

## biotechnology focus

# Engineering plant protein composition for improved nutrition

One of the goals of plant genetic engineering has been to create crops that are tailored to provide better nutrition for humans and their domestic animals. A major target has been the improvement of the amino acid composition of seed protein – in particular the lysine content of cereals and the methionine content of legume seeds. Here we focus on attempts to improve the concentration of sulphur-containing amino acids (S-amino acids) in legume seeds by the introduction of genes encoding proteins with a high methionine content.

Animals, including humans, are incapable of synthesizing ten of the 20 amino acids needed for protein synthesis, and these 'essential' amino acids must consequently be obtained from the diet. However, in order to be used efficiently for protein synthesis, amino acids derived from ingested protein must be present in balanced amounts – any that are present in amounts in excess of those defined by the first limiting amino acid are catabolized for energy production. Animal feed formulations usually include protein from diverse sources in order to balance the amino acid composition of protein in the diet – and even then they are usually supplemented with pure amino acids.



Lysine is the first limiting amino acid in cereal grains, which are commonly used as the principal energy source in the diets of humans and livestock. Conversely, legume seeds, which are important sources of protein in human and animal diets, generally contain an adequate supply of lysine, but are deficient in the S-amino acids methionine and cysteine (Table 1). Pea seed protein contains around 0.8% methionine and 1.0% cysteine (g of amino acid per 100 g of protein)<sup>1</sup>. This falls short of animal growth requirements for S-amino acids, which are in the range 3–5%, by weight, of dietary protein. Animals can convert methionine to cysteine, but not the converse, and therefore methionine can supply the complete requirement for S-amino acids, but cysteine cannot. The S-amino acid intake of animals must consequently contain a minimum amount of

methionine. For example, for optimum performance of growing pigs, dietary protein must contain 3.5%, by weight, of S-amino acids, of which 1.6% must be methionine; the remaining 1.9% can be either methionine or cysteine<sup>2</sup>.

Grain legumes or pulses are also an important source of protein for humans, particularly in countries where the diet is predominantly vegetarian. Amino acid imbalance can have severe detrimental consequences for people eating a vegetarian diet of limited diversity. The physical and mental development of children can be irreversibly retarded by deficiencies of essential amino acids.

## Improvement of nutritive value by breeding

Improvement of nutritive value, in particular the amino acid profile of seeds, has been a major, long-term goal of plant breeding programmes, especially for cereals and pulses. The biggest success was achieved when high-lysine maize mutants were identified<sup>3</sup>. However, when seed collections of pulses and their near relatives, from a wide range of origins, were screened for lines that had a higher than average level of S-amino acids, it was shown that there was very little genetic variation for this character. For example, in one survey of 45 lines of pea and related species any increase in one relatively sulphur-rich seed protein fraction (such as legumin) was invariably accompanied by a decrease in the other major sulphur-rich fraction (the albumins)<sup>4</sup>. The consequence of this was that the S-amino acid content of total seed protein remained fairly constant. The major seed-storage proteins of grain legumes vary in their contents of methionine and cysteine. However, the most abundant proteins contain few or no S-amino acids, with the result that overall seed protein S-amino acid concentration is low.

**Table 1. Methionine contents of plant proteins<sup>a</sup>**

Protein type	Protein	Methionine content (g 100 g <sup>-1</sup> protein) <sup>b</sup>	Methionine content (% of residues)	Ref.
<b>Seed proteins</b>				
	Pea seed protein	0.8	Not determined	1
	Lupin seed protein	0.6	Not determined	23
	Soybean seed protein	1.2	Not determined	19
<b>Methionine-rich proteins</b>				
	Maize 21-kDa zein	37	28	10
	Brazil nut 2S albumin	23	18	13
	Sunflower seed albumin	20	16	14

<sup>a</sup>Note that the estimates of sulphur-containing amino acid contents of all the seeds listed vary between different publications and different plant varieties; those cited are most relevant in the context of engineering plant protein composition.

<sup>b</sup>The animal growth requirement for methionine is 1.6–1.9 g 100 g<sup>-1</sup> protein.

### Improvement of nutritive value using genetic engineering

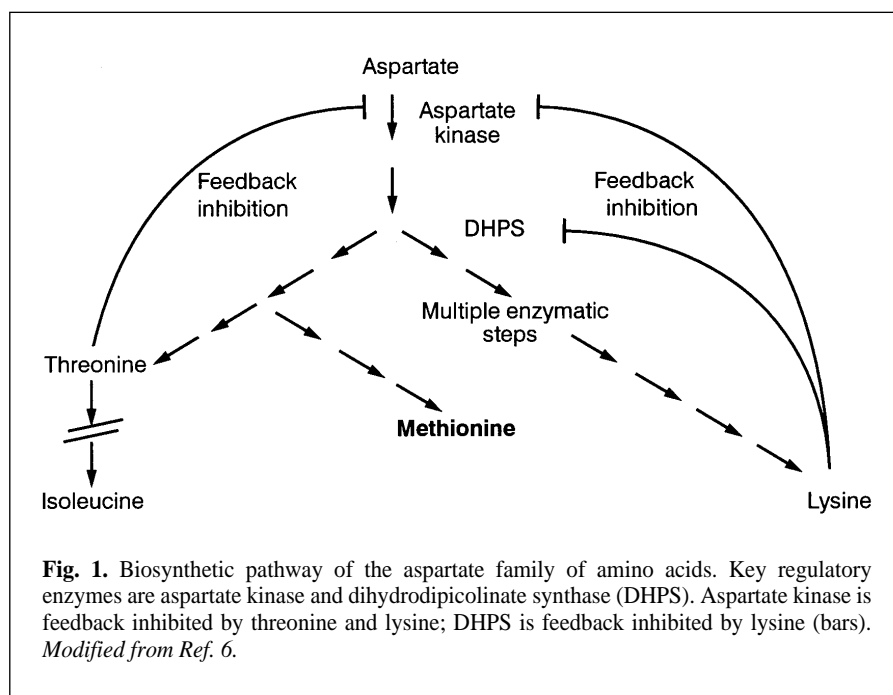
Advances in plant tissue culture techniques and gene transfer technology have opened up new possibilities for modifying the amino acid contents of plants. Attempts to modify S-amino acid content fall into two main categories. One approach has been to manipulate the regulation of amino acid biosynthesis in order to increase the abundance of methionine. Another approach has been the insertion and expression of genes encoding sulphur-rich proteins in transgenic plants.

### Manipulating methionine biosynthesis

Methionine is an amino acid of the aspartate family, which includes other nutritionally essential amino acids: lysine, threonine and isoleucine. Key control points in the aspartate pathway are catalysed by the enzymes aspartate kinase and dihydrodipicolinate synthase (DHPS). In plants, there are commonly two isozymes of aspartate kinase whose activities are feedback-inhibited by either threonine or lysine. DHPS is feedback inhibited by lysine (Fig. 1). Genes encoding feedback-insensitive forms of aspartate kinase and DHPS have been cloned and transferred to plants under the control of constitutive or organ-specific promoters. It was found that expression of a feedback-insensitive DHPS enzyme, either alone or in association with expression of a feedback-insensitive aspartate kinase, could greatly increase free lysine content in transgenic plants. Expression of these genes using a seed-specific promoter resulted in more than 100-fold increases in free lysine in transgenic soybean and canola (*Brassica napus*) seeds, equating to nutritionally significant increases in total seed lysine of 25% and 100%, respectively<sup>5</sup>. Seed-specific expression of a feedback-insensitive aspartate kinase alone resulted in a 17-fold increase in free threonine and a threefold increase in free methionine in the seeds of transgenic tobacco<sup>6</sup>. The proportions of the other amino acids, including lysine, in the seed were unchanged. No increase in protein-bound threonine was observed, but there was a slight increase in the level of protein-bound methionine. Using this approach, total seed threonine was increased by 6.5%; total seed methionine was increased by 6.8% (Ref. 5). Although these modest increases would be of little practical benefit in crop plants, they are interesting in terms of understanding the regulation of amino acid biosynthesis.

### Expressing methionine-rich proteins in transgenic plants

Gene transfer technology has been used to express a number of high-sulphur proteins in plants. One strategy has been to isolate the gene for a naturally occurring, sulphur-poor,



**Fig. 1.** Biosynthetic pathway of the aspartate family of amino acids. Key regulatory enzymes are aspartate kinase and dihydrodipicolinate synthase (DHPS). Aspartate kinase is feedback inhibited by threonine and lysine; DHPS is feedback inhibited by lysine (bars). Modified from Ref. 6.

seed protein and to modify its nucleotide sequence so that it encodes a protein with an increased S-amino acid composition.

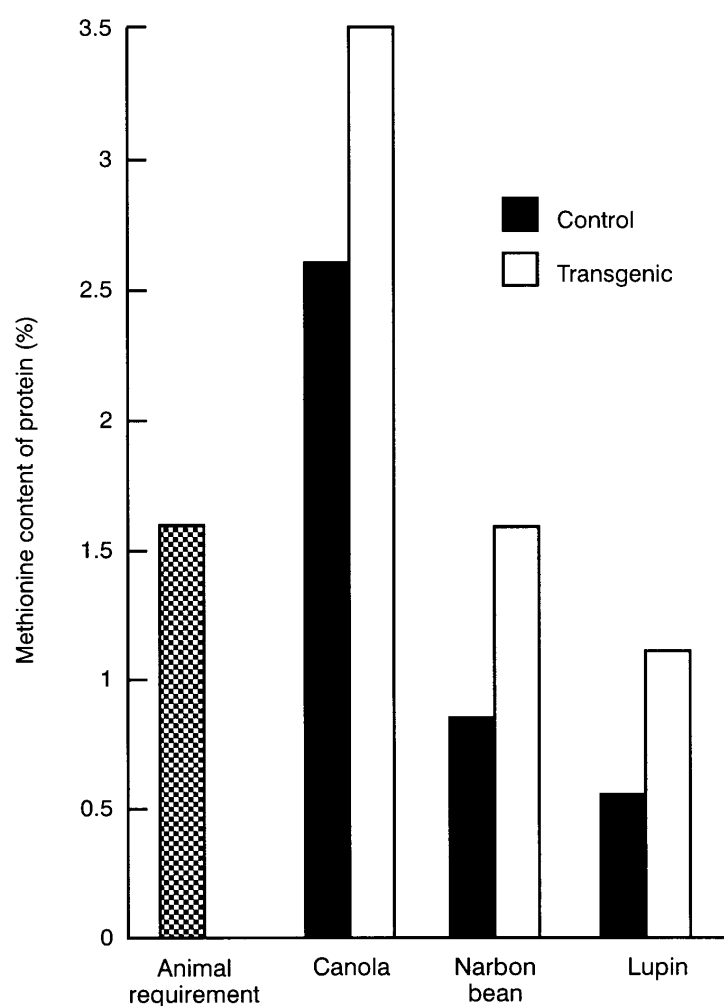
For example, a gene for  $\beta$ -phaseolin from *Phaseolus vulgaris* was modified by the addition of a 45 bp nucleotide sequence encoding a methionine-rich region from a maize 15 kDa zein seed-storage protein<sup>7</sup>. The added peptide was predicted to form an  $\alpha$ -helical structure and was inserted into an  $\alpha$ -helical region of phaseolin. The modification increased the number of methionine residues in the protein from three to nine. The modified phaseolin gene was expressed at the same level as the wild-type gene in the seeds of transgenic tobacco as measured by mRNA abundance. However, the modified, high-methionine phaseolin accumulated to a much lower concentration than the wild-type protein. It was concluded that the high-methionine phaseolin was unstable in the developing seed<sup>7</sup>.

Similar approaches were used to create other modified seed-storage proteins, and these were expressed at varying levels in the seeds of transgenic plants<sup>8,9</sup>. The proteins used in all these cases initially contained very low numbers of methionine residues and, even after the sequence modification, methionine only constituted around 2% of the total number of amino acids in the protein. In order to raise the methionine content of the total seed protein to >1.6%, by weight, the modified proteins would have had to be expressed at unrealistically high levels making this an impractical approach at this stage.

A variation on the strategy of modifying existing genes is the creation of entirely synthetic gene sequences encoding artificial proteins

with a high methionine content. Genes encoding novel proteins that contain large amounts of the essential amino acids lysine, tryptophan and methionine have been synthesized and expressed in *E. coli* to test for stability (see Note added in proof). The proteins were designed to have an  $\alpha$ -helical coiled-coil structure that should allow considerable variation in amino acid sequence. When expressed in the seeds of transgenic tobacco, a protein containing 31% lysine residues and 22% methionine residues resulted in up to a 20% increase in seed methionine (see Note added in proof). If expressed at a similar level in legume seeds, such a protein could, potentially, have a significant effect on the overall methionine content.

A synthetic gene encoding a protein rich in several essential amino acids, including 13% methionine residues, was expressed in transgenic sweet potato (*Ipomoea batatas*), resulting in a nutritionally significant increase in essential amino acid concentrations in the storage roots (C.S. Prakash, M. Egnin and J. Jaynes, pers. commun.). Unexpectedly, the transgenic plants also contained more total protein in leaves and in roots than did control plants. Sweet potato constitutes an important part of the diet of many people in developing countries, so the target of this work is to improve human nutrition. The agronomic performance of the transgenic sweet potato is being assessed in a field trial. Although animal feeding experiments will be necessary to confirm the digestibility of the synthetic protein and the availability of its essential amino acids, this work shows great promise for improvement of nutritive value of a staple crop in the developing world.



**Fig. 2.** Methionine contents of seeds modified using genetic engineering compared with an estimate of the dietary requirement of growing pigs for methionine. The pig is chosen as an example of an intensively farmed animal that is a consumer of livestock feeds. The methionine contents are expressed as a percentage of total protein (g of methionine per 100 g of protein) for canola (*Brassica napus*)<sup>17</sup>, narbon bean (*Vicia narbonensis*)<sup>22</sup> and narrow leaf lupin (*Lupinus angustifolius*)<sup>23</sup>. The methionine requirement of pigs is expressed as a percentage of dietary protein (g of methionine per 100 g of protein)<sup>2</sup>. For optimal growth, the pig requires 3.5% of dietary protein to be composed of sulphur-containing amino acids, of which at least 1.6% must be methionine. The remaining 1.9% can be methionine or cysteine.

Genes encoding naturally occurring proteins that are unusually rich in methionine (Table 1) have also been used for altering the amino acid composition of transgenic plants. The most methionine-rich proteins so far identified as candidates for this approach are a 21-kDa zein in which 28% of the amino acid residues are methionine<sup>10</sup>, and a 10-kDa zein containing 23% methionine residues<sup>11</sup> from maize. A 10-kDa prolamin with 20% methionine residues<sup>12</sup> occurs in rice seeds. Two promising candidates from dicots are a 2S seed albumin from Brazil nut (*Bertholletia excelsa*) [Brazil nut albumin (BNA)] containing 18% methionine residues<sup>13</sup> and a 2S albumin from sunflower (*Helianthus annuus*) [sunflower seed albumin (SSA)]<sup>14</sup> containing

16% methionine residues. In both of these 2S albumins, cysteine constitutes 8% of the amino acids.

The methionine-rich proteins from monocots have generally been used for expression in transgenic monocot seeds and the proteins from dicots have been most commonly used in transgenic dicots. Although the cereal grains are not particularly deficient in S-amino acids, they have been modified with high-sulphur proteins in order to increase the S-amino acid content of blended stock feeds containing both cereal and legume grains. The main monocot target for this approach has been maize, and the main dicot targets have been soybean, lupin, narbon bean, canola and potato.

### Enhancement of methionine content in maize seeds

A gene encoding the methionine-rich, 10-kDa zein has been transferred to maize. Analysis of first-generation ( $F_1$ ) seed of 28 different transformed lines showed that, in many lines, kernels that inherited the transgene had elevated levels of the 10-kDa zein and increased seed methionine. The zein accumulated to levels up to 0.9% of seed protein, and seed methionine was increased by as much as 30% (Ref. 15). High levels of the 10-kDa zein did not always correlate with high methionine levels and some alterations in the patterns of accumulation of other sulphur-containing zeins were evident. These findings suggest that the highly expressed introduced protein competed with endogenous sulphur-rich proteins for limited sulphur reserves.

### Enhancement of methionine content in dicot seeds

A chimeric gene encoding BNA under the control of the tissue-specific, developmentally regulated promoter from a *P. vulgaris* phaseolin gene was initially expressed in transgenic seeds of tobacco. The foreign protein accumulated to approximately 8% of salt-extractable seed protein, resulting in an increase of 30% in seed methionine<sup>16</sup>. The same chimeric gene was also transferred to canola<sup>17</sup>. Like maize, canola was used as a transgenic host not because of an intrinsic deficiency in S-amino acids but because of its use in feed mixes along with sulphur-deficient soybean meal. Canola lines expressing BNA at levels up to 4% of total seed protein in pooled samples of  $F_1$  seed were produced. The methionine content of salt-extractable protein from the segregating populations of BNA-containing seeds was increased by up to 33% (Fig. 2).

Chimeric BNA genes have now been introduced into some of the primary targets of this technology, the large-seeded legumes. These plants were initially difficult to transform compared with other dicots; however, protocols have now been developed for soybean, narbon bean, peas, *P. vulgaris*, chickpea and lupin.

A chimeric gene controlled by a *P. vulgaris* phaseolin gene promoter and encoding BNA has been transferred to soybean<sup>18</sup>. Transgenic lines expressed BNA at more than 10% of total seed protein, resulting in nearly 50% increases in seed methionine content (R. Jung *et al.*, pers. commun.). The methionine content of soybean protein is usually reported to be approximately 1.2%, by weight (Ref. 19). Therefore it can be deduced that the methionine content of the transgenic soybeans would be approximately 1.8%, by weight, which is within the range of methionine concentrations required by animals for optimal growth.



In soybean seeds expressing BNA at high levels, there were reductions in the amounts of the endogenous proteins that normally contained the bulk of the seed S-amino acids. For example, the amount of Bowman-Birk proteinase inhibitor was decreased in the transgenic seed in comparison with the parental, non-transgenic seed. By contrast, there was an increase in the level of the sulphur-poor,  $\beta$ -subunit of  $\beta$ -conglycinin. It therefore appears that S-amino acids were preferentially sequestered into the transgene product, resulting in down-regulation of endogenous genes encoding sulphur-rich proteins and a concomitant up-regulation of endogenous genes encoding sulphur-poor proteins. Similar effects on the seed-protein profile have been seen in legumes grown under sulphur-deficient conditions<sup>20</sup>.

The BNA protein has also been used to enrich the S-amino acid content of narbon beans. Expression in transgenic narbon beans of a chimeric BNA gene controlled by the promoter of the *LeB4* legumin gene from *Vicia faba* resulted in accumulation of BNA up to 4.8% of SDS-soluble seed protein in homozygous lines<sup>21</sup>. Seed of the highest expressing line had double the methionine content of wild-type narbon beans<sup>22</sup>. This brings the methionine concentration of the transgenic narbon beans into the range required for optimal animal nutrition (Fig. 2).

It was recently discovered that the potent allergenicity of Brazil nuts for some people is attributable to the BNA protein. Not surprisingly, this allergenicity is maintained in BNA expressed in transgenic seeds<sup>18</sup>. When BNA-containing transgenic seeds are intended for animal consumption, this allergenicity may be of little concern; however, the transgenic protein is likely to be unacceptable in human foods.

Another sulphur-rich protein that has been expressed in transgenic dicot seeds is SSA (Ref. 14). A chimeric gene controlled by the promoter of a pea vicilin gene and encoding SSA was introduced into narrow leaf lupin (*Lupinus angustifolius*)<sup>23</sup>. Lupins are pulses with high protein and fibre contents that are used in feeds for both ruminant and non-ruminant animals. SSA accumulated to approximately 5% of salt-extractable seed protein in a homozygous transgenic lupin line and total seed methionine content was doubled relative to the untransformed line (Fig. 2). In a feeding trial with rats, it was demonstrated that the biological value of the transgenic lupin seeds for animals was increased by 15%. Biological value is a measure of the efficiency of conversion of feed protein into body protein by an animal. Transgenic lupins expressing SSA are currently undergoing field trials to evaluate their potential for commercial release in Australia.

A chimeric gene encoding SSA has also been transferred to pea and chickpea. In transgenic seeds of both plants, SSA accumulated to 2–5% of extractable seed protein and was associated with a significant increase in seed methionine. Surprisingly, the transgenic seeds also had increased amounts of total protein (H. Schroeder *et al.*, unpublished). Therefore, although transgenic pea and chickpea seeds containing SSA had more S-amino acids than controls, their protein was not enriched with respect to these amino acids. This contrasts with the situation in transgenic soybean, narbon bean and lupin expressing high-methionine proteins, where methionine levels were specifically increased.

#### Enhancement of leaf protein quality for ruminant animals

The performance of ruminant animals can be improved by supplementing the diet with methionine. Although leaf proteins contain a moderate proportion of methionine, the process of ruminant digestion involves wastage of amino acids from feed protein. Sheep, in particular, have a high requirement for S-amino acids to fuel the growth of wool, which contains fibre proteins rich in cysteine. It has been demonstrated that SSA is resistant to degradation in the rumen and should therefore be an efficient medium for delivery of S-amino acids direct to the small intestine of the animal, bypassing the wasteful step of conversion to microbial protein in the rumen<sup>24</sup>.

Lupins are fed to sheep in Western Australia in times of pasture shortage, so transgenic lupins expressing SSA should be of practical use for improvement of wool production. However the vegetative parts of pasture legumes and grasses would be more desirable targets for S-amino acid supplementation for increased wool production. Previous attempts to express vacuolar seed proteins, such as pea vicilin, in the leaves of transgenic plants met with limited success until the protein-coding sequence was modified to include a peptide signal that targeted the protein to the endoplasmic reticulum of the cell<sup>25</sup>. A chimeric gene encoding SSA with a similar modification was transferred to subterranean clover (*Trifolium subterraneum*), one of the most widely cultivated pasture legumes in Australia. The SSA accumulated to about 0.75% of total protein in the leaves of  $F_1$  progeny of a highly expressing transgenic line<sup>26</sup>. This work demonstrated the feasibility of this approach for modifying the S-amino acid content of vegetative plant material for the benefit of grazing animals. A transgenic subterranean clover line containing SSA equivalent to approximately 1.2% of soluble leaf protein is being bulked up for feeding trials with sheep.

#### Marketing transgenic plants

Transgenic grains with methionine concentrations within the range required for optimal animal nutrition have been produced and are currently under evaluation for commercial release. Transgenic lupins containing SSA and with double the seed methionine of controls have been assessed in a feeding trial with chickens. The results demonstrated that the additional methionine was available to the birds and that the transgenic grain had improved nutritive value relative to the parental lupin variety (V. Ravindran *et al.*, unpublished). The widespread use of these and other transgenic grains will require the development of practices to preserve the identity of improved material during harvest, transport and feed formulation. Following regulatory approval of the transgenic grains for human consumption, the introduction of high-methionine pulses into some developing countries should also increase the standard of human nutrition.

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#### Note added in proof

See Keeler, S.J. *et al.* (1997) *Plant Mol. Biol.* 34, 15–29

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## web reviews

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### Jump stations

#### Lehle Seeds

<http://www.arabidopsis.com/index.html>  
Lehle Seeds is a commercial supplier of *Arabidopsis* seeds, but the teaching section of this 'everything *Arabidopsis*!' site contains a large number of links to useful resources, and covers plant biology and genetics rather than being restricted to *Arabidopsis*. An excellent jump station.

#### American Society of Plant Physiologists (ASPP) Education

<http://aspp.org/education/educatio.htm>  
The ASPP has a strong presence on the web and its education site is a useful source of course information and policy statements.

### General education

#### Association for Biology Laboratory Education (ABLE)

<http://www.zoo.utoronto.ca/zooweb/able/>

The purpose of ABLE is as an information exchange for university lab biology teachers. For example, there are lab-based studies ranging from responses of stomata to their micro-environment to the effects of inbreeding.

#### Syllabus Top 20 Education Sites

<http://www.syllabus.com/toptwenty.html>

Includes links to, for example, university departments, information on distance learning, government publications and museums.

### Learning resources

#### Plants in Motion

<http://sunflower.bio.indiana.edu/~rhngart/plantmotion/PlantsInMotion.html>

The first step towards allaying the 'plants are boring' tag is to show that plants do, in fact, move. This site will show this!

#### A Survey of the Plant Kingdoms

[http://www.mancol.edu/science/biology/plants\\_new/intro/start.html](http://www.mancol.edu/science/biology/plants_new/intro/start.html)

Taxonomy for the easily bored, including text, diagrams and images. Fun to investigate.

### Virtual Crops Project

<http://trc.ucdavis.edu/coursepages/plb105/students/Projhome.html>

A nice way to start looking at plant anatomy, allowing the reader to home in on the part of the plant of interest. Rice and tomato have been included so far.

#### C-fern

<http://www.bio.utk.edu/cfern/>

Contains teaching material to do with the fern *Ceratopteris*...including 'Why would you want to use a fern'.

#### MendelWeb

<http://www.netspace.org/MendelWeb/>

Everything you ever wanted to know about Mendel's 1865 publication.

### Queries

#### Plant Education Newsgroup

<http://www.bio.net/hypermail/PLANT-EDUCATION/>

There are rather too many questions on house-plant maintenance, but otherwise a helpful and active newsgroup.

