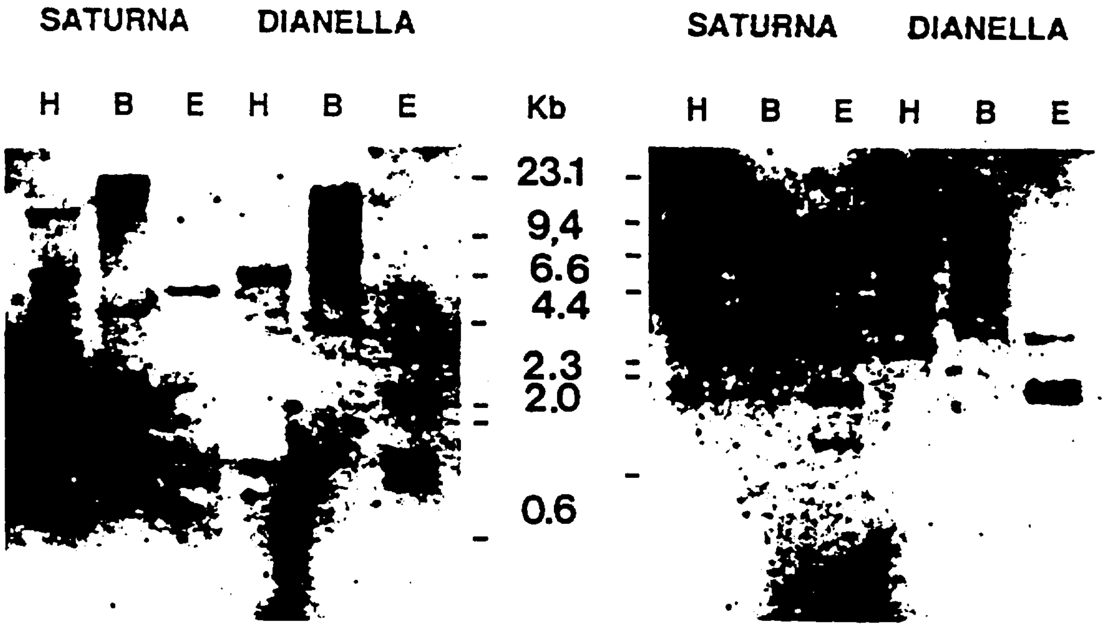

SATURNA
DIANELLA
H B E H B E
Kb
23.1
9.4
6.6
4.4
2.3
2.0
0.6
SATURNA
DIANELLA
H B E H B E

Fig. 20

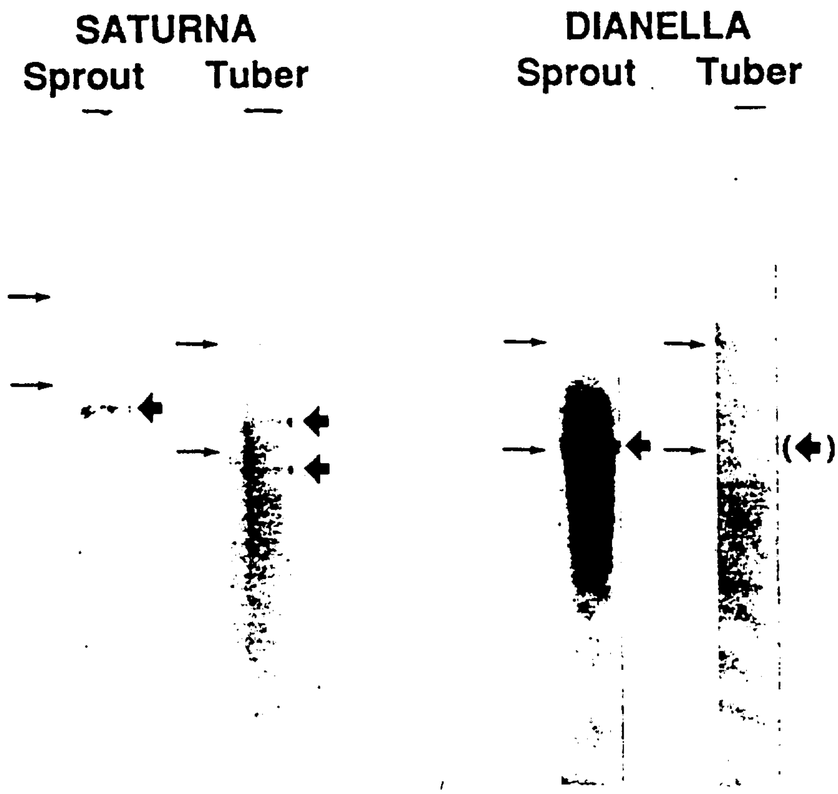


Fig. 21

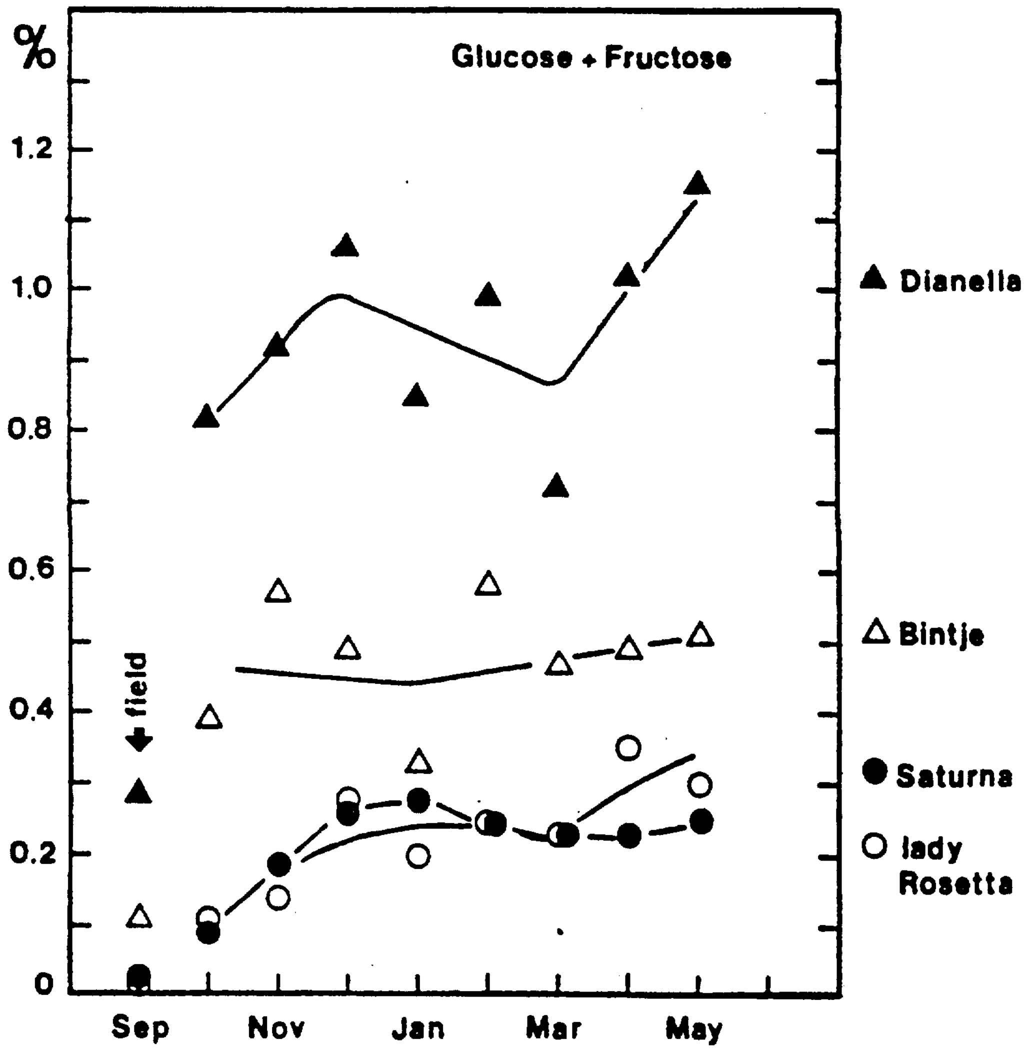
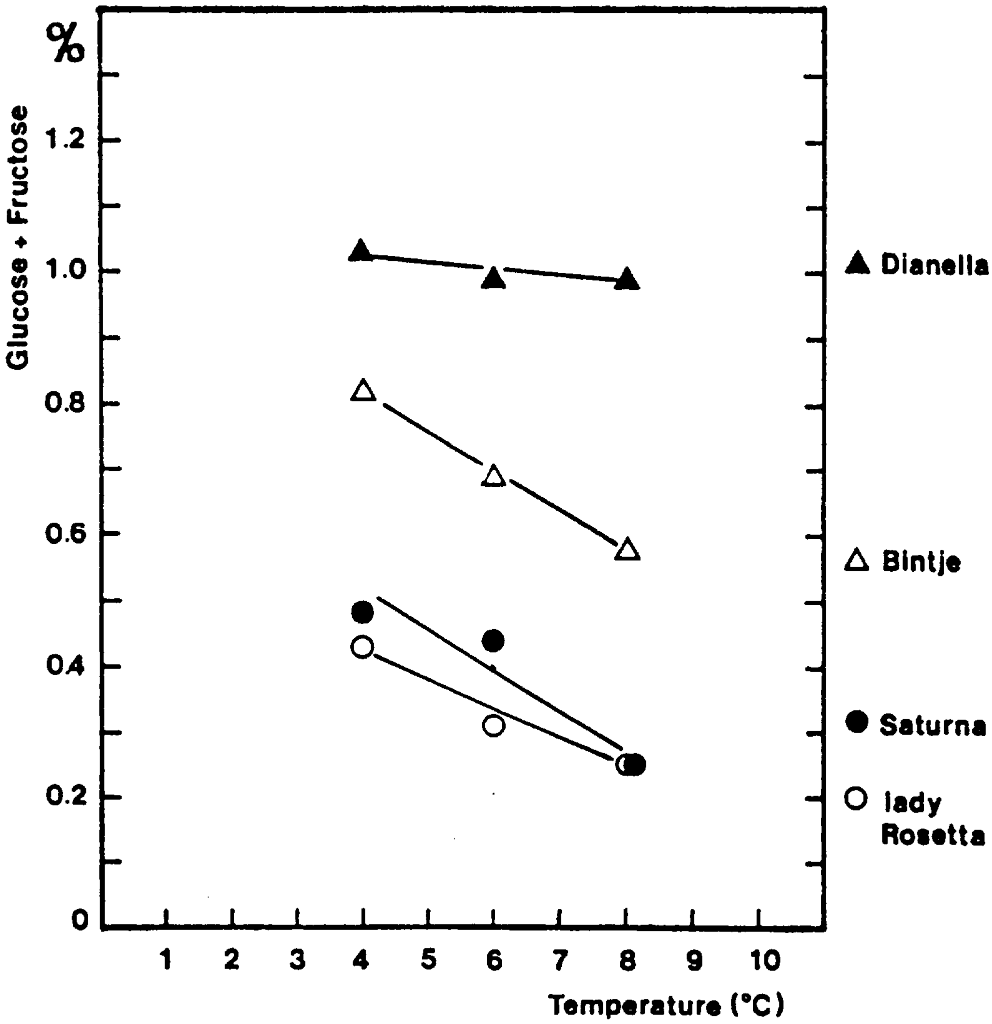

%
Glucose + Fructose
1.2
1.0
0.8
0.6
0.4
0.2
0
field
Sep
Nov
Jan
Mar
May
Dianella
Bintje
Saturna
lady Rosetta

Fig. 22



%
Glucose + Fructose
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
10
Temperature (°C)
Dianella
Bintje
Saturna
lady Rosetta

Fig. 23

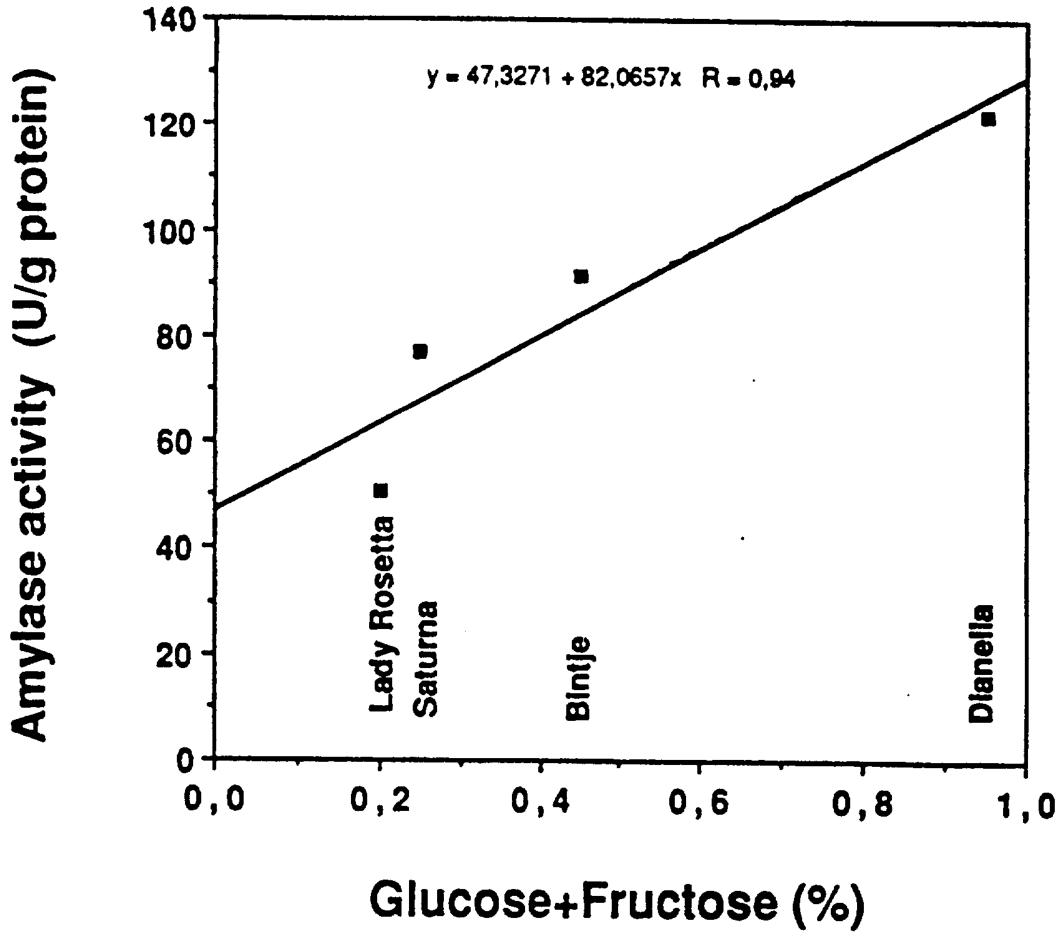

140
120
100
80
60
40
20
0
y = 47,3271 + 82,0657x R = 0,94
Lady Rosetta
Saturna
Bintje
Dianella
0,0
0,2
0,4
0,6
0,8
1,0
Amylase activity (U/g protein)
Glucose+Fructose (%)

Fig. 24

Claims

1. A DNA fragment comprising a nucleotide sequence as shown in Fig. 1, 2, 3, 4 or 5, a subsequence thereof which comprises at least 15 nucleotides and which hybridizes to at least one of said nucleotide sequences under high stringency hybridization conditions comprising hybridization at 67°C in 2xSSC and final washing at 67°C in 1xSSC or under medium high stringency hybridization conditions comprising hybridization at 67°C in 3xSSC and final washing at 67°C in 1xSSC, or a homologue of said nucleotide sequence which encodes a polypeptide identical to a polypeptide encoded by said nucleotide sequence and which hybridizes to at least one of said nucleotide sequences under high or medium high stringency hybridization conditions as defined above.

2. The DNA fragment according to claim 1 comprising a nucleotide sequence selected from:
(a) a nucleotide sequence starting at nucleotide 541 and terminating at nucleotide 1761 of the nucleotide sequence shown in Fig. 1 encoding a potato α-amylase precursor, or a subsequence or homologue thereof,
(b) a nucleotide sequence starting at nucleotide 596 and terminating at nucleotide 1761 of the nucleotide sequence shown in Fig. 1 encoding a potato α-amylase, or a subsequence or homologue thereof,
(c) a nucleotide sequence starting at nucleotide 2 and terminating at nucleotide 1219 of the nucleotide sequence shown in Fig. 2 encoding a potato α-amylase precursor, or a subsequence or homologue thereof,
(d) a nucleotide sequence starting at nucleotide 53 and terminating at nucleotide 1219 of the nucleotide sequence shown in Fig. 2 encoding a potato α-amylase, or a subsequence or homologue thereof,
(e) a nucleotide sequence starting at nucleotide 6 and terminating at nucleotide 1052 of the nucleotide sequence shown in Fig. 3 encoding a partial potato α-amylase, or a subsequence or homologue thereof,
(f) a nucleotide sequence starting at nucleotide 3 and terminating at nucleotide 647 of the nucleotide sequence shown in Fig. 4 encoding a partial potato α-amylase, or a subsequence or homologue thereof,
(g) nucleotides 1330-1624 as shown in Fig. 1, and
(h) nucleotides 387-591 as shown in Fig. 4.

3. The DNA fragment according to claim 1 comprising a nucleotide sequence selected from:
(a) the nucleotide sequence shown in Fig. 1 and being contained in the cDNA clone AmyZ3 deposited in *E. coli* K-12 as DSM 5275, or a subsequence or homologue thereof,
(b) the nucleotide sequence shown in Fig. 1 and being contained in the cDNA clone AmyZ4 deposited in *E. coli* K-12 as DSM 5276, or a subsequence or homologue thereof,
(c) the nucleotide sequence shown in Fig. 3 and being contained in the cDNA clone AmyZ1 deposited in *E. coli* K-12 as DSM 5882, or a subsequence or homologue thereof,
(d) the nucleotide sequence shown in Fig. 4 and being contained in the cDNA clone AmyZ6 deposited in *E. coli* K-12 as DSM 5883, or a subsequence or homologue thereof, and
(e) the nucleotide sequence shown in Fig. 2 and being contained in the cDNA clone AmyZ7 deposited in *E. coli* K-12 as DSM 5884, or a subsequence or homologue thereof.

4. A DNA fragment from a dicotyledonous plant which has a GC content in the range of 35-50%, preferably in the range of 40-50%, calculated as an average for the coding region and which hybridizes to a DNA sequence according to any of claims 1-3 under high stringency hybridization conditions comprising hybridization at 67°C in 2xSSC and final washing at 67°C in 1xSSC, under medium high stringency hybridization conditions comprising hybridization at 67°C in 3xSSC and final washing at 67°C in 1xSSC, or under low stringency conditions such as 6xSSC and 67°C and washing conditions of 4xSSC and 67°C.

5. The DNA fragment according to any of claims 1-4 which is a complete α-amylase gene comprising regulatory regions, promoter regions, coding regions, transcribed non-coding regions including introns and transcription termination regions, or a part thereof.

6. The DNA fragment according to claim 5 which is an α-amylase pseudo-gene.

7. The DNA fragment according to any of claims 1 to 6 which is derived from a dicotyledonous plant, in particular a member of the family *Solanaceae*, preferably from the genus *Solanum*, preferably from *Solanum tuberosum*.

8. The DNA fragment according to any of claims 1-5 or 7 which encodes a polypeptide having α-amylase activity.

9. The DNA fragment according to any of claims 1 to 8 which is a cDNA, a genomic DNA, a synthetic DNA or a combination thereof.

10. A DNA fragment encoding a fusion protein which comprises a DNA fragment according to any of claims 1-9 in reading frame with at least one second DNA fragment encoding a second polypeptide or a part thereof, said second DNA fragment being e.g. a signal sequence which encodes a prokaryotic signal peptide.

11. A polypeptide encoded by a DNA fragment according to any of claims 1-10, the polypeptide being free of naturally co-occuring enzymes.

12. A polypeptide comprising an amino acid sequence as shown in any of Figures 1-4, a subsequence of said amino acid sequence having α-amylase activity and comprising at least one active site, or a polypeptide derived from a dicotyledonous plant and having a homology of at least about 70% with said amino acid sequence.

13. The polypeptide according to claim 12 comprising an amino acid sequence as shown in Fig. 1 or 2, the polypeptide having the same enzymatic properties as the polypeptide shown in Fig. 1 or 2.

14. A DNA fragment which encodes an mRNA molecule capable of hybridizing to an mRNA transcribed from a DNA fragment according to any of claims 1-10, thereby inhibiting the translation thereof.

15. A DNA fragment in antisense orientation with respect to a DNA fragment according to any of claims 1-10, the DNA fragment in antisense orientation being regulated by a promoter capable of initiating synthesis of an mRNA molecule that hybridizes to an mRNA transcribed from a DNA fragment according to any of claims 1-10.

16. The DNA fragment according to claim 14 or 15 which encodes an anti-AmyZ4 or anti-AmyZ6 mRNA.

17. A single-stranded DNA or RNA fragment which is complementary to either strand of a DNA fragment of any of claims 1-10.

18. A vector which is capable of replicating in a host organism and which carries a DNA fragment according to any of claims 1-10 or 14-17.

19. A host organism harbouring a vector according to claim 18.

20. The host organism according to claim 19 which is capable of replicating or expressing the inserted DNA fragment according to any of claims 1-10 or 14-17.

21. A host organism which carries a DNA fragment according to any one of claims 1-10 or 14-17 wherein the DNA fragment is part of the genome of the organism.

22. The host organism according to claim 21 which is capable of replicating or expressing said inserted DNA fragment.

23. The host organism according to any of claims 19-22 which is a cell line, preferably a plant cell line; a plant; or a microorganism, e.g. a bacterium of the genus *Escherichia*, preferably *E. coli*, or of the genus *Bacillus*, preferably *B. subtilis*, a yeast, preferably of the genus *Saccharomyces*, preferably *S. cereviciae*, or a fungus, preferably of the genus *Aspergillus*.

24. An *E. coli* bacterium selected from:
    (a) *E. coli* K-12 harbouring the plasmid pAmyZ3 and being deposited at DSM under the accession number DSM 5275,
    (b) *E. coli* K-12 harbouring the plasmid pAmyZ4 and being deposited at DSM under the accession number DSM 5276,
    (c) *E. coli* K-12 harbouring the plasmid pAmyZ1 and being deposited at DSM under the accession number DSM 5882,
    (d) *E. coli* K-12 harbouring the plasmid pAmyZ6 and being deposited at DSM under the accession number DSM 5883, and
    (e) *E. coli* K-12 harbouring the plasmid pAmyZ7 and being deposited at DSM under the accession number DSM 5884.

25. A genetic construct for inhibiting the translation of an mRNA molecule encoded by a DNA fragment according to any of claims 1-10, said construct comprising:
    (1) a regulatory sequence functionally connected to
    (2) a DNA fragment according to any of claims 14-17 encoding an RNA molecule capable of inhibiting the translation of a DNA fragment according to any of claims 1-10; and
    (3) a transcription termination DNA sequence, functionally connected to the DNA fragment (2).

26. A genetic construct for producing a polypeptide according to any of claims 11-13, said construct comprising:
    (a)
        (1) a regulatory sequence functionally connected to
        (2) a DNA fragment according to any of claims 1-10 encoding the polypeptide; and
        (3) a transcription termination DNA sequence, functionally connected to the DNA fragment (2);
    (b)
        (1) a regulatory sequence functionally connected to
        (2) a DNA fragment according to claim 5 comprising an α-amylase gene without a promoter; or
    (c)
        (1) a regulatory sequence functionally connected to
        (2) a DNA fragment according to claim 5 comprising an α-amylase gene without a promoter or a transcription termination sequence; and
        (3) a transcription termination DNA sequence, functionally connected to the DNA fragment (2).

27. The genetic construct according to claim 25 or 26, in which the regulatory sequence is a plant promoter, preferably a constitutive or regulatable plant promotor, said constitutive promotor being e.g. a NOS promoter, an ubiquitin promoter or a plant virus promoter, preferably the CaMV promoter, said regulatable plant promoter being regulatable e.g. by at least one factor selected from the group consisting of a developmental, a chemical, a physical or a physiological factor.

28. The genetic construct according to claim 27, in which the plant promoter is a plant α-amylase promoter, preferably a potato α-amylase promoter, or a patatin promoter.

29. The genetic construct according to any of claims 25-28, wherein the transcription termination sequence is a plant transcription termination sequence.

30. A genetically modified plant comprising in its genome a genetic construct according to any of claims 25-29.

31. The genetically modified plant according to claim 30 comprising in its genome at least one genetic construct according to claim 25, said plant having a reduced α-amylase activity compared to a corresponding non-modified plant.

32. The genetically modified plant according to claim 30 comprising in its genome at least one genetic construct according to claim 26, said plant having an increased α-amylase activity compared to a corresponding non-modified plant.

33. The genetically modified plant according to claim 30 in which the α-amylase activity is regulatable at low temperatures, said plant comprising in its genome at least one genetic construct according to claim 25 and a promoter which at temperatures of below about 8°C activates transcription of the DNA fragment(s) contained in the construct(s).

34. The genetically modified plant according to claim 31 or 33, wherein the genetic construct is active in the same tissue or cell as the tissue or cell in which at least one α-amylase gene is expressed.

35. The genetically modified plant according to any of claims 30-34 which is a dicotyledonous plant, e.g. a member of the family *Solanaceae*, preferably of the genus *Solanum*, preferably *Solanum tuberosum*.

36. A vector system comprising at least one vector which carries a genetic construct according to any of claims 25-29, and which is capable of introducing the genetic construct into the genome of a plant, preferably a member of the family *Solanaceae*, preferably of the genus *Solanum*, preferably into *Solanum tuberosum*, said vector system being e.g. a binary vector system.

37. The vector system according to claim 36, which contains a virulence function capable of infecting the plant and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct, said vector system e.g. comprising an *Agrobacterium tumefaciens* Ti or Ri plasmid or an *Agrobacterium rhizogenes* Ri plasmid.

38. A microorganism capable of infecting a plant, preferably *Agrobacterium* spp., e.g. an *Agrobacterium tumefaciens* strain, which microorganism harbours a vector according to claim 36 or 37.

39. A method of constructing a genetically modified plant according to any of claims 30-35 comprising the steps of transferring a genetic construct according to any of claims 25-29 into the genome of a plant so as to obtain a genetic material comprising the construct, and subsequently regenerating it into a genetically modified plant.

40. The method according to claim 39, in which the genetic construct is transferred into the plant or into a part thereof by means of a microorganism according to claim 38, by direct injection or by electroporation.

41. The DNA fragment according to any of claims 1-10, which carries a label, e.g. a label selected from the group consisting of fluorophores, radioactive isotopes, and complexing agents such as biotin.

42. A method of quantifying the amount of an α-amylase messenger present in an organism, preferably a plant, preferably a dicotyledonous plant, said method comprising the steps of hybridizing a nucleic acid containing sample obtained from said organism with a DNA fragment according to any of claims 1-10 or 41 in denatured form or a RNA copy thereof under conditions favorable to hybridization between the denatured DNA fragment or RNA copy and the nucleic acid of the sample and determining the amount of hybridized nucleic acid.

43. A method of isolating an α-amylase gene or α-amylase cDNA from a genomic or a cDNA library of an organism, preferably a plant, preferably a dicotyledonous plant, said method comprising the steps of hybridizing a nucleic acid containing sample obtained from the library with a DNA fragment according to any of claims 1-10 or 41 in a denatured form or a RNA copy thereof under conditions favorable to hybridization between the DNA fragment or RNA copy and the nucleic acid of the sample, and recovering the hybridized clone so as to obtain said α-amylase gene or cDNA of said organism.

44. A method of detecting the presence of an α-amylase gene or a part thereof in an organism, preferably a plant, preferably a dicotyledonous plant, said method comprising the steps of subjecting a sample comprising a DNA fragment which comprises said α-amylase gene or a part thereof to amplification by use of a DNA fragment according to any of claims 1-10 in accordance with the polymerase chain reaction technique.

45. A method of estimating the tendency of plant breeding material to form reducing sugar, comprising the steps of determining the amount of α-amylase messenger(s) or gene(s) in a sample of the plant according to the method of claim 42 and correlating the amount with a previously established standard.

46. The method according to any of claims 42-45, in which the organism is a dicotyledonous plant, preferably *Solanum*, preferably *Solanum tuberosum* or *S. melongena* (aubergine), *Lycopersicon*, preferably *Lycopersicon esculentum* (tomato), *Capsicum*, preferably *Capsicum annuum* (pepper), *Nicotiana*, preferably *Nicotiana tabacum* (tobacco), *Ipomoea*, preferably *Ipomoea batatus* (sweet potato), *Brassica*, *Medicago*, *Trifolium* spp. (clover), *Glycine max* (soybean), *Arachis*, or a root crop such as *Beta*, preferably *Beta vulgaris* (sugar beet).

47. A method of analyzing an organism for alleles comprising a DNA fragment according to any of claims 1-10 or a homologue thereof, comprising the steps of hybridizing nucleic acid of said organism with a DNA fragment according to any of claims 1-10 and evaluating the hybridization pattern in accordance with the principles of restriction fragment length polymorphism.

Description

α-Amylase, which is involved in the hydrolysis of starch, is present in all plants. α-Amylase in monocotyledonous plants such as barley and wheat has been extensively investigated and α-amylase genes from these monocotyledonous plants have been isolated. α-amylase from dicotyledonous plants has also been investigated, but only at the enzymatic level. It has not been possible to isolate or characterize α-amylase genes from dicotyledonous plants.

The present invention concerns DNA fragments related to the encoding of α-amylase in dicotyledonous plants and to important developments based on the provision of such DNA fragments.

The DNA fragments according to the invention and variants thereof, e.g., an α-amylase encoding part of a nucleotide sequence shown in Fig. 1-5, as well as subsequences and analogues thereof constitute the basis for transgenic plant strategies for modifying various essential properties of plants, e.g. of the genus *Solanum tuberosum*, such as deliberately decreasing α-amylase activity to reduce the risk of Maillard reactions and taste modification/degradations in connection with the production of potato chips etc., or for deliberately increasing α-amylase activity in potatoes which are to be used for fermentation for the production of spirits. These strategies, as well as other economically important transgenic plant strategies resulting in modification of the properties of plants made possible through the present invention are explained in greater detail below.

Besides their very important use for direct consumption, potatoes are used for many different industrial purposes such as in a raw material in the production of potato chips and in the production of alcohol. In both types of production, the degradation of starch in the potatoes is of importance and thus, α-amylase involved in the degradation of starch is an important enzyme, as explained below.

In the production of potato chips, the degradation of starch to reducing sugars is critical. A high degree of starch degradation resulting in a high content of reducing sugars causes a problem, since reducing sugar is subjected to the so-called Maillard reactions during the frying of the potatoes in vegetable oil, which causes the chips to acquire an undesirable brown colour and an unpleasant burned taste, accompanied by undesirable alterations in the flavour and texture of the potato chips.

On the other hand, a high content of reducing sugar resulting from a high α-amylase activity is desirable when potatoes are to be used for the production of alcohol.

The present invention now opens up the possibility of regulating α-amylase activity by transgenic plant strategies. This and other aspects of the invention will appear from the following description.