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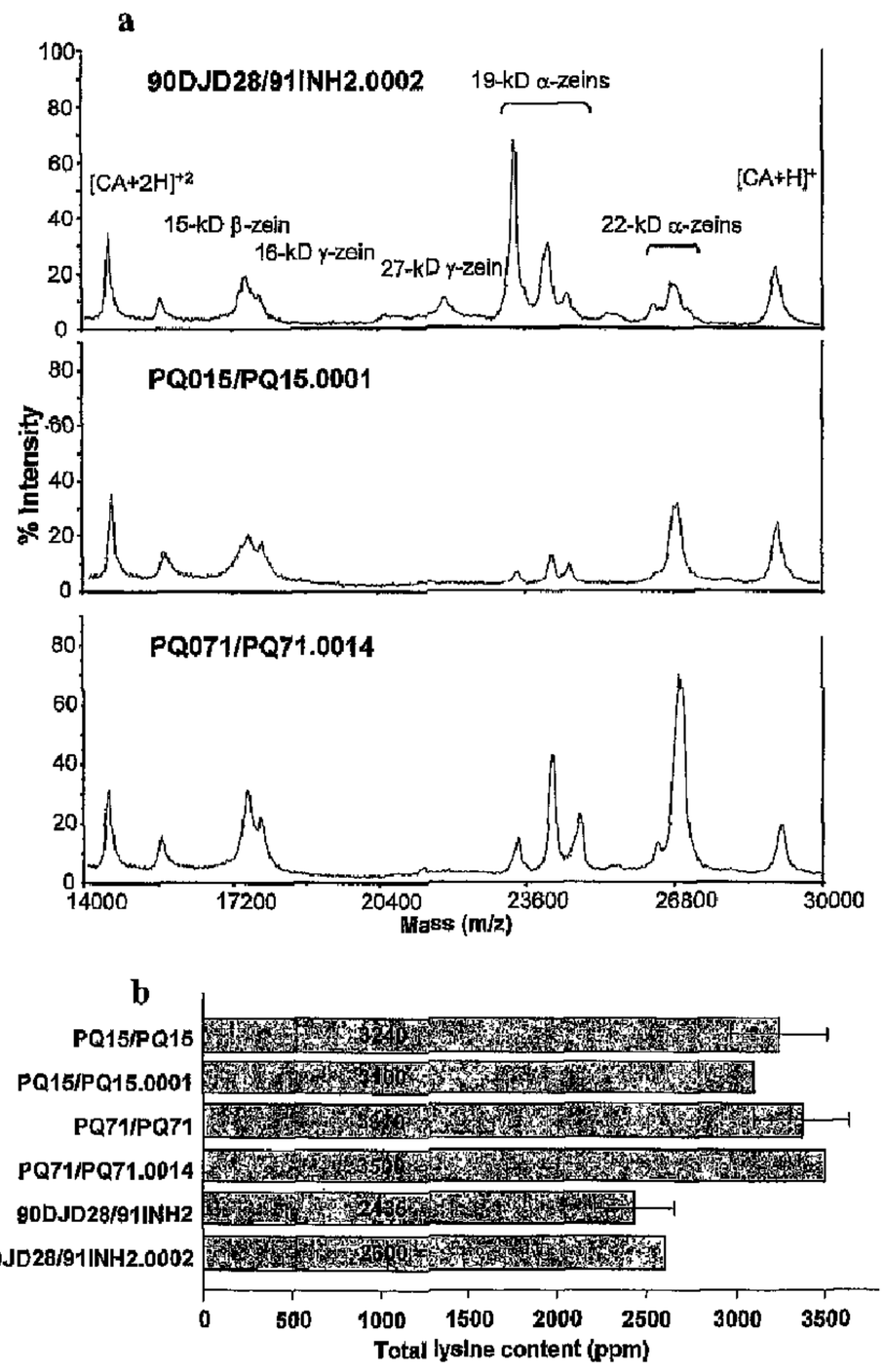
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(54) **Titre : GRAINE DE MAIS PRESENTANT UN TAUX DE LYSINE AMELIORE DE FACON SYNERGIQUE**
(54) **Title: MAIZE SEED WITH SYNERGISTICALLY ENHANCED LYSINE CONTENT**



(57) **Abrégé/Abstract:**
The present invention provides a transgenic maize plant having in its genome transgenic DNA including sequence for zein reduction and sequence for lysine biosynthesis, whereby expression of the transgenic DNA results in a synergistically increased lysine content of seed of the transgenic maize plant. The invention further provides a method for providing maize seed with synergistically increased lysine content.

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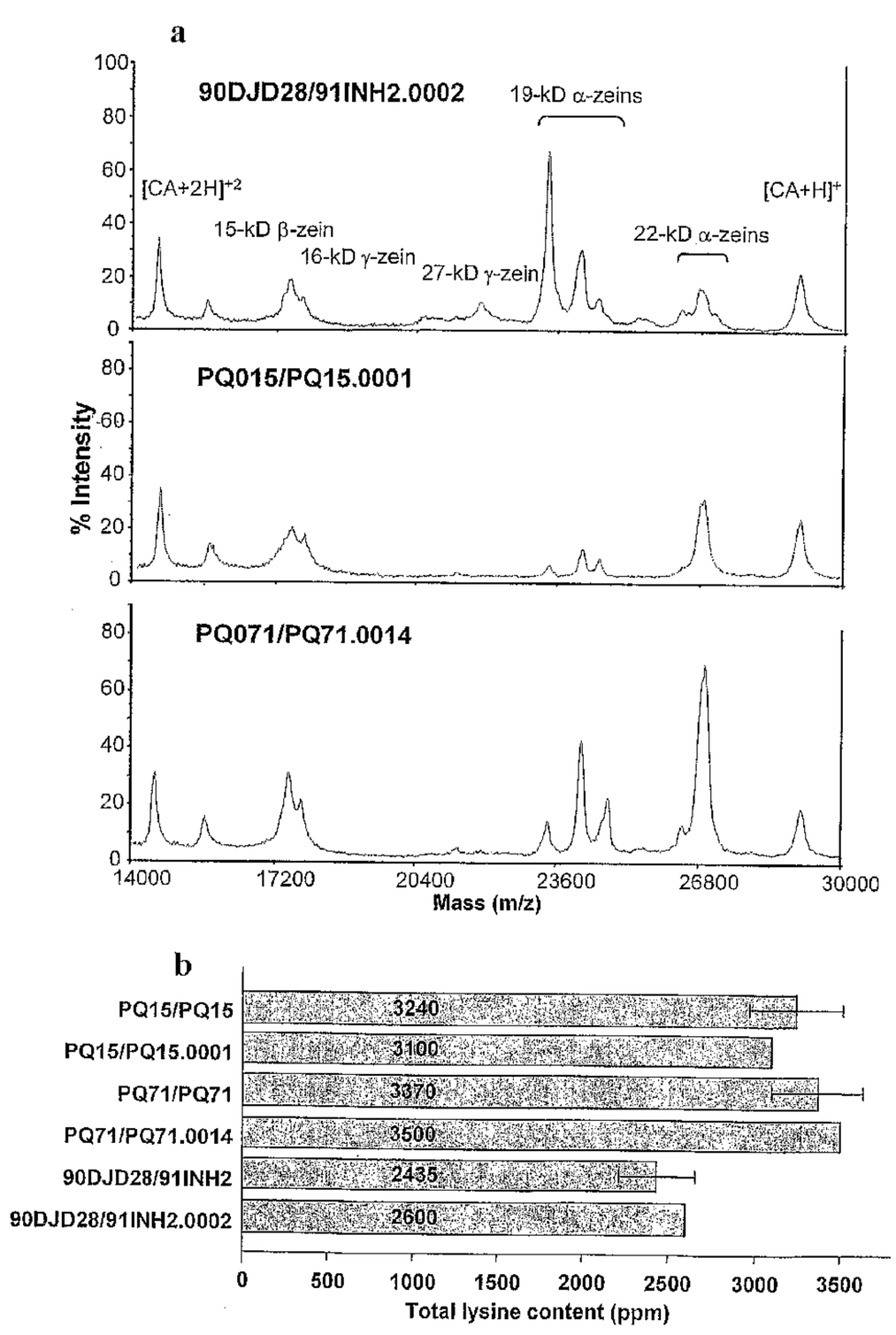
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(54) Title: MAIZE SEED WITH SYNERGISTICALLY ENHANCED LYSINE CONTENT

(57) Abstract: The present invention provides a transgenic maize plant having in its genome transgenic DNA including sequence for zein reduction and sequence for lysine biosynthesis, whereby expression of the transgenic DNA results in a synergistically increased lysine content of seed of the transgenic maize plant. The invention further provides a method for providing maize seed with synergistically increased lysine content.



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DESCRIPTION

MAIZE SEED WITH SYNERGISTICALLY ENHANCED LYSINE CONTENT

FIELD OF THE INVENTION

Disclosed herein are seeds for transgenic maize having elevated amino acid levels, particularly of lysine, recombinant DNA constructs for gene suppression, methods of making and using such constructs, and transgenic organisms, including plants, expressing such constructs.

BACKGROUND OF THE INVENTION

Zea mays, commonly known as maize or corn, is a grain important both as human food and as animal feed. Maize seeds or kernels are naturally low in lysine content due to its protein composition. The majority of maize seed proteins are the zeins or prolamins, which are found in the endosperm and account for more than half of the total seed proteins. Zeins or prolamins are rich in proline, alanine and glutamine, but almost completely devoid of the essential amino acids, lysine and tryptophan. Due to the relative abundance of zeins, the contribution of lysine and tryptophan from other seed proteins is diluted. Exogenous lysine is often added to animal feed as a necessary supplement. It is therefore of interest to increase the level of lysine in maize seed.

Amino acid levels in transgenic plants can be manipulated by various genetic techniques, including, but not limited to, modification of endogenous gene expression and/or expression of exogenous recombinant DNA in the transgenic plant. Coordinated decrease and increase of gene expression of more than one gene using transgenic constructs is disclosed in U. S. Patent Application Publication 2004/0126845. Methods and recombinant DNA constructs useful for producing anti-sense-oriented RNA for gene suppression in transgenic organisms are disclosed in U.S. Patent Publication 2005/0176670. U.S. Patent Publication No. 2006/0064772 discloses DNA cassettes for suppressing at least one gene in a cell of a eukaryote, such DNA cassettes comprising a promoter operably linked to transcribable DNA including at least an intron, a polyadenylation signal, and a polyadenylation site, where there is embedded in the intron heterologous DNA which is

derived from at least one gene targeted for suppression and which is transcribable to RNA capable of suppressing the at least one gene.

SUMMARY OF THE INVENTION

The present invention discloses a transgenic maize plant having in its genome transgenic DNA including sequence for zein reduction and sequence for lysine biosynthesis, whereby expression of the transgenic DNA results in a synergistically increased lysine content of seed of the transgenic maize plant. In one aspect of this invention, the sequence for zein reduction includes sequence for gene suppression of at least one zein synthesis gene. In another aspect of this invention, the sequence for lysine biosynthesis includes an exogenous lysine synthesis gene. In a further aspect of this invention, the sequence for lysine biosynthesis includes sequence for gene suppression of a lysine catabolic enzyme endogenous to the transgenic maize plant. Both primary transformed transgenic plants and their transgenic progeny are disclosed and claimed by the present invention.

The invention also provides a method for providing maize seed with synergistically increased lysine content, including:

(a) providing a transgenic maize plant having in its genome transgenic DNA including sequence for zein reduction and sequence for lysine biosynthesis,

(b) expressing the transgenic DNA in seed of the transgenic maize plant, the expressing resulting in a synergistically increased lysine content of the seed, and

(c) harvesting the seed with synergistically increased lysine content.

Embodiments of the method of the invention include providing a transgenic maize plant by transformation, as well as providing a transgenic maize plant by genetic crossing techniques. Other specific embodiments of the invention are disclosed in the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a depicts matrix-assisted laser desorption ionization time-of-flight mass spectra analyses of seed from first parent transgenic maize plants PQ015/PQ15.0001 and PQ071/PQ71.0014, which have in their genome a sequence for zein reduction, showing the reduction in 19-kiloDalton alpha-zeins compared to the wild-type maize line 90DJD28/91INH2.0002 which has a genotype substantially similar to the first transgenic maize plants but lacks the sequence for zein reduction. See **Example 1**. X-axis is shown as

m/z (mass to charge) ratios. Y-axis is shown as percent intensity. Mass spectra were normalized by a standard protein peak, $[CA+H]^+$ (carbonic anhydrase), near 29,000 *m/z*. The 15,615 Dalton peak between $[CA+H]^{2+}$ and 15-kiloDalton beta-zein was believed to be a contaminant from carbonic anhydrase. **Figure 1b** depicts the total lysine content of first parent transgenic maize plants PQ015/PQ15.0001 and PQ071/PQ71.0014 compared to the wild-type maize lines 90DJD28/91INH2 and 90DJD28/91INH2.0002. The error bars on total lysine contents of PQ015/PQ15, PQ071/PQ71, and 90DJD28/91INH2 represent the confidence intervals (alpha = 0.05, *n* =20).

Figure 2a-d depicts results of characterization assays of seed from M27908+.0028, representative of a second parent transgenic maize plant (M27908+) having in its genome transgenic DNA including sequence for lysine biosynthesis (*cordapA*), and seed from M27908-.0006, representative of the wild-type maize line M27908- which has a genotype substantially similar to M27908+ but lacks the sequence for lysine biosynthesis. See **Example 1**. **Figure 2a** depicts results of free lysine analyses. The error bars are the standard deviations of sibling ears. **Figure 2b** depicts results of cordapA Western analysis of individual mature kernels. An embryo-specific expression of CordapA was observed, and the homozygosity of the ear, M27908+.0028 was confirmed. **Figure 2c** depicts CordapA ELISA results, which further confirmed the embryo-specific accumulation of CordapA. The error bars are the standard deviations of experimental replicates. **Figure 2d** depicts results of a lysine insensitive DHDPS activity assay. The concentration of lysine used in the DHDPS activity assay was 1 millimolar.

Figure 3 depicts representative matrix-assisted laser desorption ionization time-of-flight mass spectra analyses of seed from F1 progeny transgenic maize plants PQ15/CordapA and PQ71/CordapA, showing similar zein profiles to the respective parent transgenic maize plants PQ015/PQ15.0001 ("PQ15") and PQ071/PQ71.0014 ("PQ71"), which have in their genome a sequence for zein reduction. See **Example 1**. Mass spectra of seed from CordapA and Control are comparable to 90DJD28/91INH2.0002. X-axis is shown as *m/z* (mass to charge) ratios. Y-axis is shown as percent intensity.

Figure 4 depicts CordapA ELISA results for dissected seed from F1 progeny transgenic maize plants, PQ15/CordapA and PQ71/CordapA, confirming embryo-specific expression of the sequence for lysine biosynthesis, *cordapA*. See **Example 1**. In PQ15/CordapA and PQ71/CordapA, the expression of CordapA is higher than in the control F1, CordapA, which lacks the sequence for zein reduction. The error bars represent the confidence intervals (alpha = 0.05, *n* >20). N. D., not detected.

Figure 5a-e depicts Pearson's correlations between free lysine and other components of the lysine biosynthesis pathway in the F1 progeny transgenic maize plants, PQ15/CordapA and PQ71/CordapA and the parent transgenic plant CordapA. See **Example 1, Table 3**. **Figure 5a** shows correlation between free lysine and free aspartate. **Figure 5b** shows correlation between free lysine and free asparagine. **Figure 5c** shows correlation between free lysine and free glutamate. **Figure 5d** shows correlation between free lysine and free saccharopine. **Figure 5e** shows correlation between free lysine and CordapA expression. Each data point represents bulked ground ears harvested from a plot and two plots for each of the three F1s are used. Numbers within each graph correspond to r^2 values of linear regressions followed by significance levels (*P*).

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the manufacture or laboratory procedures described below are well known and commonly employed in the art. Conventional methods are used for these procedures, such as those provided in the art and various general references. Where a term is provided in the singular, the inventors also contemplate aspects of the invention described by the plural of that term. The nomenclature used herein and the laboratory procedures described below are those well known and commonly employed in the art. Where there are discrepancies in terms and definitions used in references, the terms used in this application shall have the definitions given herein. Other technical terms used herein have their ordinary meaning in the art that they are used, as exemplified by a variety of technical dictionaries. The inventors do not intend to be limited to a mechanism or mode of action. Reference thereto is provided for illustrative purposes only.

Transgenic Plants

The present invention provides a transgenic maize plant having in its genome transgenic DNA including sequence for zein reduction and sequence for lysine biosynthesis, whereby expression of the transgenic DNA results in a synergistically increased lysine content of seed of the transgenic maize plant.

The transgenic DNA can be introduced into the genome of a maize plant by any one or more suitable means, including, but not limited to, direct delivery of DNA such as by PEG-mediated transformation of protoplasts, electroporation, agitation with silicon carbide fibers,

Agrobacterium-mediated transformation, acceleration of DNA-coated particles, and other like methods known in the art. Maize cells, as well as those of virtually any other plant species, may be stably transformed by such methods, and these cells developed into transgenic plants of the invention. Preferred methods of plant transformation include, but are not limited to, microprojectile bombardment as illustrated, for example, in U. S. Patent Nos. 5,015,580, 5,550,318, 5,538,880, 6,160,208, 6,399,861, and 6,403,865 and *Agrobacterium*-mediated transformation as illustrated, for example, in U. S. Patent Nos. 5,635,055, 5,824,877, 5,591,616, 5,981,840, and 6,384,301.

The transgenic DNA includes sequence for zein reduction and sequence for lysine biosynthesis, which sequences are different from each other and can be introduced into the genome of the transgenic plant by means of one or more nucleic acid constructs (that is to say, introduced simultaneously in one construct or individually using more than one construct, separately or in parallel). Preferably, the sequence for zein reduction and sequence for lysine biosynthesis are expressed primarily in the seed of the transgenic plant, for example, by use of a seed-specific promoter in the construct or constructs, such as promoters from seed genes such as, but not limited to, napin (U. S. Patent No. 5,420,034), maize L3 oleosin (U. S. Patent No. 6,433,252), zein Z27 and glutelin 1 (Russell *et al.* (1997) *Transgenic Res.*, 6:157-166), globulin 1 (Belanger *et al.* (1991) *Genetics,* 129:863-872), and peroxiredoxin antioxidant (Per1) (Stacy *et al.* (1996) *Plant Mol Biol.,* 31:1205-1216).

Expression of the transgenic DNA results in a synergistically increased lysine content of seed of the transgenic maize plant. The synergistically increased lysine content is an increase in the lysine content that is greater than the sum of the increase in lysine due to the individual effect of the sequence for zein reduction and the increase in lysine due to the individual effect of the sequence for lysine biosynthesis. Expression of the sequence for zein reduction and sequence for lysine biosynthesis preferably does not result in undesirable traits (for example, lower yields, increased susceptibility to pests, diseases, or environmental stress, and decreased seed processing or storage qualities).

The sequence for zein reduction can include any sequence that results in the reduction or decrease of one or more zeins in the maize seed; such as sequence for gene suppression of at least one zein synthesis gene. In one non-limiting embodiment, the sequence for zein reduction includes a sequence for reducing (for example, by gene suppression) seed levels of an alpha-zein, such as a 19-kiloDalton alpha-zein or a 22-kiloDalton alpha-zein. In other embodiments, the sequence for zein reduction can include a sequence for reducing seed levels

of any one or more of the zein proteins of interest, such as the alpha-, beta-, gamma-, and delta-zeins.

Reduction of zein seed levels can be by any suitable mechanism, including, but not limited to, gene suppression of one or more endogenous zein synthesis genes, or deletion or mutation of one or more endogenous zein synthesis genes, or replacement of one or more endogenous zein synthesis genes with a non-endogenous zein synthesis gene or gene homologue that results in substantially decreased zein seed levels, or transcriptional suppression of zein synthesis gene expression. Gene suppression includes, but is not limited to, any of the well-known methods for suppressing an RNA transcript or production of protein translated from an RNA transcript, including post-transcriptional gene suppression and transcriptional suppression (see, for example, Matzke *et al.* (2001) *Curr. Opin. Gen. Dev.,* 11:221-227 (2001), and Meister & Tuschl (2004) *Nature,* 431:343-349). Non-limiting methods for effecting gene suppression include gene suppression mediated by inserting a recombinant DNA construct with anti-sense oriented DNA (see, for example, U. S. Patent Nos. 5,107,065 and 5,759,829; gene suppression by integration of DNA arranged as an inverted repeat resulting from co-insertion of several copies of a transfer DNA into plants by *Agrobacterium*-mediated transformation (see, for example, Redenbaugh *et al.* in "Safety Assessment of Genetically Engineered Flavr Savr™ Tomato, CRC Press, Inc. (1992), Stam *et al.* (1997) *Plant J.,* 12:63-82, and Sanders and Hiatt (2005) *Nature Biotechnol.,* 23:287-289); gene suppression by inserting a recombinant DNA construct with sense-oriented DNA (see, for example, U. S. Patent Nos. 5,283,184 and 5,231,020), gene suppression by transcribing RNA from both a sense and an anti-sense oriented DNA using two separate transcription units (see, for example, U. S. Patent No. 5,107,065); gene suppression by providing transformation constructs that are capable of generating an RNA that can form double-stranded RNA along at least part of its length (see, for example, published European Patent Application EP 0426195 A1, Sijen *et al.* (1996) *The Plant Cell,* 8:2277-2294, International Patent Publication Nos. WO98/53083, WO 99/53050, WO 99/49029, and U. S. Patent Application Publication Nos. 2003/0175965, 2003/0036197, 2003/0018993, and 2002/0048814); and gene suppression by transcriptional suppression such as promoter *trans* suppression (see, for example, Mette *et al.* (1999) *EMBO J.,*18:241-148, and Mette *et al.* (2000) *EMBO J.,* 19:5194-5201). Gene suppression can also be obtained by means of a sequence encoding a DNA or RNA aptamer, as is known in the art (see, for example, Toulme *et al.* (2004) *FEBS Lett.,* 567: 55-62, Lee *et al.* (2004) *Nucleic Acids Res.,* 32:95-100, and Nimjee *et al.* (2005) *Ann. Rev. Med.,* 56:555-

583).

One recombinant DNA construct useful in introducing sequence for zein reduction into the transgenic plant of the invention is described at length in U.S. Patent Publication No. 2005/0176670. In one non-limiting embodiment, the sequence for zein reduction can include a recombinant DNA construct for suppressing at least one zein gene, which includes, in 5' to 3' order, a promoter element operably linked to an anti-sense-oriented DNA element from the at least one zein gene and a sense-oriented DNA element, wherein the sense-oriented DNA element is shorter than the anti-sense-oriented DNA element, and sense-oriented RNA transcribed by the sense-oriented DNA element is complementary to the 5'-most end of anti-sense-oriented RNA transcribed by the anti-sense-oriented RNA transcribed by the anti-sense-oriented DNA element, wherein the transcribed RNA forms a loop of anti-sense-oriented RNA for suppressing the at least one zein gene. In another embodiment, the sequence for zein reduction can be introduced in the transgenic plant of the invention by providing in cells of the transgenic plant a recombinant DNA construct which is transcribed to RNA that forms a loop of anti-sense-oriented RNA for suppressing at least one zein gene.

The sequence for lysine biosynthesis can include sequence for any gene or genes that confer an increase in lysine levels in the transgenic plant of the invention. Thus, the sequence for lysine biosynthesis can include an exogenous lysine synthesis gene sequence encoding enzymes for synthesis of lysine or its precursors, and/or sequence for gene suppression of a lysine catabolic enzyme endogenous to the transgenic maize plant. Lysine synthesis genes include genes encoding or controlling expression of enzymes for synthesis of lysine or its precursors, such as aspartate kinase (AK) and dihydrodipicolinic acid synthase (DHDPS), and homologues of these genes. Lysine catabolic genes include, but are not limited to, the maize LKR/SDH gene encoding lysine-ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH), and its homologues. Useful homologues of these lysine synthesis or catabolic genes may be identified from nucleic acid or protein sequence databases, for example, by using comparison tools known to those in the art, such as, but not limited to, algorithms such as BLAST (Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402).

In one preferred embodiment, the sequence for lysine biosynthesis includes sequence for at least one exogenous lysine synthesis gene, preferably a substantially feedback-insensitive lysine synthesis gene. Substantially feedback-insensitive lysine synthesis genes

include, but are not limited to an aspartate kinase that is substantially insensitive to inhibition by lysine such as an *E. coli* AKIII (see U. S. Patent Nos. 6,459,019 and 5,773,691 and U. S. Patent Application Publication No. 2003/0056242), or a dihydrodipicolinic acid synthase that is substantially insensitive to inhibition by lysine, such as an *E. coli* DHDPS (see U. S. Patent Nos. 5,288,300, 6,459,019, and 5,773,691 and U. S. Patent Application Publication No. 2003/0056242) or a *Corynebacterium* DHDPS or *cordapA* (see U. S. Patent Nos. 6,459,019 and 5,773,691 and U.S. Patent Application Publication No. 2003/0056242). See also U.S. Patent Publication No. 2005/0132437 to Dizigan *et al.*, "High Lysine Maize Compositions and Methods for Detection Thereof", filed 2 December 2004 which discloses a *Corynebacterium* DHDPS or *cordapA*, which would be a suitable substantially feedback-insensitive lysine synthesis gene.

In another preferred embodiment, the sequence for lysine biosynthesis includes sequence for gene suppression of at least one lysine catabolic enzyme endogenous to said transgenic maize plant, such as a sequence for gene suppression of the LKR or SDH enzymes endogenous to the maize plant. In yet another preferred embodiment, the sequence for lysine biosynthesis includes both sequence for at least one exogenous lysine synthesis gene and sequence for gene suppression of at least one lysine catabolic enzyme endogenous to said transgenic maize plant. In still yet another preferred embodiment, the sequence for lysine biosynthesis includes both sequence encoding a substantially feedback-insensitive lysine synthetic enzyme and a sequence for gene suppression of a lysine catabolic enzyme endogenous to the transgenic maize plant.

The constructs and methods described in U.S. Patent Publication No. 2006/0064772 are particularly useful in introducing sequence for lysine biosynthesis into a transgenic plant of the present invention. In one specific and particularly preferred embodiment, the transgenic plant of the invention has in its genome a sequence for lysine biosynthesis including (a) intron sequence within which is embedded a sequence for gene suppression of at least one lysine catabolic enzyme endogenous to said transgenic maize plant, and, optionally, (b) sequence encoding a substantially feedback-insensitive lysine synthetic enzyme.

The present invention contemplates and claims both maize plants directly regenerated from cells which have been transformed with transgenic DNA including sequence for zein reduction and/or sequence for lysine biosynthesis, as well as progeny of such plants, for example, inbred progeny and hybrid progeny of transformed maize plants. This invention

contemplates transgenic maize plants produced by direct transformation with transgenic DNA including sequence for zein reduction and/or sequence for lysine biosynthesis, and transgenic plants made by crossing a plant having transgenic DNA of the invention to a second plant lacking the construct. In one embodiment of the present invention, the transgenic maize plant can include a progeny transgenic maize plant from genetic crossing of a first parent transgenic maize plant having in its genome transgenic DNA including sequence for zein reduction, and a second parent transgenic maize plant having in its genome transgenic DNA including sequence for lysine biosynthesis, wherein the progeny transgenic maize plant from the genetic crossing has in its genome the sequence for zein reduction and the sequence for lysine biosynthesis; seed of the progeny transgenic maize plant has a synergistically increased lysine content when compared to seed from the first and second parent transgenic maize plants.

In a preferred embodiment, seed from the first parent transgenic maize plant having in its genome transgenic DNA including sequence for zein reduction has a higher lysine content than seed from a maize plant with a genotype substantially similar to the first transgenic maize plant but lacking the sequence for zein reduction, and seed from the second parent transgenic maize plant having in its genome transgenic DNA including sequence for lysine biosynthesis also has a higher lysine content than seed from a maize plant with a genotype substantially similar to the first transgenic maize plant but lacking the sequence for lysine biosynthesis. Seed of the progeny transgenic maize plant has a synergistically increased lysine content when compared to seed from the first and second parent transgenic maize plants.

In another embodiment, seeds of transgenic maize plants of the present invention can be harvested from fertile transgenic plants and be used to grow progeny generations of transgenic maize plants of this invention, including hybrid or inbred plant lines that contain in their genome the transgenic DNA including sequence for zein reduction and sequence for lysine biosynthesis and that produce seed with a synergistically increased lysine content.

Method for Providing High-Lysine Maize Seed

The present invention discloses and claims a method for providing maize seed with synergistically increased lysine content, including:

(a) providing a transgenic maize plant having in its genome transgenic DNA including sequence for zein reduction and sequence for lysine biosynthesis,

(b) expressing the transgenic DNA in seed of the transgenic maize plant, the expressing resulting in a synergistically increased lysine content of the seed, and

(c) harvesting the seed with synergistically increased lysine content.

The transgenic maize plant useful in the method of the invention is described above at length under the heading "Transgenic Plants". Preparation of nucleic acid constructs for transformation of plant cells and production of the transgenic plant makes use of techniques well known in the art. See, for example, methodologies disclosed in Sambrook and Russell, "Molecular Cloning: A Laboratory Manual", third edition, Cold Spring Harbor Laboratory Press, 2001. One versed in the art would be familiar with techniques for transforming plant cells to provide a transgenic plant useful in the method of the invention. See, for example, microprojectile bombardment methods as disclosed in U.S. Patent Nos. 5,550,318, 5,538,880, 6,160,208, and 6,399,861, and *Agrobacterium*-mediated transformation methods as described in U.S. Patent No. 5,591,616. Useful techniques for transforming plant cells using site-specific integration include the cre-lox system disclosed in U.S. Patent No. 4,959,317 and the FLP-FRT system disclosed in U.S. Patent No. 5,527,695.

Transformation of plant cells to yield transgenic plants useful in the method of this invention is preferably practiced in tissue culture on media and in a controlled environment. Practical transformation methods and materials for making transgenic plants useful in the method of this invention, e. g., various media and recipient target cells, transformation of immature embryos, and subsequent regeneration of fertile transgenic plants, are disclosed in U.S. Patent Nos. 6,194,636 and 6,232,526.

After delivery of the transgenic DNA to recipient plant cells, transformed cells are generally identified for further culturing and plant regeneration. To improve the ability to identify transformants, one may employ a selectable or screenable marker gene, where the potentially transformed cell population can be assayed by exposing the cells to a selective agent or agents or screened for the desired marker gene trait. Non-limiting examples of screenable markers include a gene expressing a colored or fluorescent protein such as a luciferase or green fluorescent protein (GFP), or a gene expressing a *beta*-glucuronidase or *uidA* gene (GUS) for which various chromogenic substrates are known. Non-limiting examples of selectable markers include those conferring resistance to antibiotics such as kanamycin and paromomycin (*nptII*), hygromycin B (*aph IV*) and gentamycin (*aac3* and *aac*C4) or resistance to herbicides such as glufosinate (*bar* or *pat*) and glyphosate (*aroA* or EPSPS); particularly useful examples of such selectable markers are illustrated in U. S. Patent Nos. 5,550,318, 5,633,435, 5,780,708, and 6,118,047.

The method of the invention may provide the transgenic maize plant by genetic crossing of a first parent transgenic maize plant having in its genome transgenic DNA including sequence for zein reduction and a second parent transgenic maize plant having in its genome transgenic DNA including sequence for lysine biosynthesis, wherein the genetic crossing results in a progeny transgenic maize plant having in its genome the sequence for zein reduction and the sequence for lysine biosynthesis.

Expression of the transgenic DNA preferably is substantially localized in seed of the transgenic maize plant, for example, substantially localized in the embryo, in the endosperm, or in both the embryo and endosperm, using, for example, promoters described above under the heading "Transgenic Plants". Such expression results in a synergistically increased lysine content of the seed, most preferably a synergistically increased lysine content of the seed at the time of the seed's harvest from the transgenic maize plant. The seed may be harvested and used for any purpose of interest, including, for example, for planting purposes, or for processing into human food products, animal feed products, oils, starches, pharmaceuticals, and various industrial products. Exemplary discussions of the uses of maize can be found, for example, in U. S. Patent Nos. 6,194,636, 6,207,879, 6,232,526, 6,426,446, 6,429,357, 6,433,252, 6,437,217, and 6,583,338 and PCT Publications WO 95/06128 and WO 02/057471.

EXAMPLES

Example 1

This non-limiting example illustrates a method of the invention for providing maize seed with synergistically increased lysine content, and further illustrates a transgenic maize plant of the invention having in its genome transgenic DNA including sequence for zein reduction and sequence for lysine biosynthesis, whereby expression of the transgenic DNA results in a synergistically increased lysine content of seed of the transgenic maize plant. More specifically, this example describes the genetic crossing of first parent transgenic maize plants (zein reduction lines PQ15 and PQ71, which have in their genome a sequence for zein reduction and show reduction in zein accumulation) and second parent transgenic maize plants (high-lysine line M27908+, which has in its genome a sequence for lysine biosynthesis and shows elevated free lysine caused by expression of an exogenous *cordapA* gene). These genetic crosses demonstrated that synergistically increased lysine content could be obtained in maize seed from an exemplary transgenic maize plant of the invention.

TRANSGENIC PLANTS

Zein reduction lines

A vector designed to reduce zein was constructed and used for transformation as described in detail by Huang *et al.* (2004) *J. Agric. Food Chem.,* 52:1958-1964.

Briefly, a DNA fragment containing nucleotides 965 – 868 (in the antisense orientation) of the maize 19-kiloDalton alpha-zein gene, *Z4* (GenBank accession No. V01472), was isolated by PCR. This zein gene coding region fragment was inserted, in the antisense orientation, into an expression cassette that also contained the gamma-zeinA promoter (corresponding to nucleotides 19 – 1118 of the sequence with GenBank assession No. S78780) to drive high mRNA synthesis, and the 3'untranslated region of the *Agrobacterium tumefaciens* nopaline synthase gene, to provide polyadenylation sequence. These expression cassettes were introduced into immature maize embryos by microprojectile bombardment.

Regenerated R0 transgenic plants were pollinated with wild-type pollen to produce F1 seeds, which were screened for the opaque phenotype and analyzed by SDS-PAGE gels to evaluate the zein profiles. Among the R0 lines found to display a 19-kiloDalton alpha-zein reduction phenotype, two R0 lines, "PQ15" and "PQ71" (containing 3 copies and 6 copies, respectively, of the transgene) were used in the genetic crossing study. The selected ears for this study, PQ015/PQ15.0001 and PQ071/PQ71.0014, exhibit MALDI-TOF (Matrix-assisted laser desorption ionization time-of-flight) mass spectra that are representative of PQ15 and PQ71 (see **Figure 1a**). PQ15 has the most dramatic reduction in 19-kiloDalton alpha-zeins and a slight increase in 22-kiloDalton alpha-zeins, while PQ71 maintains a modest reduction in 19-kiloDalton alpha-zeins, with a large increase in 22-kiloDalton alpha-zeins. Both selected ears also have higher total lysine content than wild-type ears (see **Figure 1b**).

High lysine lines

Binary plasmids were designed to express *cordapA*, the lysine-insensitive dihydrodipicolinic acid synthase (DHDPS) from *Corynebacterium glutamicum* (see U. S. Patent No. 5,773,691). Construction and use of these binary plasmids is described in detail by Huang *et al.* (2004) *Transgenic Res.* 13:451-461. Briefly, the binary plasmids contained left and right T-DNA border regions flanking an expression cassette that included the maize *glbl* promoter, a rice actin intron, the *cordapA* coding region with a maize DHDPS leader peptide, and the maize *glbl* terminator. This expression cassette is designed for embryo-specific expression of *cordapA*. The binary plasmids also included a selectable

marker conferring glyphosate resistance, *epsps-cp4* (5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium tumefaciens* strain CP4). Conventionally, the selectable marker (*epsps-cp4*) would be typically placed adjacent to the gene of interest (*cordapA*), allowing both the *cordapA* expression cassette and the *epsps-cp4* selectable marker to be co-integrated when transformed into a plant cell, thus enhancing recovery of plants containing the *cordapA* transgene by selection under glyphosate. Instead, alternative binary vectors were constructed (see Figure 3 in Huang *et al.* (2004) *Transgenic Res.*, 13:451-461), where the *epsps-cp4* marker was either (1) placed in would normally be considered the "backbone" of the vector, rather than within the T-DNA region and adjacent to the gene of interest, *cordapA* (vectors pMON65178 and pMON65179), or (2) located between a second set of left and right T-DNA border regions (vector pMON65180).

R0 plants that were identified as positive for *cordapA* were assayed by Southern blot to determine the frequency of plants containing *cordapA* unlinked to the selectable marker, *epsps-cp4*. The vector that produced the highest percentage (35.6%) of unlinked events was pMON65178, which contained *epsps-cp4* in the backbone of the vector and adjacent to the *cordapA* expression cassette. R1 seedlings derived from R0 plants with unlinked insertions were tested for the segregation of *epsps-cp4* and *cordapA* by PCR.

Marker-free segregants that were *cordapA*-positive and *epsps-cp4*-negative were identified from 93% of unlinked events transformed by pMON65178. pMON65178 included right border and left border sequences flanking an expression cassette containing the maize *glb1* promoter, a rice actin intron, the *cordapA* coding region (nucleotides 3 through 903 of the sequence disclosed as SEQ ID NO: 6 in U. S. Patent No. 6,459,019)
with a maize DHDPS leader peptide, and the maize *glb1* terminator, with
the *epsps-cp4* marker placed in the plasmid "backbone" and adjacent to the T-DNA region. Other functional *cordapA* genes could be used, such as, but not limited to, the full length 917 base pair sequence disclosed as SEQ ID NO: 6 in U. S. Patent No. 6,459,019, or the *Corynebacterium* dapA gene sequence published by Bonnassie *et al.* (1990) *Nucleic Acids Res.*, 18:6421, or lysine-insensitive homologues of these genes which may be identified by sequence comparison tools known in the art, for example, a BLAST algorithm. One of the marker-free *cordapA* transgenic lines generated by this approach was M27908. Seeds derived from this event have an average of 2505 ppm free lysine content compared to seeds from its wild-type segregant, which contain an average of 71 ppm free lysine.

Transgenic lines M27908+.0028 and M27908-.0006 were used as the *cordapA* and wild-type control in the crossing experiment, which contain free lysine levels of 2706 and 64 ppm, respectively (**Figure 2a**). The embryo-specific expression of *cordapA* was confirmed by Western blots and ELISA assays (**Figure 2b** and **Figure 2c**). Transgenic M27908+.0028 embryos also displayed elevated DHDPS activity, even in the presence of lysine (**Figure 2d**).

Genetic crossing of reduced-zein and high-lysine maize lines

Genetic crossing was carried out with first parent transgenic maize plants (zein reduction lines PQ15 and PQ71, which have in their genome a sequence for zein reduction) and second parent transgenic maize plants (high-lysine line M27908+, which has in its genome a sequence for lysine biosynthesis). The zein reduction lines, PQ15, PQ71 and their wild-type control, 90DJD28/91INH2, were used as females and pollinated with the high-lysine line, M27908+, and its wild-type control, M27908-, in the field. **Table 1** lists the resulting six different F1 genotypes, their designations, and the numbers of ears harvested and analyzed. All the transgenic kernels that resulted from the crosses of homologous transgenic parents were hemizygous. More than twenty F1 ears from two separate plots from each cross were harvested and analyzed; only ears with about 150 to about 400 kernels per ear were selected for analysis.

Table 1

Ear designation	Female parent	Male parent	No. of ears analyzed		
			Plot 1	Plot 2	Total
PQ15/CordapA	PQ15/PQ15.0001	M27908+.0028	12	14	26
PQ15	PQ15/PQ15.0001	M27908-.0006	10	12	22
PQ71/CordapA	PQ71/PQ71.0014	M27908+.0028	10	13	23
PQ71	PQ71/PQ71.0014	M27908-.0006	12	11	23
CordapA	90DJD28/91INH2.0002	M27908+.0028	17	11	28
Control	90DJD28/91INH2.0002	M27908-.0006	13	15	28

ANALYSES:

Kernel dissection

To facilitate the kernel dissection, mature dry kernels were submerged in water and stored at 4 degrees Celsius overnight. Fifty kernels per ear were dissected and lyophilized to remove water. After recording the dry weights of the separated embryo and endosperm, the samples were ground to powder. Water imbibition was not found to significantly affect the following amino acid and proximate analyses.

CordapA extraction and activity assay

Dried embryo and endosperm powders were extracted with a 1:3 ratio of tissue to hexane several times at 50 to 55 degrees Celsius for 3 to 10 minutes. After hexane extraction, the powder was lyophilized and stored at 4 to 6 degrees Celsius. CordapA and native DHDPS were extracted with 20 millimolar Tris-HCl, pH 8.0, 100 millimolar KCl, and 10 millimolar sodium pyruvate. A 1:5 ratio of tissue to CordapA extraction buffer was used with shaking for 1 hour at 4 degrees Celsius. The extract was microcentrifuged for 5 minutes at 14,000 x g. For the assay (adopted from Frisch *et al.* (1991) *Plant Physiol.,* 96:444-452), the extract supernatant was further diluted six-fold in extraction buffer. Twenty microliters of the diluted extract was added to the wells of a microplate. To begin the reaction, 180 microliters of reaction buffer was added which contained 200 millimolar Tris-HCl, pH 8.0, 16 millimolar sodium pyruvate, 1 millimolar ASA (L-aspartic acid-beta-semialdehyde hydrate trifluoroacetate) and plus or minus 1 millimolar lysine monohydrochloride. The ASA was obtained from Gateway Chemical Technology, St. Louis and dissolved in 4 normal HCl to yield a 140 millimolar stock solution. Before use, the 140 millimolar ASA stock was diluted to 14 millimolar by adding 100 microliters of the 140 millimolar ASA stock to 100 microliters of 1 molar Tris-HCl, pH 8.0 and 800 microliters of 1 normal NaOH. The reaction was allowed to proceed for 1 hour at 37 degrees Celsius and then stopped by addition of 100 microliters of endpoint colorimetric reagent. This reagent was made by dissolving 20 milligrams ortho-aminobenzaldehyde in 0.3 milliliters of ethanol, which was then added to 11.7 milliliters of 0.22 molar citrate-0.55 molar phosphate buffer pH 5.5. Color was allowed to develop for 10 minutes at room temperature and the absorbance read at 520 nanometers with a SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA).

CordapA Western blot

Twenty milligrams of ground endosperm or embryo were incubated in 200 microliters SDS-borate buffer (60 millimolar $Na_2B_4O_7 \cdot 10\ H_2O$ pH 10, 4% SDS, 1.5 milligrams/milliliter dithiothreitol) at 65 degrees Celsius for 1.5 hours. Samples were spun at 14000 rpm for 5 minutes and 60 microliters supernatant transferred to a 96-well PCR plate containing 30 microliters 3X SDS-PAGE loading buffer (Bio-Rad, Hercules, CA). The sample plate was heated at 98 degrees Celsius for 10 minutes then cooled and stored at 4 degrees Celsius until loading. Twenty-four well pre-cast SDS-PAGE (4-20%) gels (Bio-Rad) were loaded with 13 microliters of sample each along with purified recombinant CordapA and prestained MW

standard (Bio-Rad) for reference. Gels were run at constant voltage (90 volts) until the dye front reached the bottom of the gel; the gels were then transferred to PVDF membrane (Bio-Rad). The goat anti-CordapA antibody was produced by immunizing goats against *E. coli*-expressed recombinant CordapA. The Immun-Blot Assay Kit containing rabbit anti-goat IgG alkaline phosphatase conjugate (Bio-Rad) was used for detection following the manufacturer's protocols.

CordapA ELISA

A hundred milligrams of dry dissected embryo or endosperm powder was analyzed. After adding 2.5 milliliters of cold extraction buffer (50 millimolar $Na_2B_4O_7 \cdot 10\ H_2O$, 0.75 molar KCl, 0.2% v/v Tween20, 36 millimolar NaOH, and 10 millimolar L-ascorbic acid) the sample was vortexed vigorously at 4 degrees Celsius for 30 minute. The extracts were filtered, and aliquots of samples were stored at -20 degrees Celsius. A series of dilutions (1/10, 1/100, 1/1000, 1/5000) was made and used for the analysis. A hundred microliters of diluted primary antibody (2.4 micrograms/milliliter) was added to each well of a Nunc Immuno plate (VWR, West Chester, PA) and incubated at 4 degrees Celsius. The plates were washed 3 times with PBST (137 millimolar NaCl, 8.1 millimolar $Na_2HPO_4 \cdot 7H_2O$, 1.5 millimolar KH_2PO_4, 2.7 millimolar KCl and 0.05% v/v Tween20) and incubated for 1 hour at 37 degrees Celsius with 100 microliters of TBA (100 millimolar Tris, 100 millimolar $Na_2B_4O_7 \cdot 10H_2O$ pH 7.8, 5 M $MgCl_2$, 0.05% v/v Tween20, and 0.2% (v/v) L-ascorbic acid) with 0.1% dry milk to block against non-specific protein adsorption, then washed again 3 times with PBST. Serial dilutions of CordapA protein in TBA were made (1.6, 0.8, 0.4, 0.2, 0.1, 0.05 nanograms/milliliter) as standards. One hundred microliters of each standards and sample dilutions were loaded in triplicate onto the plates and incubated for 1 hour at 37 degrees Celsius. After washing 3 times with PBST, 100 microliters of 1:10000 dilution in TBA of Biotin conjugated goat anti-CordapA primary antibody was added to each well and plates incubated for 1 hour at 37 degrees Celsius. After washing 3 times with PBST, a second incubation with a 1:10000 dilution of Neutravidin-HRP conjugated mouse anti goat IgG (KPL Inc., Gaithersburg, MD) was added. After washing 3 times with PBST, a ten-minute incubation with 100 microliters of TMB substrate/H_2O_2 (1:1) (VWR) was followed, and the reaction was stopped by addition of 100 microliters of 6 molar H_3PO_4. Absorbance was read at 450 nanometers using a SpectraMax Plus microplate reader (Molecular Devices).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Individual kernels were powdered and extracted with an ethanol buffer (70% ethanol, 25 millimolar ammonium hydroxide, and 10 millimolar dithiothreitol) at a ratio of 40 milligrams kernel powder to 1 milliliter buffer. Samples were incubated for 1 hour in a 60 degrees Celsius water bath, with periodic shaking and centrifuged for 15 minutes at 1000 rpm. One microliter of each sample was then mixed with 8 microliters of matrix (10.4 milligram/milliliter of 2-(4'-hydroxyazobenzene)-benzoic acid (HABA) in 70% acetonitrile with 0.3% trifluoroacetic acid) using Applied Biosystems (Framingham, MA) SymBiot™ I robot. From this mixture, 0.6 microliters was deposited on a 384-position MALDI target plate (Applied Biosystems). Carbonic anhydrase (Sigma, St. Louis, MO) was included in the matrix mixture (1 millimolar) as an internal standard to normalize the spectra. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed using an Applied Biosystems Voyager-DETM PRO Biospectrometry™ Workstation with a 337 nanometer nitrogen laser. A detailed description of the MALDI-TOF MS method for analyzing zein proteins has been published by Adams *et al.* (2004) *J. Agric. Food Chem.,* 52:1842-1849.

Amino acid analysis

Ground meal samples (50 milligrams) from pools of whole kernels or dissected embryo and endosperm were extracted with 5% TCA at 4 degrees Celsius overnight. Extracts were filtered, diluted if necessary, and analyzed for free amino acids by the OPA method, which uses ortho-phthaldialdehyde to derivatize samples before injection onto a C18, reverse phase HPLC column (Zorbax Eclipse-AAA, XDB C-18, 4.6 millimeters x 75 millimeters, 3.5 micrometers, Agilent Technologies, Palo Alto, CA) followed by a guard column (Zorbax Eclipse-AAA, 4.6 millimeters x 12.5 millimeters, 5 micrometers, Agilent Technologies, Palo Alto, CA). We used an Agilent HPLC (model 1100) equipped with a fluorescence detector. For total amino acid analysis, protein hydrolysis was performed prior to OPA derivation. Samples (60 milligrams) were hydrolyzed with 10 milliliters of 6 normal HCl and 10 microliters of phenol at 110 degrees Celsius for 24 hours under argon. Aliquots were dried down and redissolved in 0.1 normal HCl for OPA derivation and subsequent HPLC analysis. Lysine measurements were repeated by LC-MS/MS. The lysine metabolites, alpha-aminoadipic delta-semialdehyde ("AAA") and saccharopine ("Sac") were also measured.

Composition analysis

The proximate contents of bulked kernels from each ear in **Table 2** were determined by near-infrared transmission (NIT) analysis (as described by Dyer and Feng (1997)

Feedstuffs, 69:16-25) using an Infratec 1221 Grain Analyzer (Foss, Eden Prairie, MN). The total protein content of dissected embryo and endosperm presented in **Table 2** was determined with a FP-528 Protein/Nitrogen Determinator (LECO, St. Joseph, MI) following AOCS (American Oil Chemical Society) official method Ba 4f-00.

LKR/SDH extraction and activity assay

This extract preparation procedure was adapted and modified from Goncalves-Butruile *et al.* (1996) *Plant Physiol.*, 110:765-771, Gaziola *et al.* (1999) *J. Agric. Food Chem.*, 47:1268-1275, and Kemper *et al.* (1998) *Eur. J. Biochem.*, 253:720-729.

Fresh frozen ground corn kernels of the germplasms of interest (300-600 milligrams) were placed into 2 milliliter tube with Lysing matrix E (Q-Biogen, Irvine, CA) pre-mixed with 150 microliters (dry volume) of polyvinylpyrrolidone. A volume of 625 microliters of extract buffer (50 millimolar KH_2PO_4:K_2HPO_4 pH 7.4, 125 millimolar NaCl,2.0 millimolar $MgCl_2$, 1.0 millimolar EDTA, 250 micromolar $CaCl_2$, 10% Glycerol, CHAPS 0.1%, 8 millimolar NaF , 4 millimolar DTT , and one complete protease tablet (Roche, Indianapolis, IN) was added on top of ground kernels and matrix. Samples were homogenized using a Fast Prep machine (Q-Biogen). Homogenates were placed on ice, with occasional mixing/agitation, for 15-20 minutes before centrifugation at 14,000 x g for 15 minutes. The supernatant (550 to 625 microliters) was removed from packed debris and placed in a separate tube. This sample was fractionated by PEG precipitation (PEG 8000 50% w/v in water) at 7.5 and 15%. The 15% pellet (which contained the LKR and SDH activity) was resuspended in fresh extract buffer (150–300 microliters) and LKR and SDH activity analyzed. All procedures were performed at 4 degrees Celsius or on ice (except homogenization at room temperature).

LKR and SDH activity were analyzed spectrophotometrically (see, for example, protocols described by Goncalves-Butruile *et al.* (1996)) for 10 to 20 minutes by observing the rate of oxidation of NADPH or the rate of reduction of NAD^+ respectively, at 340 nanometers and 30 degrees Celsius in a Tecan Safire2 (Tecan US, Research Triangle Park, NC). The reaction sample volume was 200 microliters for both assays. The LKR assays contained 25 millimolar lysine, 13.3 millimolar alpha-ketoglutaric acid, 325 micromolar NADPH, and 10 to 30 microliters of extract in 100 millimolar KH_2PO_4:K_2HPO_4 pH 7.0, 1 millimolar EDTA, and 10 millimolar $MgCl_2$. Negative controls contained everything except alpha-ketoglutaric acid. The SDH assays contained 2.15 millimolar L-saccharopine, 2.5 millimolar NAD^+ and 10 to 30 microliters of extract in 100 millimolar KH_2PO_4:K_2HPO_4 pH

8.4, 20millimolar TAPS, and 1 millimolar EDTA. Negative controls contained everything except L-saccharopine. Specific activities are reported in nanomoles NADPH oxidized or NAD^+ reduced per minute per milligram of protein. Protein concentrations were determined by using the Bradford reagent from Bio-Rad.

RESULTS

Mass spectral zein analysis

Since the parents of the F1 crosses were both homozygous for their respective transgenes, the F1 kernels were uniformly hemizygous for both transgenic loci. To ensure the expression of transgenic loci in the F1 generation, the zein composition of individual kernels was examined by MALDI-TOF mass spectral analysis. The representative spectra for F1 kernels from each cross were found to be similar to the zein reduction lines from which they originated (**Figure 3**). In addition, all *CordapA* containing F1s retained the embryo-specific expression of *CordapA* (**Figure 4**). While the presence of the *CordapA* transgene did not change the zein profiles in these F1s, its expression is noticeably higher in the zein reduction F1s.

Whole kernel proximate analyses

Table 2 gives the results of the near-infrared transmission (NIT) proximate analysis and size of bulked kernels produced by transgenic and wild-type ears. The data presented are means plus or minus confidence intervals (alpha = 0.05). The sizes were measured in 100-kernel weights in grams. Total protein weights are presented as total protein weights of 100 kernels in grams, which were calculated by multiplying protein content by 100-kernel size. The relatively small differences observed between PQ15/*CordapA* and PQ15, PQ71/*CordapA* and PQ71, and *CordapA* and Control, suggested that *CordapA* did not contribute to any significant change in kernel proximate composition. On the other hand, the impact of zein reduction in the kernel proximate composition was detectable. The PQ15/*CordapA* and PQ15 materials that appeared to have the most dramatic reduction in zein proteins, also appeared to have lower protein content, lower seed density and smaller seed size. When the protein weight per 100 kernels was calculated, PQ15/*CordapA* and PQ15 had an average of 25% reduction compared to their non-zein reduction counterparts, *CordapA* and Control. The slightly higher protein contents of PQ71/*CordapA* and PQ71 materials were offset by their slightly smaller sizes, and their protein weights per 100 kernels were indistinguishable from those of *CordapA* and Control. The oil content of the PQ15/*CordapA* and PQ15 seeds were

noticeably higher than others (consistent with observations for the previous generation), although it was unclear whether this was caused by the zein reduction since the other zein reduction line, PQ71, had normal oil content.

Table 2

% ± CI	PQ15/CordapA	PQ15	PQ71/CordapA	PQ71	CordapA	Control
Oil	5.7 ± 0.2	5.4 ± 0.3	4.4 ± 0.1	4.5 ± 0.2	4.6 ± 0.1	4.5 ± 0.1
Protein	9.2 ± 0.5	9.4 ± 0.4	10.6 ± 0.8	11.6 ± 0.7	9.5 ± 0.5	10.1 ± 0.6
Starch	69.0 ± 0.4	69.0 ± 0.4	69.3 ± 0.6	69.5 ± 0.5	70.7 ± 0.4	70.3 ± 0.6
Moisture	10.7 ± 0.4	10.5 ± 0.2	10.2 ± 0.2	10.7 ± 0.1	11.0 ± 0.1	11.1 ± 0.2
Density	1.22 ± 0.01	1.23 ± 0.01	1.29 ± 0.01	1.31 ± 0.01	1.30 ± 0.00	1.31 ± 0.01
Size	25.11 ± 1.04	23.97 ± 1.10	26.81 ± 1.10	27.71 ± 1.04	29.49 ± 1.37	30.68 ± 1.41
Protein weight	2.32 ± 0.19	2.25 ± 0.15	2.85 ± 0.24	3.22 ± 0.26	2.79 ± 0.19	3.13 ± 0.28

Whole kernel free amino acid and lysine metabolite analysis

The whole kernel free amino acid analysis results are given in **Table 3**, with the data in parts per million (ppm) and presented as averages of two plots plus or minus a standard deviation. The levels of lysine and its metabolites, alpha-aminoadipic delta-semialdehyde (AAA) and saccharopine (Sac), as measured by LC-MS/MS, are also given.

Table 3

Ave ± SD	PQ15/CordapA	PQ15	PQ71/CordapA	PQ71	CordapA	Control
Ala	94 ± 11	140 ± 25	94 ± 11	129 ± 29	88 ± 11	108 ± 9
Arg	88 ± 1	80 ± 14	85 ± 4	79 ± 21	69 ± 8	47 ± 1
Asn	284 ± 3	515 ± 11	247 ± 4	424 ± 122	174 ± 13	194 ± 9
Asp	261 ± 26	365 ± 66	229 ± 31	299 ± 39	121 ± 11	125 ± 5
Glu	239 ± 9	412 ± 85	217 ± 19	323 ± 67	176 ± 16	227 ± 11
Gln	88 ± 10	123 ± 14	77 ± 5	126 ± 42	65 ± 21	74 ± 2
Gly	51 ± 1	58 ± 5	52 ± 1	59 ± 4	51 ± 2	54 ± 2
His	41 ± 1	41 ± 3	38 ± 1	38 ± 4	37 ± 2	30 ± 0
Ile	15 ± 2	19 ± 3	13 ± 0	16 ± 1	14 ± 1	17 ± 1
Leu	27 ± 2	31 ± 5	24 ± 2	26 ± 5	22 ± 1	19 ± 1
Lys	2908 ± 218	64 ± 6	2498 ± 52	67 ± 16	1838 ± 1	43 ± 4
Met	15 ± 2	17 ± 4	15 ± 1	17 ± 1	14 ± 1	13 ± 1
Phe	20 ± 1	27 ± 2	18 ± 0	22 ± 2	18 ± 1	18 ± 1
Ser	80 ± 7	125 ± 19	73 ± 8	107 ± 15	73 ± 6	90 ± 0

Ave ± SD	PQ15/CordapA	PQ15	PQ71/CordapA	PQ71	CordapA	Control
Thr	22 ± 4	46 ± 11	21 ± 5	38 ± 8	17 ± 2	21 ± 0
Trp	19 ± 1	17 ± 1	15 ± 0	15 ± 1	16 ± 1	14 ± 1
Tyr	55 ± 4	66 ± 1	74 ± 24	74 ± 4	50 ± 3	39 ± 1
Val	33 ± 2	45 ± 9	28 ± 1	38 ± 7	27 ± 1	30 ± 0
Total	4335 ± 300	2187 ± 281	3814 ± 166	1892 ± 390	2965 ± 100	1158 ± 13
AAA*	80 ± 1	13 ± 0	59 ± 5	9 ± 2	81 ± 3	9 ± 1
Sac*	588 ± 37	25 ± 6	488 ± 52	13 ± 4	459 ± 6	17 ± 1
Lys*	3148 ± 144	55 ± 1	2704 ± 45	64 ± 20	1918 ± 14	31 ± 5

*measured by LC-MS/MS

The whole kernel free lysine content of all of the F1 materials containing the *CordapA* transgene was very high, and was accompanied by increases in the lysine metabolites, alpha-aminoadipic delta-semialdehyde and saccharopine. Unexpectedly, zein reduction appeared to synergistically increase the accumulation of free lysine caused by the expression of *CordapA* (2908 and 2498 ppm in PQ15/*CordapA* and PQ71/*CordapA* vs. 1838 ppm in *CordapA*). Other than lysine, among the amino acids examined, asparagine, aspartate, glutamate and glutamine had significant increases in the PQ15 and PQ71 lines. However, when combined with *CordapA*, as in PQ15/*CordapA* and PQ71/*CordapA*, the levels of these amino acids resembled those in Control material.

Whole kernel total amino acid analysis

The whole kernel total amino acid analysis results are given in Table 4, with the data in parts per million (ppm) and presented as averages of two plots plus or minus a standard deviation. Asparagine and aspartate are combined ("Asx"); glutamine and glutamate are combined ("Glx").

Table 4

Ave ± SD	PQ15/CordapA	PQ15	PQ71/CordapA	PQ71	CordapA	Control
Ala	6085 ± 120	6270 ± 0	6915 ± 233	8395 ± 516	6330 ± 212	6885 ± 233
Arg	4525 ± 35	4620 ± 113	4700 ± 269	5405 ± 233	4140 ± 198	4470 ± 85
Asx	5435 ± 163	6005 ± 64	5875 ± 106	7500 ± 283	5030 ± 170	5100 ± 28
Glx	15590 ± 382	16420 ± 127	18440 ± 665	23060 ± 1499	16825 ± 544	17825 ± 502
Gly	3475 ± 35	3495 ± 120	3415 ± 163	3985 ± 134	3210 ± 85	3365 ± 35
His	1625 ± 49	1725 ± 78	1770 ± 354	2210 ± 212	1955 ± 64	1530 ± 198
Ile	2925 ± 78	2840 ±	3125 ± 49	3895 ±	2965 ± 92	3125 ±

Ave ± SD	PQ15/CordapA	PQ15	PQ71/CordapA	PQ71	CordapA	Control
		113		191		177
Leu	8960 ± 156	9125 ± 92	10700 ± 297	13045 ± 884	10540 ± 325	11570 ± 495
Lys	6160 ± 354	2930 ± 127	5475 ± 375	3320 ± 269	4290 ± 269	2575 ± 21
Phe	3005 ± 35	3045 ± 7	3340 ± 57	4075 ± 205	3435 ± 92	3705 ± 120
Ser	3825 ± 21	4110 ± 28	4320 ± 283	5245 ± 290	4065 ± 163	4400 ± 71
Thr	3050 ± 28	3155 ± 7	3280 ± 170	4105 ± 120	3075 ± 92	3240 ± 99
Tyr	3525 ± 21	3675 ± 7	3970 ± 255	4915 ± 262	3745 ± 120	4010 ± 99
Val	4220 ± 99	4140 ± 127	4465 ± 49	5530 ± 240	3850 ± 113	4055 ± 177
Total	72405 ± 1478	71555 ± 322	79790 ± 2206	94685 ± 4914	72915 ± 2539	75855 ± 1718
Lys%	8.5 ± 0.3%	4.1 ± 0.2%	6.9 ± 0.3%	3.5 ± 0.1%	5.9 ± 0.2%	3.4 ± 0.0%

As shown in **Table 4**, the most significant differences observed among the amino acids quantified in the whole F1 kernels analyzed were in the lysine contents. PQ15 and PQ71 were found to have elevated total lysine levels when compared to the Control. In addition, after the subtraction of their free lysine contents, the total lysine contents of PQ15/*CordapA*, PQ71/*CordapA* and *CordapA* were similar to those in PQ15, PQ71 and Control respectively (see **Table 3**).

Dissected embryo and endosperm—free amino acid and lysine metabolite analysis

The free amino acid analysis results for the dissected embryos and endosperms are given in **Table 5**, with the data in parts per million (ppm) and presented as averages of two plots plus or minus a standard deviation. Samples were pooled by equal amount of ground mills from each ear harvested from a plot. The levels of lysine and its metabolites, alpha-aminoadipic delta-semialdehyde (AAA) and saccharopine (Sac), as measured by LC-MS/MS, are also given.

Table 5

Ave ± SD	PQ15/CordapA		PQ15		PQ71/CordapA		PQ71		CordapA		Control	
	Em	En	Em	En	Em	En	Em	En	Em	En	Em	En
Ala	343 ± 28	61 ± 1	740 ± 4	119 ± 14	350 ± 2	41 ± 8	622 ± 7	57 ± 8	307 ± 19	39 ± 4	629 ± 33	41 ± 1
Arg	488 ± 63	27 ± 2	344 ± 5	46 ± 2	520 ± 50	20 ± 2	392 ± 96	23 ± 6	412 ± 26	18 ± 4	279 ± 57	19 ± 1
Asn	1220 ± 172	109 ± 2	2419 ± 172	203 ± 5	1074 ± 118	88 ± 19	1893 ± 392	180 ± 68	809 ± 31	49 ± 10	1130 ± 87	63 ± 3
Asp	113 ± 25	257 ± 23	158 ± 32	350 ± 69	207 ± 63	177 ± 29	237 ± 34	226 ± 44	117 ± 6	91 ± 13	218 ± 56	72 ± 1
Glu	337 ± 45	122 ± 1	672 ± 65	240 ± 38	397 ± 115	99 ± 23	445 ± 40	151 ± 60	297 ± 6	69 ± 8	573 ± 105	65 ± 8
Gln	100 ± 28	27 ± 2	103 ± 2	57 ± 6	102 ± 10	21 ± 4	90 ± 36	46 ± 26	124 ± 18	15 ± 1	95 ± 26	18 ± 1
Gly	65 ± 4	12 ± 0	88 ± 10	19 ± 1	67 ± 4	10 ± 1	91 ± 10	13 ± 1	66 ± 3	9 ± 1	103 ± 18	11 ± 1
His	166 ± 15	21 ± 1	139 ± 1	27 ± 1	169 ± 16	14 ± 1	157 ± 9	19 ± 4	179 ± 12	12 ± 3	143 ± 8	15 ± 1
Ile	83 ± 33	10 ± 0	116 ± 62	17 ± 2	84 ± 35	8 ± 1	91 ± 40	9 ± 1	102 ± 21	8 ± 2	119 ± 14	8 ± 1
Leu	81 ± 4	13 ± 1	51 ± 6	25 ± 2	73 ± 15	9 ± 2	38 ± 3	13 ± 2	79 ± 13	7 ± 1	41 ± 15	8 ± 1
Lys	23159 ± 2325	119 ± 7	302 ± 102	28 ± 1	21581 ± 1085	105 ± 28	431 ± 175	20 ± 5	16630 ± 1070	58 ± 4	238 ± 70	10 ± 3
Met	46 ± 6	6 ± 1	0 ± 0	11 ± 2	63 ± 9	3 ± 4	45 ± 9	6 ± 2	29 ± 41	3 ± 4	23 ± 32	4 ± 0
Phe	29 ± 40	14 ± 1	28 ± 40	23 ± 4	34 ± 48	10 ± 1	18 ± 25	12 ± 3	0 ± 0	10 ± 2	33 ± 46	8 ± 1
Ser	148 ± 7	25 ± 1	286 ± 21	57 ± 3	141 ± 11	18 ± 5	276 ± 56	25 ± 3	139 ± 8	17 ± 5	290 ± 13	19 ± 1
Thr	41 ± 2	13 ± 1	93 ± 11	36 ± 4	28 ± 39	11 ± 2	94 ± 10	18 ± 3	42 ± 0	8 ± 2	89 ± 9	10 ± 2
Trp	100 ± 2	9 ± 2	35 ± 49	13 ± 0	111 ± 23	8 ± 1	82 ± 1	10 ± 2	93 ± 18	3 ± 4	90 ± 28	7 ± 1
Tyr	251 ± 6	29 ± 1	176 ± 1	60 ± 5	411 ± 103	31 ± 11	214 ± 23	45 ± 7	307 ± 12	17 ± 6	220 ± 44	18 ± 1
Val	110 ± 4	17 ± 0	139 ± 18	33 ± 6	109 ± 11	12 ± 2	109 ± 13	17 ± 3	107 ± 11	12 ± 0	123 ± 2	12 ± 1
Total	26874 ± 1932	885 ± 28	5884 ± 85	1359 ± 127	25516 ± 1455	679 ± 89	5321 ± 798	886 ± 247	19835 ± 882	442 ± 71	4430 ± 682	403 ± 25
AAA	581 ± 9	8 ± 0	12 ± 17	8 ± 0	418 ± 41	6 ± 1	0 ± 0	4 ± 1	635 ± 98	6 ± 1	44 ± 31	2 ± 0
Sac	3937 ± 14	116 ± 3	95 ± 13	13 ± 1	3682 ± 631	91 ± 15	52 ± 73	11 ± 1	3625 ± 288	85 ± 9	249 ± 177	13 ± 4

As shown in **Table 5**, results from the dissected embryo and endosperm tissues were similar to the data from the analysis of whole kernels. F1 materials harboring *CordapA* had

increases in lysine and lysine metabolites, and the zein reduction F1s had higher asparagine, aspartate, and glutamate. In general, free amino acids were more concentrated (in ppm) in the embryo, which led to an amplification of some of the minor differences which would otherwise have been statistically insignificant when examined across intact kernels. For example, the expression of *CordapA* also reduced alanine and serine by approximately 50% in embryo tissues (comparing PQ15/*CordapA*, PQ71/*CordapA* and *CordapA* to PQ15, PQ71 and Control). On the other hand, most of increases of asparagine, aspartate, and glutamate in zein reduction lines were found in the endosperm (comparing PQ15 and PQ71 to Control).

Dissected embryo and endosperm—total amino acid analysis

The whole kernel total amino acid analysis results are given in **Table 6**, with the data in parts per million (ppm) and presented as averages of two plots plus or minus a standard deviation. Asparagine and aspartate are combined ("Asx"); glutamine and glutamate are combined ("Glx").

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Table 6

Ave ± SD	PQ15/CordapA		PQ15		PQ71/CordapA		PQ71		CordapA		Control	
	Em	En	Em	En	Em	En	Em	En	Em	En	Em	En
Ala	10759 ± 120	5775 ± 64	11570 ± 0	6145 ± 21	10790 ± 184	7540 ± 354	12090 ± 311	8880 ± 877	10180 ± 240	6275 ± 290	10375 ± 134	6930 ± 141
Arg	16020 ± 693	3085 ± 78	15745 ± 205	3380 ± 113	17240 ± 240	3530 ± 42	17860 ± 1541	4265 ± 375	16410 ± 735	2855 ± 120	17290 ± 622	3100 ± 57
Asx	12660 ± 14	4825 ± 7	14190 ± 170	5320 ± 184	13220 ± 240	5780 ± 212	15260 ± 1329	6955 ± 841	12575 ± 177	4415 ± 233	12710 ± 552	4795 ± 78
Glx	25315 ± 106	15385 ± 134	26360 ± 212	16720 ± 14	25705 ± 460	20985 ± 1054	27975 ± 1619	25190 ± 2659	24405 ± 643	16720 ± 764	25635 ± 318	18630 ± 410
Gly	9900 ± 141	2750 ± 14	9770 ± 127	2975 ± 106	10225 ± 120	3125 ± 64	10740 ± 495	3615 ± 290	9600 ± 255	2605 ± 78	9530 ± 325	2805 ± 21
His	5785 ± 78	1355 ± 219	4915 ± 191	1685 ± 120	6005 ± 35	1825 ± 21	5340 ± 212	2485 ± 134	5770 ± 42	1835 ± 163	4795 ± 516	1971 ± 71
Ile	5270 ± 28	2555 ± 7	5545 ± 35	2710 ± 14	5365 ± 148	3315 ± 106	5825 ± 78	3920 ± 396	4965 ± 120	2780 ± 113	5005 ± 21	3065 ± 92
Leu	11855 ± 35	9095 ± 106	12505 ± 148	9695 ± 35	11740 ± 240	12460 ± 636	12875 ± 163	14730 ± 1570	10960 ± 14	11325 ± 530	11005 ± 163	12575 ± 332
Lys	34690 ± 2291	2030 ± 14	9895 ± 92	2060 ± 99	33415 ± 686	2220 ± 14	10970 ± 636	2365 ± 233	28020 ± 240	1505 ± 49	9815 ± 163	1525 ± 21
Phe	5785 ± 92	2835 ± 21	6130 ± 14	2990 ± 14	5840 ± 156	3620 ± 127	6575 ± 262	4330 ± 396	5280 ± 127	3525 ± 148	5425 ± 35	3870 ± 85
Ser	8645 ± 233	3730 ± 71	8665 ± 134	3985 ± 78	8940 ± 156	4700 ± 198	9990 ± 679	5555 ± 587	8310 ± 580	4080 ± 170	8275 ± 205	4470 ± 71
Thr	6360 ± 28	2685 ± 7	6600 ± 85	2920 ± 71	6625 ± 78	3345 ± 49	7195 ± 134	3920 ± 424	6405 ± 148	2820 ± 141	6175 ± 191	3060 ± 57
Tyr	5670 ± 42	3455 ± 35	5850 ± 0	3630 ± 99	5960 ± 170	4250 ± 14	6290 ± 184	5045 ± 658	5585 ± 170	3740 ± 170	5330 ± 85	4120 ± 113
Val	8760 ± 99	3720 ± 14	9250 ± 14	3900 ± 42	8980 ± 410	4665 ± 148	9835 ± 318	5445 ± 544	8450 ± 170	3535 ± 134	8415 ± 247	3865 ± 49
Total	167510 ± 2376	63280 ± 99	146990 ± 1131	681150 ± 912	170050 ± 2546	81360 ± 2857	158820 ± 7962	96700 ± 9984	156915 ± 2835	68015 ± 3104	139780 ± 3140	74780 ± 1556
Lys%	20.7 ± 1.1%	3.2 ± 0.0%	6.7 ± 0.0%	3.0 ± 0.1%	19.6 ± 0.1%	2.7 ± 0.1%	6.9 ± 0.1%	2.4 ± 0.0%	17.9 ± 0.5%	2.2 ± 0.0%	7.0 ± 0.0%	2.0 ± 0.1%

Comparing PQ15/*CordapA* to PQ15, PQ71/*CordapA* to PQ71 and *CordapA* to Control, the expression of *CordapA* slightly reduced total amino acid accumulations in endosperm. In the embryo, histidine and lysine were the only amino acids that showed consistent increases in the *CordapA* containing F1s. With respect to the impact of zein reduction on various total amino acid levels, PQ71, but not PQ15, had higher total amino acid contents compared to Control. However, the increase in total lysine, which occurred only in endosperm, was consistent in both events.

Dissected embryo and endosperm—protein content and dry weight

The protein content and dry weight analysis results for dissected embryos and endosperms are given in **Table 7**, presented as means plus or minus confidence intervals (alpha = 0.05). Dry weights are given as means of sums of 50 dissected embryo or endosperm in grams.

Table 7

Ave ± CI	PQ15/CordapA		PQ15		PQ71/CordapA		PQ71		CordapA		Control	
	Em	En	Em	En	Em	En	Em	En	Em	En	Em	En
Protein %	20.59 ± 0.69	6.55 ± 0.50	18.08 ± 0.48	7.62 ± 0.46	21.42 ± 0.78	8.41 ± 0.86	19.45 ± 0.66	9.84 ± 0.77	18.19 ± 0.48	7.02 ± 0.45	17.13 ± 0.47	7.69 ± 0.67
Dry weight	1.477 ± 0.097	9.717 ± 0.414	1.395 ± 0.082	9.373 ± 0.471	1.470 ± 0.066	10.788 ± 0.449	1.440 ± 0.073	10.863 ± 0.501	1.588 ± 0.085	11.920 ± 0.530	1.643 ± 0.078	12.489 ± 0.559

In all three pair-wise comparisons, PQ15/*CordapA* vs. PQ15, PQ71/*CordapA* vs. PQ71 and *CordapA* vs. Control, the protein content appeared to increase slightly in the endosperm relative to the embryo, without affecting proportional dry weight. This was consistent with data shown in **Table 6**, where the total amino acids were higher in embryo tissues and lower in endosperm tissues in materials carrying the *CordapA* transgene. With respect to the impact of zein reduction, compared to the Control, PQ15 had similar protein contents but significantly reduced weights, while PQ71 had higher protein contents and only small reduction in weights, in both embryo and endosperm.

LKR/SDH activities of the F1 developing kernels

The measured LKR and SDH specific activities in the F1 developing kernels are presented in **Table 8**. Data are shown as means plus or minus standard deviation ($n = 3$). Each sample was prepared from kernels of a developing ear at 25 days after pollination (DAP), and was assayed in triplicate. Three developing ears per F1 were used. Specific activities for LKR and SDH are reported in nanomoles NADPH oxidized or NAD^+ reduced per minute per milligram protein.

Table 8

% ± SD	PQ15/CordapA	PQ15	PQ71/CordapA	PQ71	CordapA	Control
LKR	24.58 ± 1.17	20.57 ± 3.48	20.44 ± 1.96	29.88 ± 4.60	14.15 ± 0.90	15.56 ± 1.57
SDH	4.30 ± 1.51	4.34 ± 1.65	5.91 ± 0.97	6.01 ± 0.12	3.78 ± 0.31	4.15 ± 0.23
LKR/SDH Ratio	5.72	4.74	3.46	4.97	3.74	3.75

LKR and SDH specific activities in the F1 developing kernels were between 14.15 to 29.88 nanomoles NADPH oxidized per minute per milligram protein and 3.78 to 6.01 NAD^+ reduced per minute per milligram protein, respectively. The LKR specific activities were 5- to 10-fold higher than had previously been reported for maize developing endosperm. See, for example, Azevedo *et al.* (2004) *J. Agric. Food Chem.*, 52:4865-4871, Gaziola *et al.* (1999) *J. Agric. Food Chem.*, 47:1268-1275, and Kemper *et al.* (1999) *Plant Cell,* 11:1981-1993. Unlike in opaque mutants, the zein reduction by the transgenic approach did not decrease LKR and SDH specificity activities in these F1s.

Developing kernels of all the zein reduction F1s (PQ15/*CordapA*, PQ15, PQ71/*CordapA*, and PQ71) had similar or higher LKR and SDH specificity activities than those of F1s with normal zein levels, *CordapA* and Control. The elevated lysine concentration did not appear to

increase the LKR and SDH specificity activities (compare PQ15/*CordapA* to PQ15, PQ71/*CordapA* to PQ71 and *CordapA* to Control). The LKR/SDH ratios ranged from 3.46 to 5.72 and were also several fold higher than previously reported, consistent with the accumulation of saccharopine found in high-lysine F1s.

DISCUSSION

The F1 seeds displayed phenotypes similar to homozygous parents. F1 progeny containing the *CordapA* transgene had higher free lysine (**Table 3**) and F1 seeds derived from PQ15 and PQ71 showed reduction of zeins (**Figure 3**) and elevated total lysine contents (**Table 4**). Combining zein reduction and *CordapA* expression, as in the PQ15/*CordapA* and PQ71/*CordapA* crosses, resulted in a substantial increase (about a doubling) of the total lysine content observed in the Control material (**Table 4**).

An unexpected *CordapA*-dependent synergistic effect on the free lysine accumulation was observed. The *CordapA* F1 progeny accumulated 1838 ppm free lysine, greater than a 40-fold increase over the Control of 43 ppm (**Table 3**). Zein reduction synergistically increased free lysine levels derived from *CordapA* expression, as seen in the combinations of PQ15/*CordapA* and PQ71/*CordapA*, which had even higher free lysine content, 2908 and 2498 ppm, respectively. F1s with zein reduction alone (PQ15 and PQ71) had Control levels of free lysine, which indicated that zein reduction enhanced lysine biosynthesis in a synergistic fashion in the presence of *CordapA*. However, elevated free lysine did not contribute to increases in protein-bound lysine caused by zein reduction. Almost all of the increase in total lysine was found in the endosperm since large amounts of the lysine-poor zeins are reduced in the endosperm of transgenic seeds (**Table 6**).

It has been proposed that the high lysine content of the *opaque-2* mutant (Mertz *et al.* (1964) *Science,* 145:279-280) is due to the reduction of lysine-poor zeins and a pleiotropic increase in the lysine-rich non-zein proteins. See, for example, Damerval and Devienne (1993) *Heredity,* 70:38-51, Habben *et al.* (1993) *Plant Mol. Biol.,* 23:825-838, and Lopez-Valenzuela *et al.* (2004), *Plant Physiol.,* 135:1784-1797.

The increases in free lysine caused by zein reduction and *CordapA* expression in PQ15/*CordapA* and PQ71/*CordapA* were calculated from **Table 3**, yielding calculated free lysine values of 2865 (2908 - 43) ppm in PQ15/*CordapA* and 2454 (2498 - 43) ppm in PQ71/*CordapA*, respectively. By adding the increases in free lysine to the total lysine of PQ15 and PQ71 shown in **Table 4**, the expected total lysine contents for PQ15/*CordapA* and PQ71/*CordapA* were estimated to be 5975 and 5784 ppm. These estimates were not significantly different from the actual measurements of 6160 ± 354 and 5475 ± 375 ppm

(**Table 4**). Thus, in contrast to earlier hypotheses, higher free lysine did not stimulate the synthesis of lysine-rich non-zein proteins in the transgenic zein reduction lines. Although PQ15 had a protein content similar to Control, its smaller kernels (and thus smaller endosperm) led to it containing less overall protein on a per-kernel basis (**Table 2** and **Table 7**), suggesting that zein reduction in PQ15 had little effect on the synthesis of lysine-rich non-zein proteins. On the other hand, PQ71 has slightly smaller kernels, but a higher protein content and thus the amount of overall protein is comparable to Control (**Table 2** and **Table 7**). All together, the higher total lysine content observed in PQ15 merely reflected zein reduction, while the increase in total lysine content in PQ71 was probably caused by the synthesis of lysine-rich non-zein proteins.

Zein reduction was found to unexpectedly increase the supply of precursor amino acids for *CordapA*-enhanced lysine biosynthesis, resulting in a synergistic increase in lysine. In the zein reduction F1s (PQ15 and PQ71), three free amino acids, asparagine, aspartate, and glutamate, had the most noticeable increases among all free amino acids measured (**Table 3**). The introduction of *CordapA* to the zein-reduced lines synergistically enhanced lysine biosynthesis and concomitantly reduced asparagine, aspartate, and glutamate (PQ15/*CordapA*, PQ71/*CordapA* in **Table 2**). In plants, lysine is believed to be synthesized from aspartate through the lysine branch of the Asp family pathway; aspartate, in turn, can be synthesized from glutamate or asparagines. See, for example, Galili (2002) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 53:27-43. The unexpected changes in aspartate, asparagines and glutamate that were detected in the zein reduction F1s and the zein reduction/*CordapA* F1s further indicated the importance of these amino acids in enhancing lysine biosynthesis.

Example 2

This non-limiting example describes a recombinant DNA construct useful in introducing sequence for zein reduction into the transgenic plant of the invention, the use of which construct is described generally in U. S. Patent Application No. 11/057,062. More specifically, this example describes use of a closed loop of anti-sense-oriented RNA for suppressing at least one zein gene (for example, any one or more of the genes for alpha-, beta-, gamma-, and delta-zeins). The recombinant DNA construct for suppressing at least one zein gene, can include in 5' to 3' order a promoter element operably linked to an anti-sense-oriented DNA element from the at least one zein gene and a sense-oriented DNA element, wherein the sense-oriented DNA element is shorter than the anti-sense-oriented DNA element, and sense-oriented RNA

transcribed by the sense-oriented DNA element is complementary to the 5'-most end of anti-sense-oriented RNA transcribed by the anti-sense-oriented RNA transcribed by the anti-sense-oriented DNA element, wherein the transcribed RNA forms a loop of anti-sense-oriented RNA for suppressing the at least one zein gene. In another embodiment, the sequence for zein reduction can be introduced in the transgenic plant of the invention by providing in cells of the transgenic plant a recombinant DNA construct which is transcribed to RNA that forms a loop of anti-sense-oriented RNA for suppressing at least one zein gene.

Example 3

This example describes non-limiting constructs useful in obtaining transgenic maize plants of the invention. Expression cassettes containing heterologous DNA for gene suppression embedded in an intron are described in detail in United States Provisional Patent Application No. 60/638,256. Similar expression cassettes useful in the practice of the present invention are apparent to one versed in the art. For example, a suitable vector useful in obtaining a transgenic plant of the invention includes an expression cassette containing a seed specific promoter and leader, operably linked to an maize heat shock protein intron in which is embedded sequence (such as an inverted repeat of DNA) for suppressing at least one zein synthesis gene, and optionally sequence for suppressing at least one endogenous lysine catabolic enzyme (e. g., maize LKR), followed by targeting sequence and sequence encoding a lysine synthesis gene (e. g., an *E. coli* AKIII, or a *Corynebacterium* DHDPS), and finally DNA with polyadenylation signal and site. The vector can additionally contain a selectable or screenable marker, e. g., a gene encoding glyphosate herbicide resistance.

All of the materials and methods disclosed and claimed herein can be made and used without undue experimentation as instructed by the above disclosure. Although the materials and methods of this invention have been described in terms of preferred embodiments and illustrative examples, it will be apparent to those of skill in the art that variations may be applied to the materials and methods described herein. The scope of the claims should not be limited by the preferred embodiments set forth herein, but should be given the broadest interpretation consistent with the description as a whole.

DEMANDE OU BREVET VOLUMINEUX

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CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 31

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JUMBO APPLICATIONS/PATENTS

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The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. A cell of a transgenic maize plant having in its genome transgenic DNA encoding an antisense sequence complementary to all or a portion of a 19 kiloDalton or 22 kiloDalton alpha-zein messenger RNA (mRNA) and a transgenic DNA for the expression of lysine insensitive aspartate kinase (AK) or lysine insensitive dihydrodipicolinic acid synthase (DHDPS), whereby expression of said transgenic DNA results in a synergistically increased lysine content of seed of said transgenic maize plant, wherein said antisense sequence reduces the expression of its targeted 19 kiloDalton or 22 kiloDalton alpha-zein messenger RNA.

2. The cell of claim 1, wherein said transgenic maize plant is a progeny transgenic maize plant from genetic crossing of a first parent transgenic maize plant having in its genome transgenic DNA encoding an antisense sequence complementary to all or a portion of 19 kiloDalton or 22 kiloDalton alpha-zein mRNA and a second parent transgenic maize plant having in its genome transgenic DNA for the expression of lysine insensitive aspartate kinase (AK) or lysine insensitive dihydrodipicolinic acid synthase (DHDPS), wherein said progeny transgenic maize plant from said genetic crossing has in its genome said transgenic DNA comprising an antisense sequence complementary to all or a portion of 19 kiloDalton or 22 kiloDalton alpha-zein mRNA and the transgenic DNA for the expression of lysine insensitive aspartate kinase (AK) or lysine insensitive dihydrodipicolinic acid synthase (DHDPS).

3. A method for providing maize seed with synergistically increased lysine content, comprising:

(a) providing a transgenic maize plant having in its genome transgenic DNA encoding an antisense sequence complementary to all or a portion of a 19 kiloDalton or 22 kiloDalton alpha-zein messenger RNA (mRNA) and a transgenic DNA for the expression of lysine insensitive aspartate kinase (AK) or lysine insensitive dihydrodipicolinic acid synthase (DHDPS),

(b) expressing said transgenic DNA in seed of said transgenic maize plant, said expressing resulting in a synergistically increased lysine content of said seed, and

(c) harvesting said seed with synergistically increased lysine content.

4. The method of claim 3, wherein said providing step (a) comprises genetic crossing of a first parent transgenic maize plant having in its genome transgenic DNA encoding an antisense sequence complementary to all or a portion of 19 kiloDalton or 22 kiloDalton alpha-zein mRNA and a second parent transgenic maize plant having in its genome transgenic DNA for the expression of lysine insensitive aspartate kinase (AK) or lysine insensitive dihydrodipicolinic acid synthase (DHDPS), said genetic crossing resulting in a progeny transgenic maize plant having in its genome said transgenic DNA comprising the antisense sequence complementary to all or a portion of 19 kiloDalton or 22 kiloDalton alpha-zein mRNA and the transgenic DNA for the expression of lysine insensitive aspartate kinase (AK) or lysine insensitive dihydrodipicolinic acid synthase (DHDPS).

Figure 1a

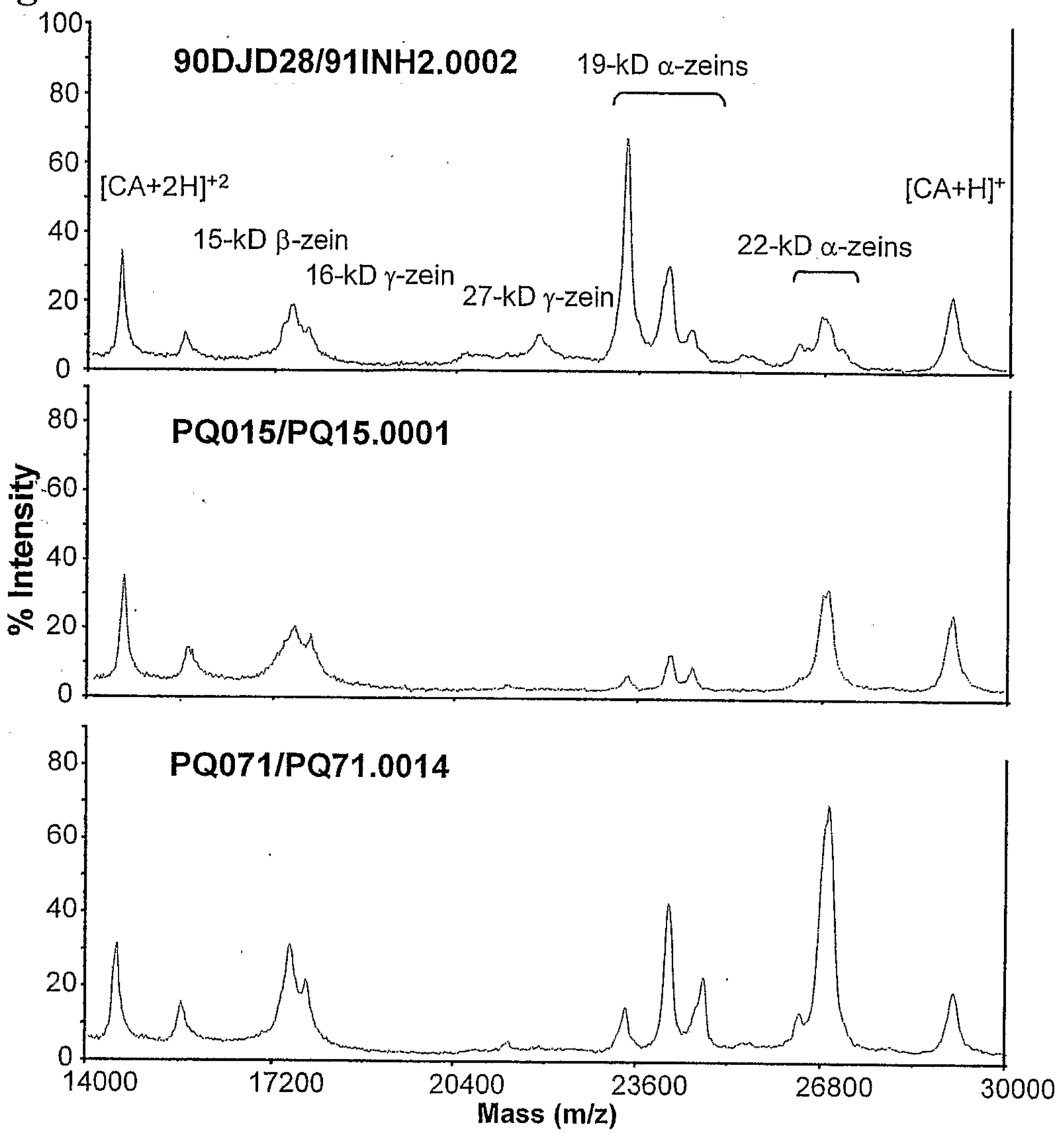


Figure 1b

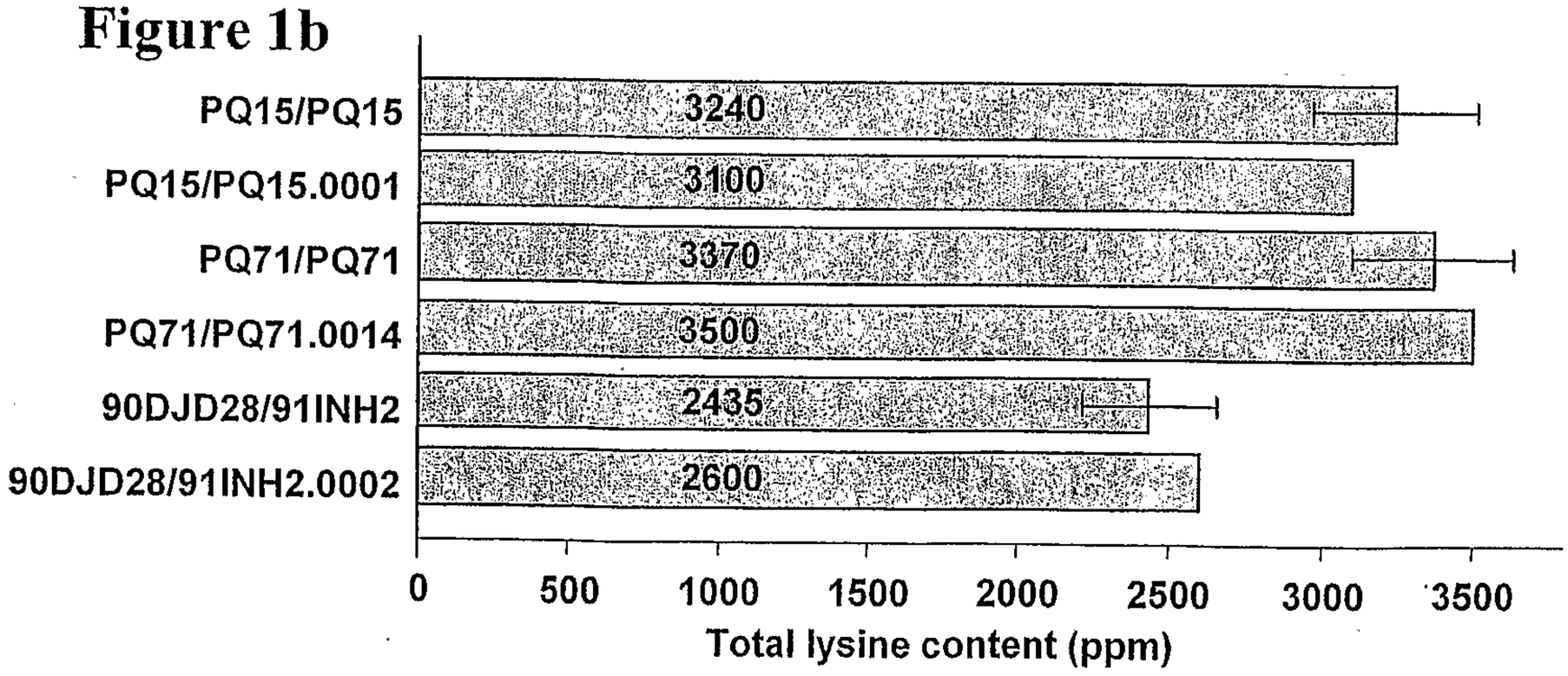


Figure 2a

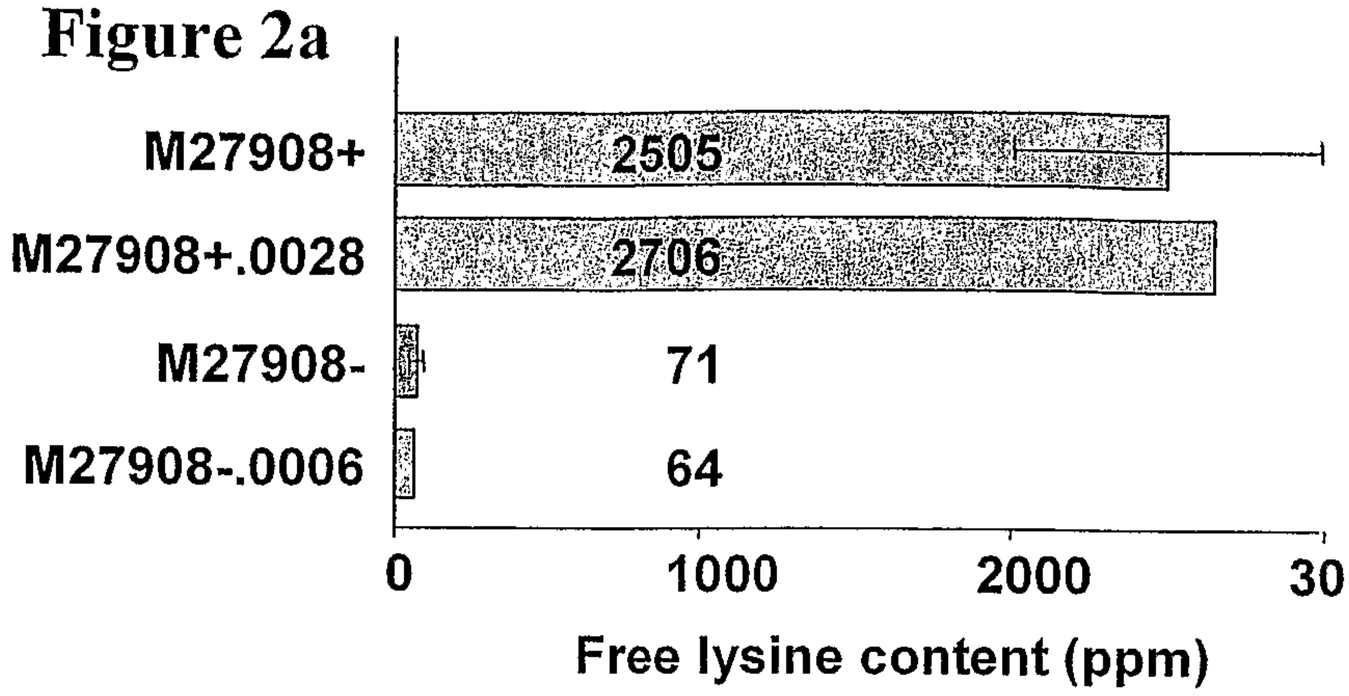
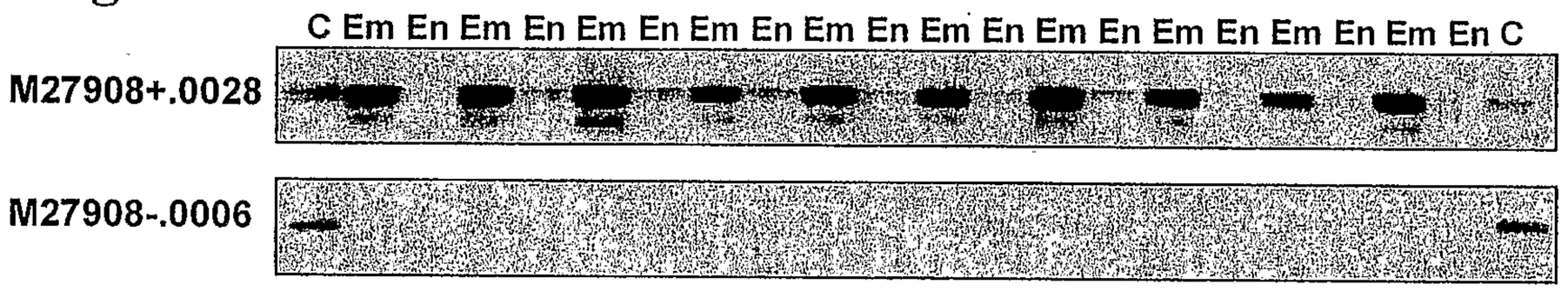


Figure 2b



C: Control recombinant CordapA from *E. coli*;
Em: Embryo; En: Endosperm

Figure 2c

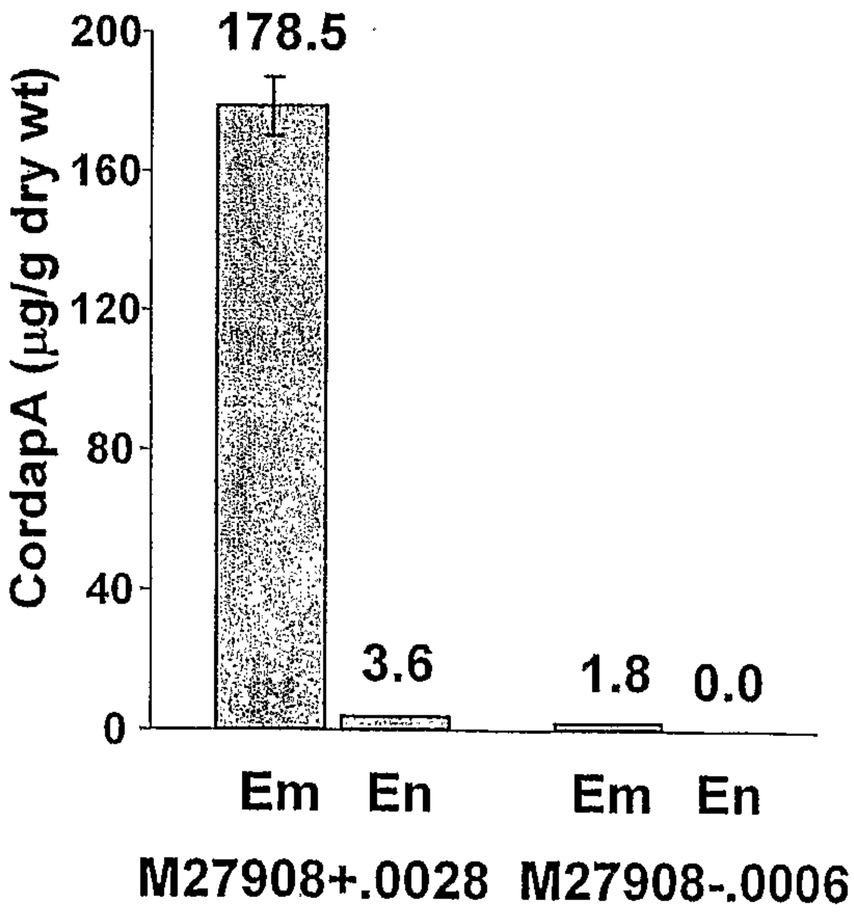
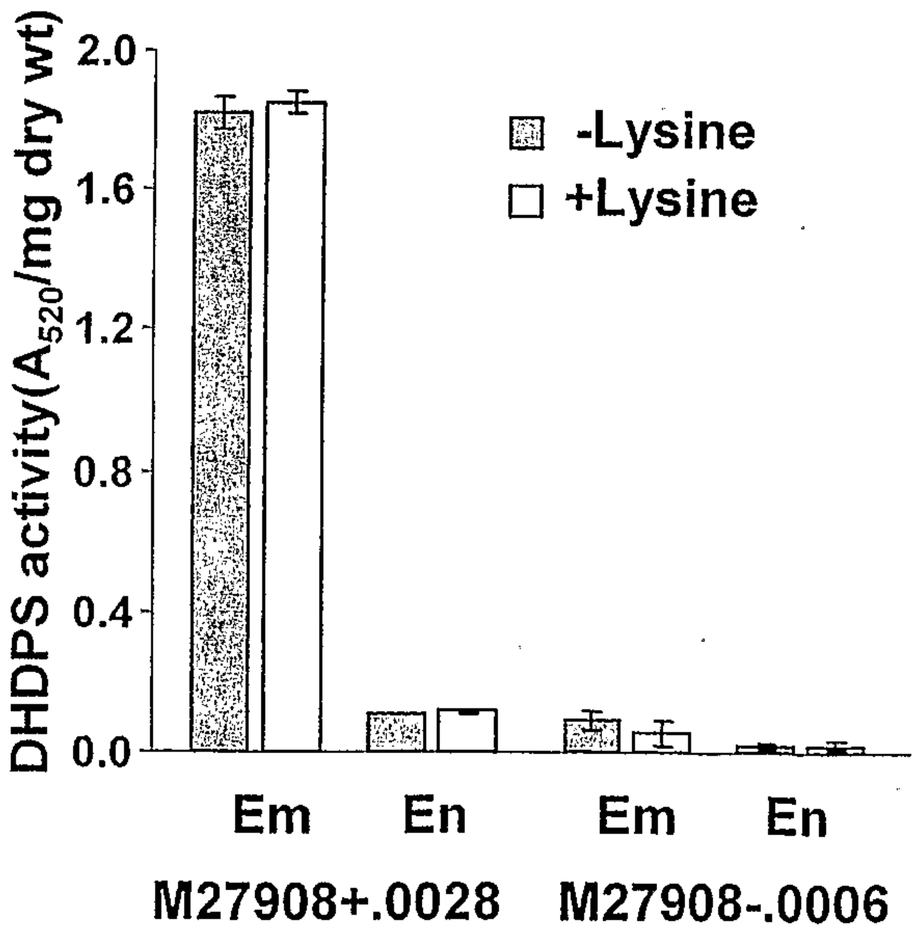


Figure 2d



Docket 38-15(53571)A

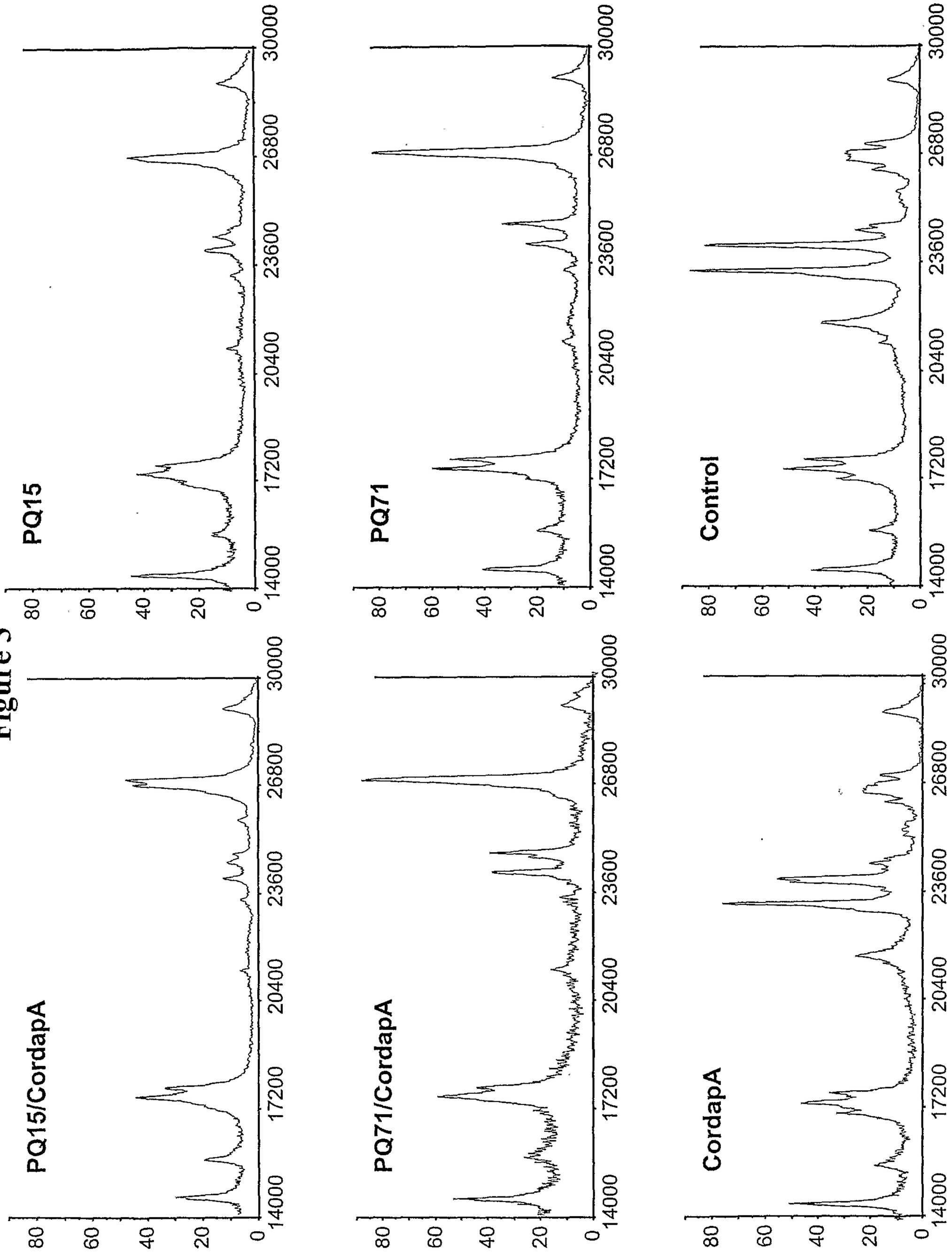


Figure 3

Figure 4

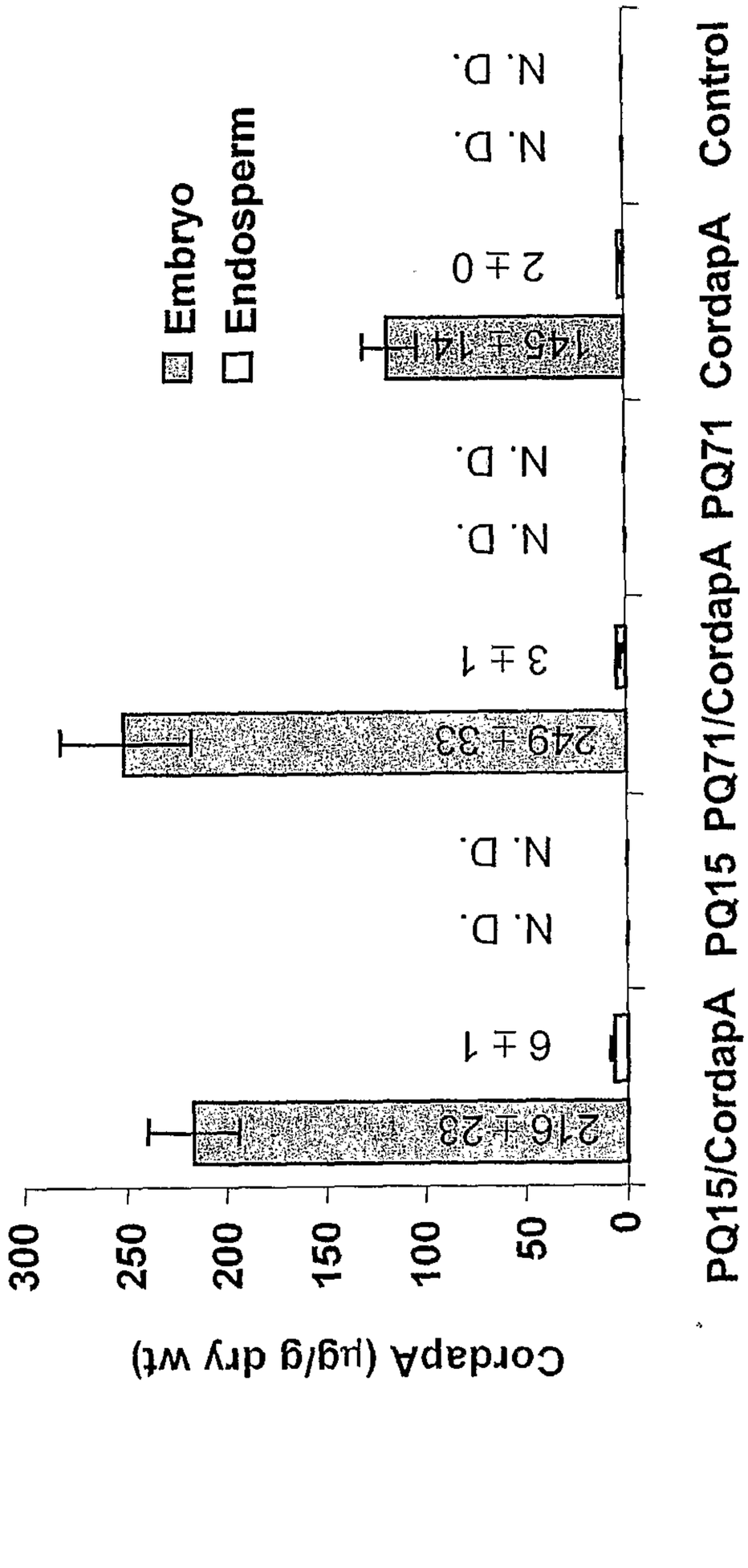
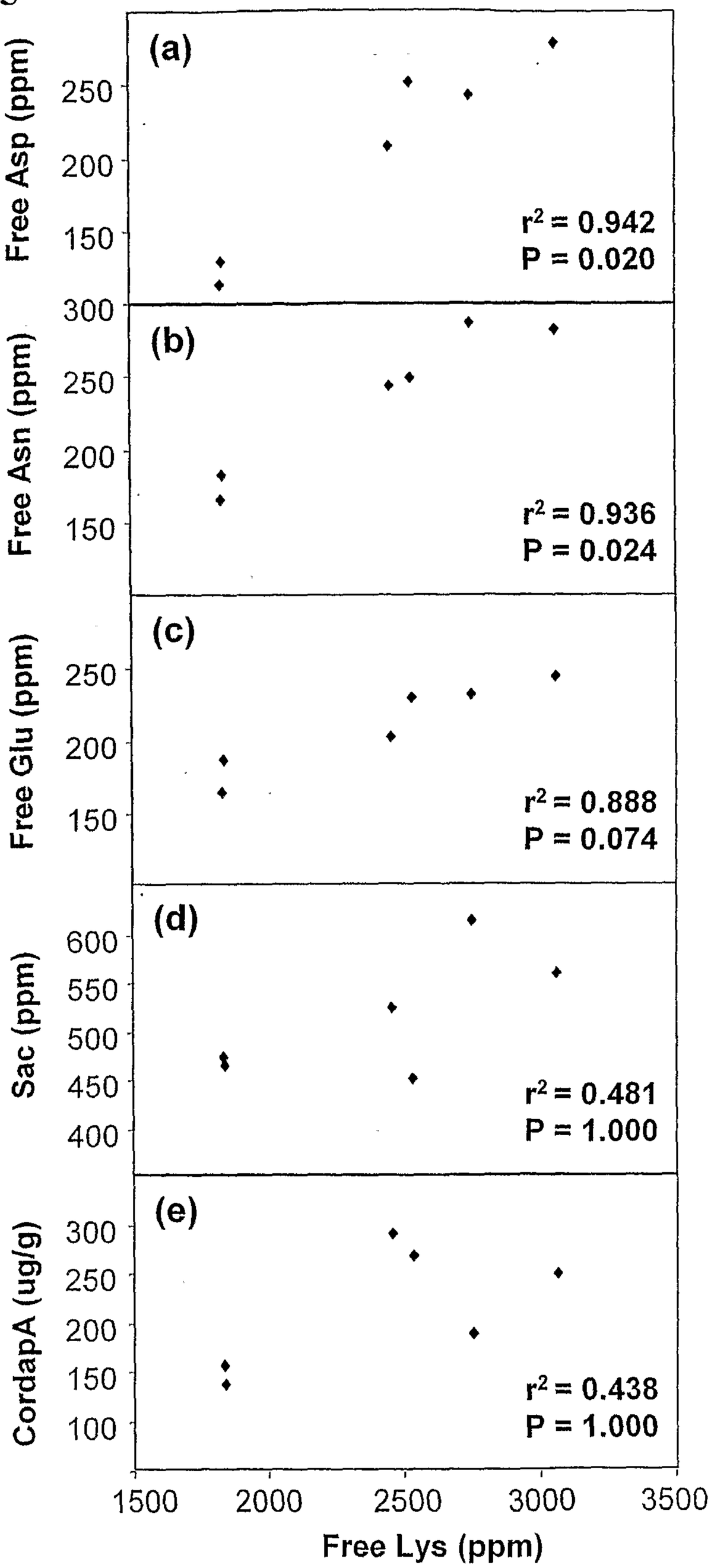
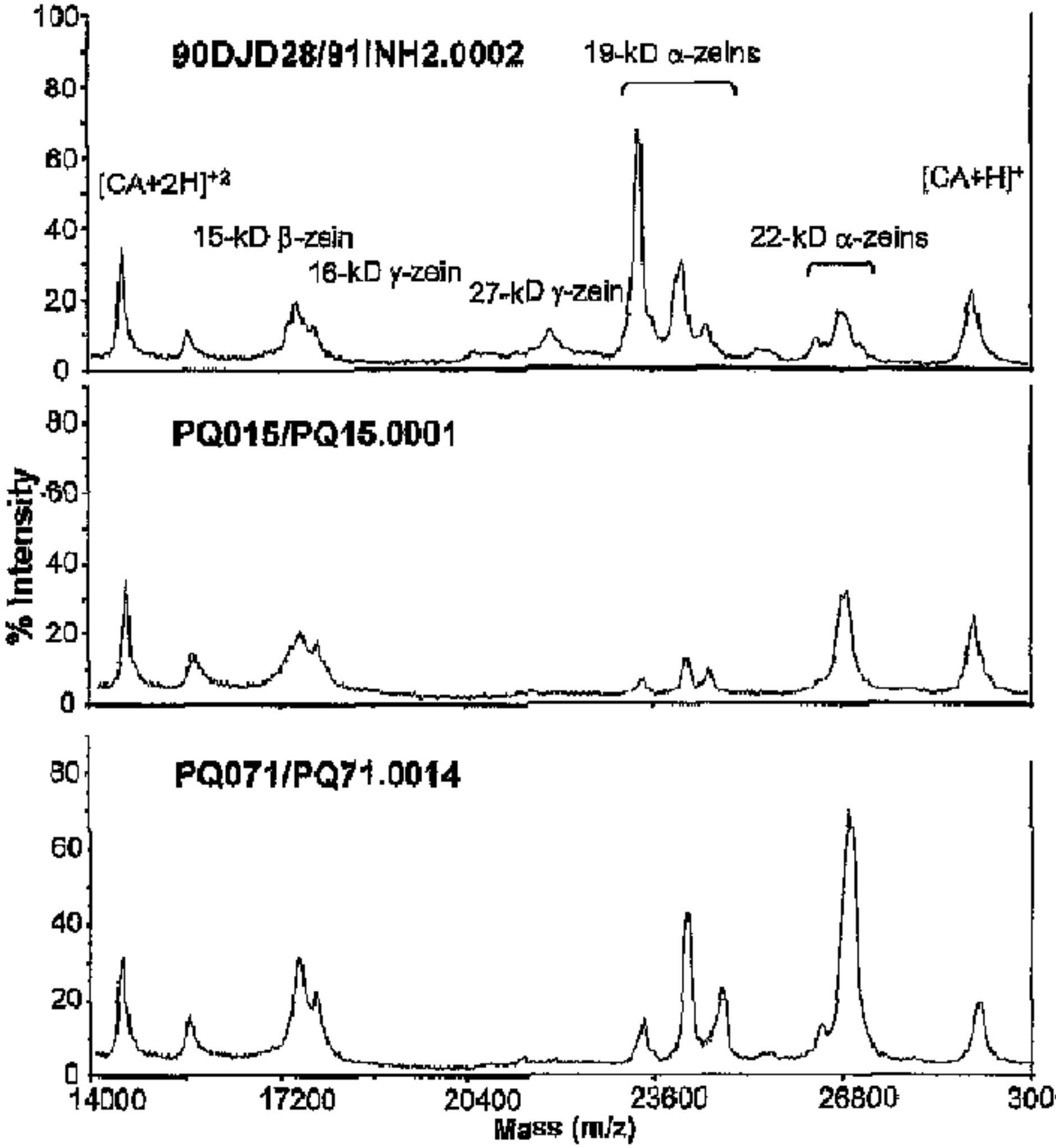

Embryo
Endosperm
CordapA (µg/g dry wt)
300
250
200
150
100
50
0
216 ± 23
6 ± 1
N. D.
N. D.
249 ± 33
3 ± 1
N. D.
N. D.
145 ± 14
2 ± 0
N. D.
N. D.
PQ15/CordapA
PQ15
PQ71/CordapA
PQ71
CordapA
Control

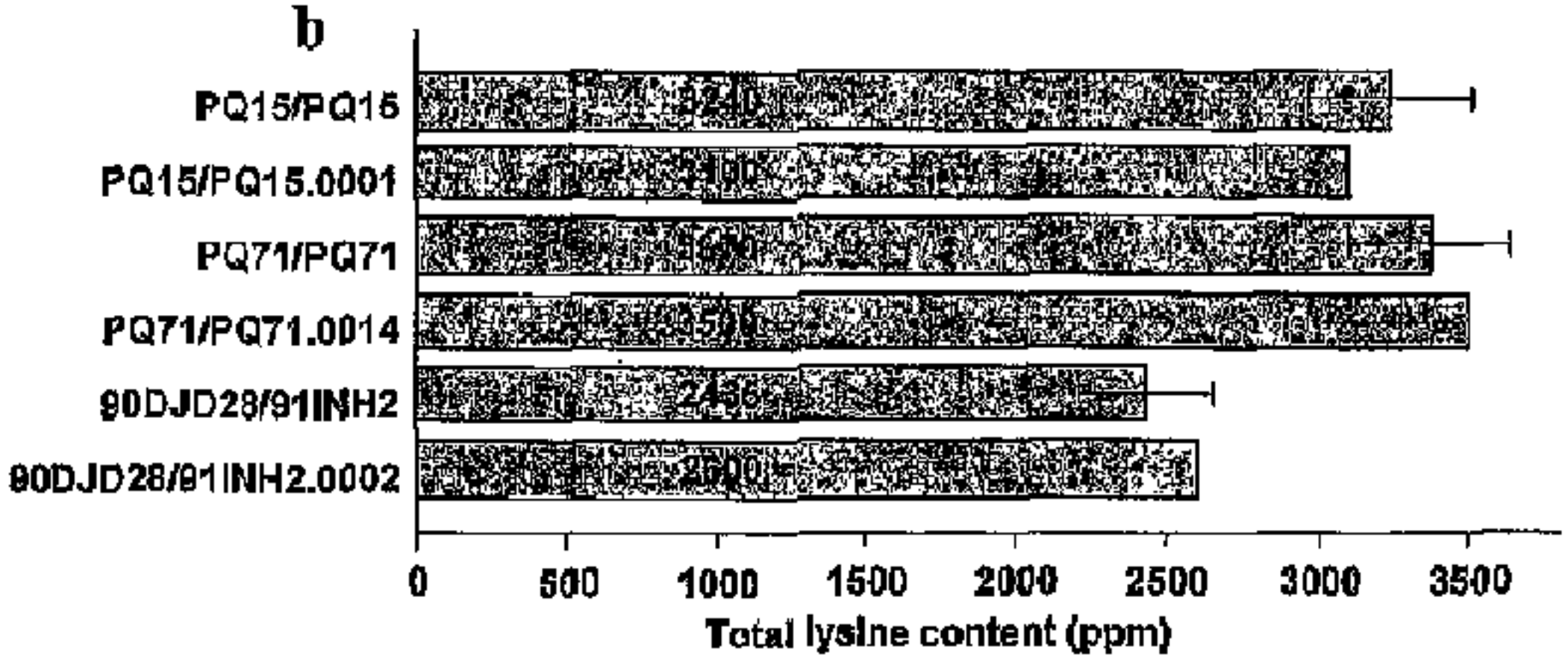
Figure 5


(a)
Free Asp (ppm)
250
200
150
r2 = 0.942
P = 0.020
(b)
Free Asn (ppm)
300
250
200
150
r2 = 0.936
P = 0.024
(c)
Free Glu (ppm)
250
200
150
r2 = 0.888
P = 0.074
(d)
Sac (ppm)
600
550
500
450
400
r2 = 0.481
P = 1.000
(e)
CordapA (ug/g)
300
250
200
150
100
r2 = 0.438
P = 1.000
1500
2000
2500
3000
3500
Free Lys (ppm)

a


90DJD28/91INH2.0002
19-kD α-zeins
[CA+2H]+2
[CA+H]+
15-kD β-zein
16-kD γ-zein
27-kD γ-zein
22-kD α-zeins
PQ015/PQ15.0001
PQ071/PQ71.0014
% Intensity
Mass (m/z)

b


PQ15/PQ15
PQ15/PQ15.0001
PQ71/PQ71
PQ71/PQ71.0014
90DJD28/91INH2
90DJD28/91INH2.0002
Total lysine content (ppm)