SCIENTIFIC REPORT

AR0104: An assessment of standing ability in peas: analysis of stem strength and tissue architecture

1. INTRODUCTION

Peas are adapted to scramble and have little ability to stand erect compared with, for example, faba beans. Hence, to the farmer, they have a 'design fault' in that the canopy of the crop readily collapses at the end of the season when the seed load is high: a phenomenon often referred to as lodging. This makes the pea crop susceptible to disease and reduces the harvest index. In the past the *afila* gene (a character that converts leaflets to tendrils) was introduced into modern breeding varieties in order to reduce the canopy load. At least two factors contribute to the lack of standing ability: the strength of the straw - pea stems easily buckle - and an inherent 'weakness' at the base of the stem, which confers the 'scrambling' characteristics. During these investigations we focussed on the development and structure of pea stems to improve our understanding of the standing ability of the crop. This research will provide a basis for further research by conventional breeding or genetic manipulation.

A study of the forces involved in the process of canopy collapse (Holland, 1988) showed that it is weaknesses in the stem and petiole that leads to its collapse. In his study, Holland concluded that the best way to improve peas would be to improve the mechanical attributes of stems and petioles. By increasing the stem wall (considering the stem as a pipe) relative to the total cross-sectional area of the stem and by increasing the outer diameter of the stems, one would improve standing ability. It was thought that by increasing those cell-types with higher values for Young's modulus, relative to ones with lesser amounts of the substances would also be advantageous. Hence, we planned to identify the true basis of straw strength in pea by examining in detail such architectural features since, in the past it has been studied by simple agronomic features alone. In employing a number of advanced techniques, some of which were suitable for the screening of large numbers of genotypes, an attempt was made to further elucidate the genetic basis for characters that confer an improved standing ability. From the genotypes screened, several target genes were identified and discrete molecular analyses carried out.

2. OVERALL AIMS AND OBJECTIVES

This project aimed to identify characters contributing to standing ability in peas by analysing in detail, cell wall components and stem architecture. It used a multidisciplinary approach using morphological, chemical, physical, microscopical (including infrared microspectroscopy) techniques coupled to genetic variation with the ultimate goal of identifying the genetic basis of straw strength. The rationale for this project included the identification of the potential of using any knowledge acquired for biorational manipulation of standing ability by conventional plant breeding or by genetic manipulation to the benefit of pea growers and users in the food and feed industries.

The research was in accordance with MAFF Forward Objectives, A3 (a) (ii), the Pea Review (27.7.97) taken from ROAME A Programme 8 Policy document in which standing ability has already been identified as a way of significantly improving the crop yield potential and harvestibility.

Seven scientific objectives were identified in the original proposal as being of importance in meeting the aims of the project (as stated elsewhere), and to this end a range of phenotypes were measured using a number of methodologies. Initially, a growth analysis was undertaken using the simple measurements of the size and shape of stems and the timing of collapse. In order to gain an idea of the architectural arrangement of the cells in the stem and the chemistry of its components, more sophisticated analyses were carried out using cytological, histochemical and Fourier-transformation infra-red (FTIR) spectroscopy methodologies. Mechanical measurements were also made of the hardness and bendability of stems. Finally a series of comparisons between lines was made together with a number of crosses to identify particular genes of interest. A number of candidate genes were chosen for detailed molecular analysis and future functional studies.

3. RESULTS AND DISCUSSION

3.1 Genotypes

A large number of genotypes from the JIC germplasm collection and elsewhere were used for the different aspects of this work. Tables 1 and 2 list them and how they have been used. The reasons for employing particular genotypes are given in the relevant section.

Table 1. Pea genotypes used in this project and their description

Accession (JI unless stated otherwise)	Description
64	*Pisum elatius* from Turkey. Extreme, wild-type scrambling pea
73	Thin stemmed line used for production of JIC RILs
205+	Tall isoline developed by Reid and Murfet (Tasmania)
205-	Dwarf isoline developed by Reid and Murfet (Tasmania)
305	'Rogue' of JI884
516	'Maro' (wild type line for JI1014 'rogue')
820	A line denoted as 'stiff-strawed' (later found to be an allele of *fa* in this project)
825	*fasciata* (*fa*); type line fasciation locus which causes massive stem broadening
826	*bifurcation* (*bif*); type line for bifurcation of stem
884	Feltham First (wild type for JI305 'rogue' or tare-leaf line)
981	Line observed to have stiff straw by M. Ambrose, JIC
1014	'Progreta' a 'rogue' of JI516
1183	A line used in JIC 1970's breeding programme to introduce stem stiffness
2122	*arthitic-2* (*art2*); a line showing stem swelling at nodes
2313	*nodulation3* (*nod3*); a supernodulating mutant
2665	*bifurcation2* (*bif2*); a second bifurcation locus
2669	an allele of *bif*
2671	*fasciata* (*fas*); a type line believed to represent another fasciation locus
2771	*fasciata2* (*fa2*); a second fasciation locus
3012	*arthitic-1* (*art1*); a line showing stem swelling at nodes
3024	*bulbous base* (*blb*); a line showing mild swelling at the base of the stem
3031	*symbiosis28* (*sym28*); a hyper(super)nodulating line also showing fasciation
3032	*symbiosis29* (*sym29*); a hyper(super)nodulating line
Woodcock	A cultivar from Unilever plc having good standing ability
1689	A selection from C. Rameau, INRA, Versailles, showing basal stem swelling (found to be an allele of *blb* in this study)
M3T 946	A selection from C. Rameau, INRA, Versailles, showing altered stem structure

3.2 Growth Analysis

Measurements of stem cross-sectional area of a number of genotypes was carried out as part of the developmental analysis to try and obtain and idea of stem structure during growth. They showed that there is an initial decrease in area followed by an almost proportional increase with progression towards the upper stem (Figure 1). Such a structure is an inverted pyramid and cannot be self-supporting. In the example given here, JI64, the area increases and then decreases with new nodes initiated after flowering.

In a comparison between two extreme phenotypes – the scrambling JI64 (a wild type) and the 'stiff-straw' (later found to be fasciated line) - the stem area of the upper internodes of JI64 was much less than that of JI820, with the increase in area continuing with internode number whereas the stem area of JI64 decreased

late in development (Figure 2) illustrating the broadening effect of fasciation on the stem during flowering.

3.3 Time Lapse Photography

To determine the position of the initial failure in pea plants, seedlings were grown under controlled conditions until they collapsed. Time Lapse Video allowed us to establish that the point of initial collapse was in the basal region of the stem in all genotypes examined and the cross-sectional area of this region was measured. In addition, a field of conventional peas was photographed throughout a growing season. Figure 3 shows the effect of the width of the base on the length of time the plants remained erect. The mutant

Table 2. Experimental application of genotypes

	Developmental analysis	Time lapse video	Histology including lignin	Immuno-histology	FTIR	Sugar analysis	Mechanical testing	Genetic analysis	Molecular analysis
64	✓	✓	✓	✓	✓	✓	✓		✓
73	✓	✓							
205+	✓	✓		✓		✓	✓		
205-	✓	✓		✓		✓	✓		
305	✓	✓			✓				
516	✓	✓			✓				
799	✓	✓							
820	✓	✓	✓	✓	✓	✓	✓	✓	✓
825								✓	✓
826								✓	
884	✓	✓		✓	✓				
981	✓	✓		✓	✓				
1014	✓				✓				
1183	✓	✓	✓	✓	✓	✓	✓		
2122	✓	✓				✓	✓		
2313								✓	
2665								✓	
2669								✓	
2671									
2771								✓	✓
3012									
3024	✓	✓	✓					✓	
3031								✓	
3032								✓	
Woodcock	✓	✓							
1689		✓	✓					✓	
M3T 946		✓	✓					✓	
Vicia faba		✓							

1689/1 (the outlier in the figure) remained erect for 38 days in comparison with JI64, which remained upright for 17 days. With the data for 1689/1 removed, the remainder show a linear relationship between cross-sectional area and days upright or weight of tissue above bend point (Figure 3 inset) indicating a purely mechanical phenomenon occurred. 1689/1 has been found here to be an allele of *blb* (see Section 3.9), so these data indicate that *blb* may be a useful gene to increase the early standing ability of peas since it may give the crop more time to establish its network of tendrils.

The site of the initial stem failure was observed to be at the basal region of the stem, above the first scale leaf, in all genotypes examined. Histological analysis of this region has shown that the architecture is between that of root and stem, the so-called the transition zone documented by Gourley (1931), as described

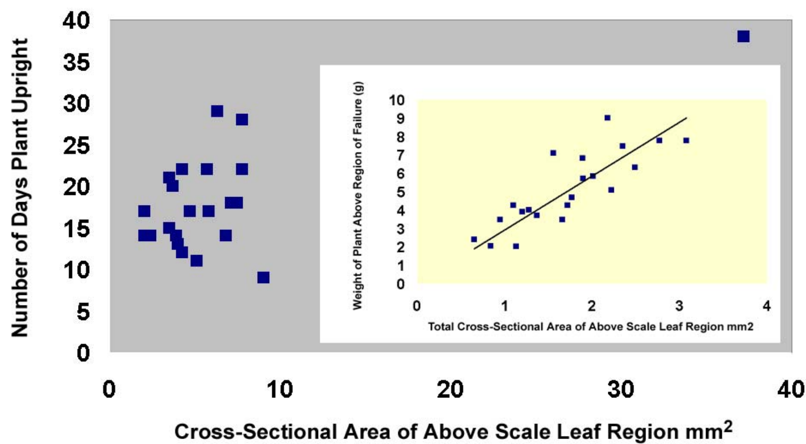


Figure 3. The effect of stem cross-sectional area above scale leaf on number of days the plant remains upright or the weight of stem it can bear (inset) .

in the vascular tissue architecture section of this report (Figure 4B).

3.4 Vascular Architecture

To examine tissue architecture in stems of pea, thin sections were prepared from the stems and stained with Toluidene Blue, which stains lignified tissue blue. The use of this stain also permitted the visualisation of stem cell anatomy. Vascular elements are located centrally in the root (Figure 4A). In the basal region of the stem, the fibrovascular elements are positioned in the centre of the stem where they form a root-like core (Figure 4B). This so-called 'transition zone' is intermediate in structure between that of the root and the stem and thus has an inherent weakness being designed neither to perform as a root nor as a stem. This feature may be designed specifically for the scrambling habit of pea.

The phloem fibres also stain blue for lignin. Away from the core, towards the periphery of the stem there are four fibrovascular elements at each axis. Sections from internode two show the core fibrovascular elements displaying a gradual movement towards the periphery of the stem with the pith showing no lignin staining (Figure 4C-E). The typical stem arrangement was not apparent until internode 3, confirming the observation of Gourlay (1931). In the mature, upper internodes (e.g. 15; Figure 4F), this typical dicotyledonous vascular arrangement is reached with easily distinguished fibrovascular bundles forming a continuous ring separated by interfascicular fibres (Figure 4G). In pea, the internodal regions are hollow. Lignin staining (see later) was not detected in the cortex or pith, indicating that deposition is specific to xylem cells and fibres. The detailed structure of this anatomy can be seen in Figure 4G. A more detailed developmental analysis of tissue organisation was undertaken to look for differences between genotypes. Sampling points were selected to include the basal stem region, internode 2, 15 and 17 since these are where major changes have been identified. Sampling times were early establishment, start of anthesis, start of fruiting and end of fruiting. Differences were most apparent between the basal internodes and upper internodes as described above, for all the genotypes examined. The vascular patterning of those lines examined was similar in all genotypes apart from three lines that exhibited variation. Lines exhibiting a broad basal phenotype, JI3024 and 1689 (Figure 4H and I respectively), later found to be alleles at the *blb* locus, showed variations in their vascular patterning at the base of the stem, whereas a line, JI820, described as 'stiff straw', but later found to be fasciated, showed multiplication of the vascular elements (Figure 4J) and increased proximity between them in the mature stem compared to JI64. JI3024 (*blb*) showed a doubling in the elements in the transition zone, but, in 1689/1, the arrangement of vascular tissue was severely distorted, with additional elements distributed throughout the stem. Thus both fasciation loci and bulbous base locus modify the vasculature of the stem.

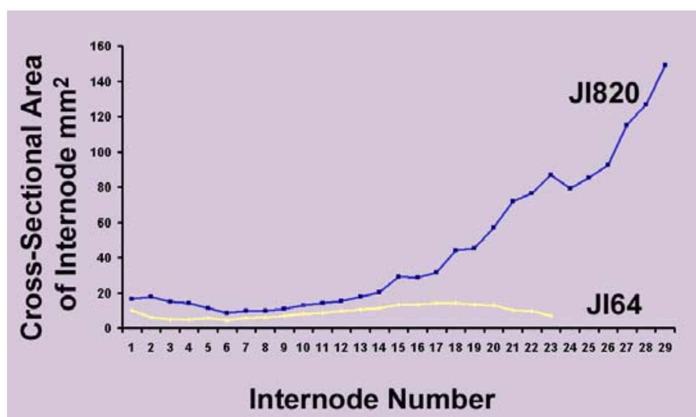


Figure 2. A comparison of John Innes pea accession lines JI820 and JI64 in terms of cross-sectional area during development.

3.5 Fourier transformed infrared Analysis

For these analyses, wild type, stiff straw and rogue (with their parent) lines were all chosen. Fresh hand cut sections from lines in the JIC germplasm were taken from the same sampling points used in the histochemical studies and analysed by FTIR micro-spectroscopy. Spectra were taken from the xylem and cortex region of the stem cross-section and analysed using the statistical method Principal Component Analysis. The results of this study

indicate that there were no clear differences within or between genotypes.

3.6 Cell Wall Chemistry

Samples from the same lines examined in the mechanical tests were analysed for sugar composition using GC-MS. Results show that there were no differences within or between genotypes

3.7 Lignin Evaluation

To examine the changes in lignin content through time, a developmental approach was used to observe differences within and between genotypes, using the same sampling pattern as for the architectural study. Two measurements to quantify lignin were taken: the area of stem occupied by lignified cells was calculated and secondly, the area which stained blue with Toluidene Blue was calculated to give an amount of lignified tissue. These measurements were then also worked out as a percentage of stem cross-sectional area. For simplicity, only the results from John Innes accession lines JI64 and JI820 are presented here. Details of the experimental procedure and sampling are the same as used in Kemsley *et al*. (2004). Sampling times were as follows: d1 – 4 weeks after planting; d2 – start of flowering; d3 – start of fruiting; d4 – end of fruiting.

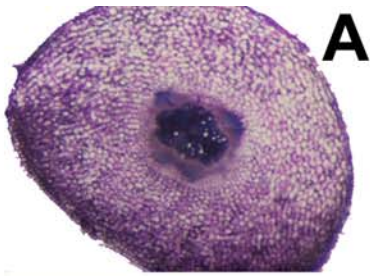
In both genotypes and in both internode 2 and 15, the area of stem occupied by lignified cells and the amount of lignified tissue both increase with developmental stage (Figure 5). The amount of lignified tissue in internode 15 is greater than in internode 2 and this is observed in both genotypes (Figure 5A). It was also observed that the amount of lignified tissue in internode 15 and internode 2 of JI820 is greater than the corresponding internodes of JI64. The values as a percentage of stem cross-sectional area indicate that it is internode 15 which has a greater area occupied by lignified tissue than internode 2 and this pattern is observed in both genotypes (Figure 5B). However, comparing the area of lignified tissue as a percentage of stem area between the two genotypes shows the amounts to be similar in both internode two and internode fifteen (Figure 5B). Therefore, the increase in lignified tissue from internode 2 to internode 15 is explained by the increase in stem area in these internodes.

For the area of lignified cells present, it is again JI820, which has the greater area in both internode 2 and 15 (Figure 5C). In JI820, it is internode 15 that has a greater area of lignified cells than internode 2, but in JI64 it is internode 2 which has a greater area of lignified cells over internode fifteen (Figure 5C). Similarly, as a percentage of stem cross-sectional area (Figure 5D) internode 15 of JI820 again has a greater amount of stem occupied by lignified tissue compared to JI64. At internode 2, however, JI64 has a greater amount of lignified cells as a percentage of stem cross-sectional area than internode 15 and also a greater amount than internode 15 and internode 2 of JI820. All these findings suggest that the structure of the stem is a parameter that has to be considered when assessing the role of the amount of lignified tissue in improving stand.

3.8 Mechanical Testing

Two types of mechanical test were employed in this investigation. The first test was a cutting test, which measured stem toughness. The second test was a three-point bend test, which measured stem strength. These tests were carried out on five genotypes JI1183, JI820, JI64, 205+ and 205- using the same sampling points as in the histochemical study with the addition of the above scale leaf region. These lines were used as extreme examples of phenotypes including tall and dwarf, stiff straw fasciated and wild type. The full details of these tests and their analysis can be found in Kemsley *et al* (2004). The abstract with the conclusion is provided below for reference.

Figure 4. Anatomy of pea stems. A-E: sections through the stem of JI64 from root (A), transition zone (B), first node (C), second node (D), third node (E) and mature stem (F) showing arrangement of tissues. G shows the arrangement of these tissues in the mature stem – e=epidermis, sf=sclerenchyma fibres, p=phloem, c=cambium, x=xylem. H and I show that arrangement of tissues in the transition zone of JI 3024 and 1689 from INRA, both alleles of *blb.* Section J is a cross-section of mature stem from JI 820 which represents the structure of a fasciated stem. All sections were stained with Toluidine Blue.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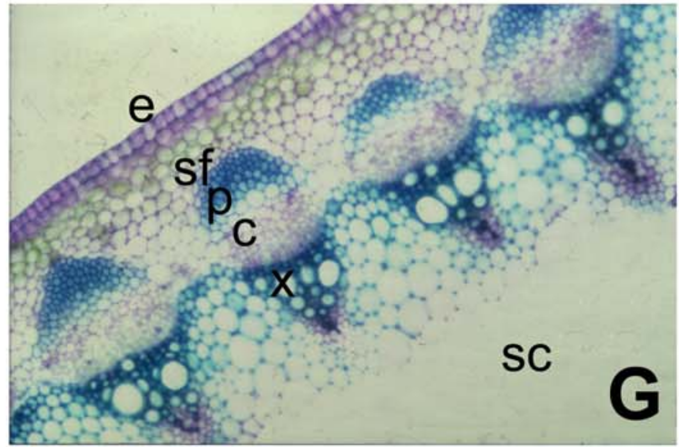
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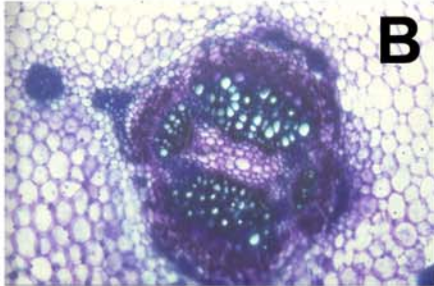
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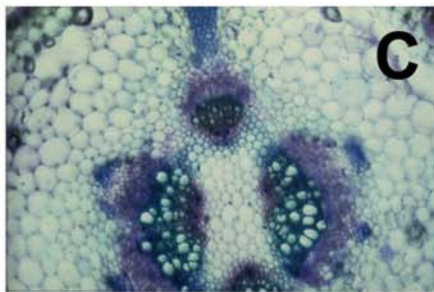
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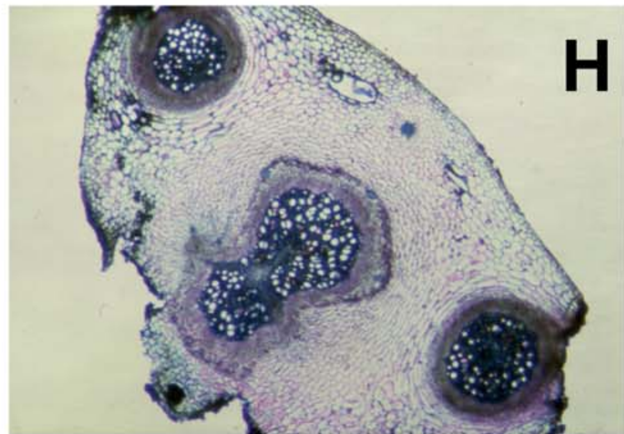
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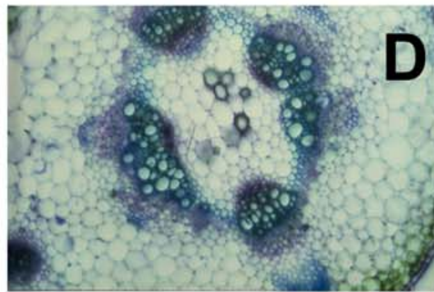
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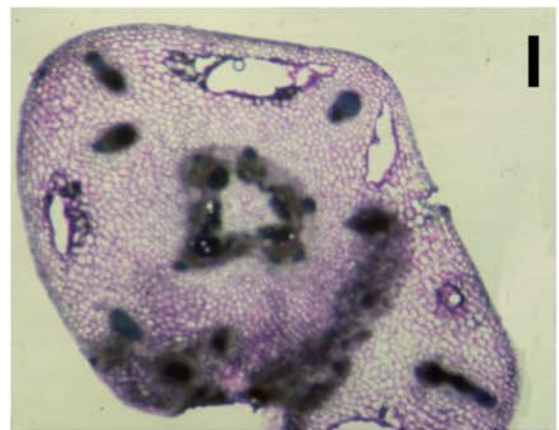
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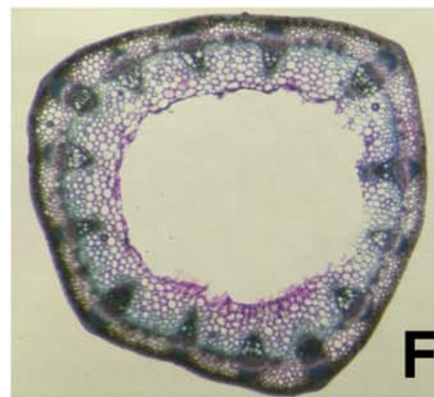
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"Mechanical tests have been used to assess the engineering properties of pea (*Pisum sativum* L) stems. Measurements were made on plants of three different genotypes at four different stages of development and at five defined locations along the stem. The force-displacement curves obtained were used to estimate values of the engineering properties of toughness and flexural modulus, from cutting and flexure mechanical tests respectively. Specimens of all genotypes showed an increase in toughness with age and generally also with stem height. However, there were marked differences in flexural modulus between genotypes. One genotype, known to exhibit a 'stiff straw' characteristic, showed a consistent increase in modulus with age and stem height, and at and beyond fruiting had substantially the greatest flexural modulus. The remaining genotypes showed decreasing flexural modulus with age. Chemometric methods were used to analyse sets of complete force-displacement curves, following suitable pre-processing to allow the application of linear algebra methods. Whereas univariate consideration of the engineering quantities allowed trends to be observed, multivariate analysis of force-distance curves was able to model empirically the genotype differences so that individual specimens could be largely correctly classified. Examination of some of the model coefficients suggested that the ability to discriminate between genotypes is related to structural features of the specimens and that cutting tests in particular are sensitive to the anatomy of the specimen. This is the first time that chemometric methods have been applied to such data and suggests the potential of mechanical tests combined with multivariate analysis to form the basis of a screening system for phenotypic properties of new lines and varieties. "

Both cutting and bending tests were able to highlight differences in the mechanical properties of pea stems. Simple analysis of conventional engineering quantities suggested that specimens of all genotypes increased in toughness with age and generally also with stem height. In particular, we were able to distinguish JI1183, a line used for 'stem stiffness' in a 1970's JI breeding programme, although we were not able to distinguish this line in other studies. JI1183 specimens exhibited generally the lowest toughness. The flexural modulus showed a very different pattern of change for the three genotypes. JI1183 showed a consistent increase with age and stem height, and at fruiting and after fruiting had a substantially greater flexural modulus than JI820 or JI64 - it was substantially stiffer. JI820 and JI64 became less stiff with age.

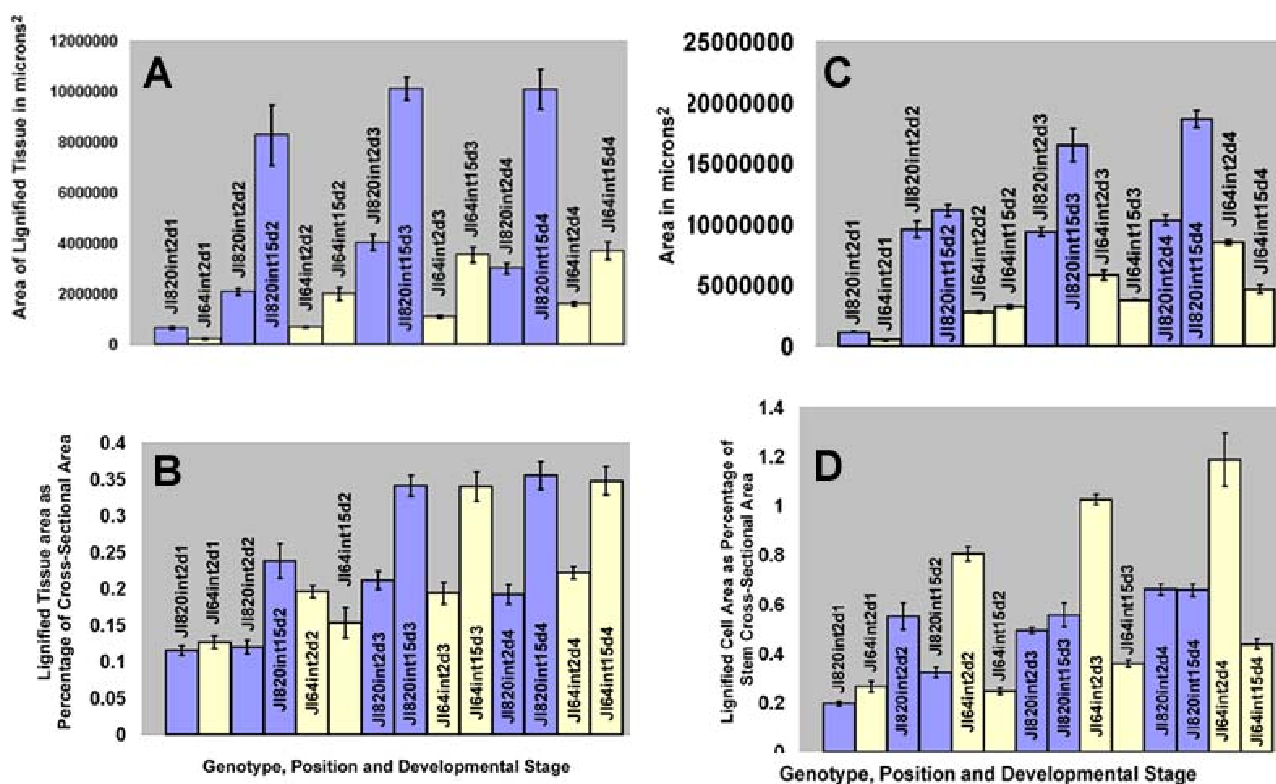

Figure 5. Evaluation of lignin content of pea stems. All data shown are for two internodes at four stages and for two genotypes. A – area of lignin-stained tissue. B – as in A but expressed as a percentage of the stem cross sectional area. C – area of lignified cells. D – as in C but expressed as a percentage of the cross-sectional area.

3.9 Genetic Analysis

For our genetic and molecular analysis, we chose a series of candidate genes based on what was practical to achieve in peas. Clearly modification of the base of the stem is of prime importance in pea from our studies. Bulbous base is an important character in this respect and we showed by crossing that a line, isolated at INRA, with a severely thickened stem base was a new allele of *blb*. A third allele with an intermediate phenotype has been isolated recently at JIC in another mutation programme (M. Ambrose, JIC, pers. comm.). The genes known to affect stem structure from this study were those associated with this character and that of fasciation, both of which have the phenotype of increased vasculature, one component of stem strength. The identity of the genes at these loci in pea is unknown and thus one has to choose other routes to reveal them. These would be map-based cloning which is extremely problematic in pea and only just being addressed or the candidate gene approach. We chose the only practical course at the time and that was the candidate gene approach. We picked genes involved in fasciation that had been identified in *Arabidopsis* since bulbous base is not evident in *Arabidopsis* nor other species to the best of our knowledge. Studies on *Arabidopsis* published towards the end of this project have also revealed further genes that affect vasculature and lignin content and that could be targeted through reverse genetic means (see Section 4).

As mentioned earlier, one of the stiff straw lines (JI820) in the collection turned out to have a fasciated phenotype. Mild fasciation has been introduced into the pea crop through conventional pea breeding, but for other reasons: it brings about simultaneity of flowering. The accession lines used in all the complementation, crossing and mapping experiments carried out during the genetic analysis of fasciation are detailed in Table 3. These details include the accession line origins, initial accession line number and the loci they represent. Representative phenotypes are shown in Figure 6. Other accession lines that were used, included JI826 and JI2665 that showed a bifurcated phenotype and were alleles of two loci *bif* and *bif2*. Bifurcation occurs when the stem of the plant splits into two or forms into two vertical ribs. We felt this was a mild form of fasciation. The fasciation phenotype itself is characterised by a broadened of the stem which often looks like a bundle of sticks or is ribbed (cf. fascia or fascicle), simultaneous flowering towards the apex of the plant and leaf doubling or leaf shape alteration. All the characteristics are normally seen at flowering, but sometimes one or two leaf alterations occur earlier. These genes all affect apical meristem growth. Genetic analysis of these lines was performed first.

Table 3. Lines used for genetic and molecular analysis of 'fasciation' loci

Line	Origins	Loci/mutations and other details	Parental line	JI accession no. of wild-type parental line
JI825		*fa*		
JI 820	Lamprecht line	*fa* or *fas* F5 from a complex cross.	Not known	-
JI2669		*bif1>37*		
JI 826	Gottschalk mutant	*bif* 1201A	Dippes Gelbe Victoria	JI 2413
JI 2665	Gottschalk mutant	*bif2* M. 157 A	Dippes Gelbe Victoria	JI 2413
JI 2771	Vassilova mutant	*fa2*11/87	Raman	JI 3133
JI3031	INRA, Dijon	*sym28* supernod. and fasciated	Frisson	JI2491
JI3032	INRA, Dijon	*sym29* supernod. (clavata-like)	Frisson	JI2491
JI2313		*nod3*		

Complementation tests between these lines showed that, of the three known fasciated lines, the two designated *fa* and *fas* were actually alleles at the same locus. One mutant classified as an allele of *bif* was

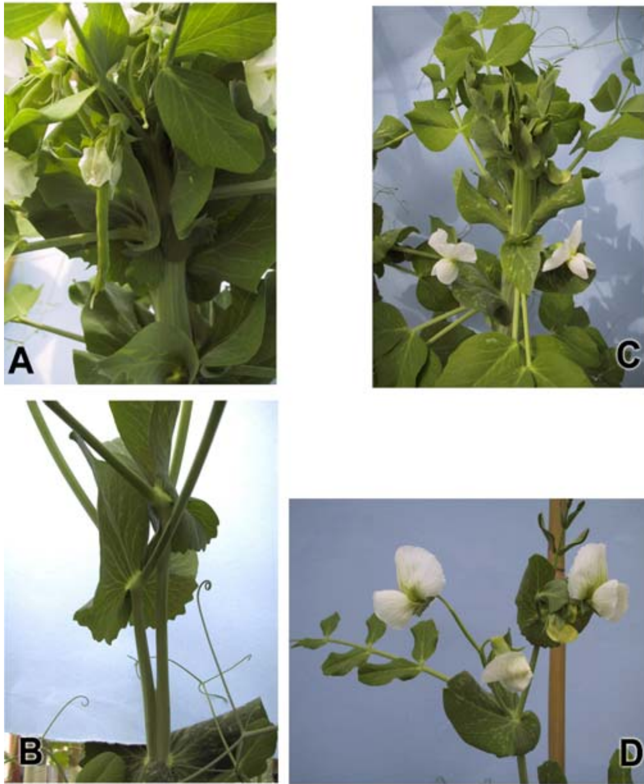

Figure 6 Phenotypes of 'fasciation' genes. A – Close up of apex of type line for fa2 (JI2771). Note broadened stem at point of flowering and multiple simultaneous inflorescences. B – type line (JI826) for *bif* showing bifurcation of stem. C – Close up of apex of stiff straw line, JI820 (*fa*). Showing broad stem and other characteristics of fasciation: multiple leaves at a single node and altered leaf shape. D – type line for *bif2* (JI2665). This line had a very weak phenotype showing only occasionally a ribbed stem (not apparent in this photograph where the apex appears quite normal) indicating the low penetrance of this characteristic.

found to be incorrectly assigned and was in fact an allele at the *fa* locus. This was confirmed by another group in a publication in Pisum Genetics during the course of this work (Swiecicki, 2001) and confirmed our hypothesis that this phenotype is a form of fasciation. Furthermore, JI820 (a stiff straw line from our collection) was found to be an allele at this locus. The second locus, *fa2,* formed a second group and the two remaining bifurcation lines formed two further and separate groups (Table 4). There are three super- (or hyper-nodulation) lines in our collection, one of which is also fasciated (JI3031). More recently another of them (JI3032) was found to encode a *clavata1*-like gene (Krusell *et al*., 2002), a member of a family of genes that also affects meristem activity (DeJong *et al*., 2001). This raised the possibility of a connection between these processes. Complementation tests, therefore, were also carried out with these new lines. The results indicated that the fasciation phenotype of JI3031 segregated in a Mendelian fashion and complemented the other fasciation loci further indicating that the 'supernodulation/fasciation' locus formed a separate complementation group. In the F2s the different forms of fasciation were separable and each acted as a single gene recessive. A separate class of putative double mutant, therefore, should be distinguishable on the basis of its phenotype and should differ from either of the other two fasciation loci. This was indeed the case. Its phenotype had high levels of penetrance, a very early expression of both stem and leaf phenotypes and an exaggerated fasciation phenotype (Figure 7). A 'sunflower-like' phenotype at the apex was also observed (Figure 7 inset). The effect of combining *fa* and this locus was clearly additive indicating two pathways were operating to modify meristem development in these plants.

3.10 Molecular Analysis

In *Arabidopsis thaliana* the fasciated phenotype was originally attributed to mutations in the genes *FAS1* and *FAS2*. A thorough characterisation of the *fas1* and *fas2* mutants, carried out by Kaya *et al.* (2000), revealed that the affected genes encode proteins with similarity to those of the human Chromatin Assembly Factor-1 complex (CAF-1). Included in this complex is a third protein identified by sequence similarity as *MSI1*, previously studied and characterised by Ach *et al.* (1997).

Due to similarities between the *A. thaliana* fasciated mutants and the pea fasciated lines, it was decided to attempt to clone the CAF-1 homologues in pea. We obtained sequences for all three pea genes including a complete cDNA sequence for one of them. These sequences will be placed in the NCBI database in the near future for reference. In order to determine the role of these genes in fasciation, we also planned to carry out studies on their expression in fasciated and non-fasciated lines. Bulbous base has no such equivalent in *A. thaliana* and its cloning will be problematic as mentioned earlier. The postembryonic development of plants is dependent on the activity of both the shoot-apical meristem (SAM) and root apical meristem (RAM) that are established during embryo development. The *FAS1*, *FAS2* and MSI1 genes that form the *FAS*-gene complex, maintain both the cellular and functional organisation of both the SAM and RAM. Given that the *FAS* gene products are subunits of the counterpart to CAF-1, they are thereby implicated in the assembly of chromatin at the DNA replication fork (Mello and Almouzni, 2001). Presumably, any mutation in the *FAS*-

gene complex would have the potential to disrupt the process of DNA replication and the integrity of the DNA-histone arrangement that forms the chromatin. In *A. thaliana* (Kaya *et al.*, 2000) *FAS* gene mutations show a disrupted organisation of the SAM, which in turn produces a fasciated phenotype.

Table 4. Complementation groups of the 'fasciation loci

Complementation Group	JI accession line	Gene	Phenotype
I	JI820, JI2671, JI2669, JI825	*fa, fas, bif1>37*	Fasciated
II	JI2771	*fa2*	Fasciated
III	JI826	*bif*	Bifurcated
IV	JI2665	*bif2*	Bifurcated

.

Figure 7. A JI3031 X JI820 'Double mutant'. Note the way in which this plant, the fasciated phenotype is expressed further down the stem than is the case in pure JI820 or JI2771 lines.

3.10.1 Cloning MSI1 *in pea.*

A PCR fragment corresponding to 1255bp of the coding region of the MSI1 cDNA of *Arabidopsis thaliana* was generated by RT-PCR using forward and reverse primers as previously described by Kaya et al., 2001. After purification and sequencing, PCR fragments were used as probes for the screening of a pea embryo cDNA library (Harrison *et al.*, 2000).

Four positive clones for the *MSI1* homologue were isolated and sequenced. Although slight differences in size and in their 5' region could be detected, all shared the same protein sequence deduced from their coding regions. It was decided to continue working with a 1601bp cDNA clone that contains the entire open reading frame (ORF) encoding *MSI1* from pea. Sequence similarity analysis showed that the isolated cDNA encodes an MSI isoform in *P. sativum*. Most similarity was observed between the deduced protein sequence of the pea cDNA clone and the tomato and *Arabidopsis MSI1* proteins. It is important to mention that the other proteins in this family, *MSI2*, *MSI3* and *MSI4* from *Arabidopsis* displayed less similarity. Phylogenetic analysis revealed that the deduced protein sequence of the *P. sativum MSI1* cDNA grouped uniquely with other known MSI1 proteins (Figure 8).

3.10.2 Mapping PsMSI1

Genomic DNA was isolated from leaves of individuals showing a fasciated phenotype in F2 populations of JI15xJI820 and JI399xJI820. Southern analysis revealed the presence of several bands, most likely corresponding to multiple copies of the gene encoding MSI1 in pea. In the parental line JI15, 4 copies were detected whereas in the parental line JI399 only 3 copies could be detected. The map positions for the *PsMSI1* loci were determined using RFLP markers. Three loci were implicated in the analysis, the alleles for which are termed *PsMSI1/1*, *PsMSI1/2* and *PsMSI1/3*. Scoring of the 3 bands, displaying polymorphisms between the parental lines was used, together with a large set of molecular and morphological markers to calculate their positions in linkage groups (N. Ellis, JIC, pers. comm.). None of the scored *psMSI1* copies mapped to the position calculated for the *fa* locus (Rameau *et al.*, 1998), indicating that this gene is not responsible for the fasciated phenotype conferred through *fa*. Two of these loci, *PsMSI1/1* and *PsMSI1/3*,

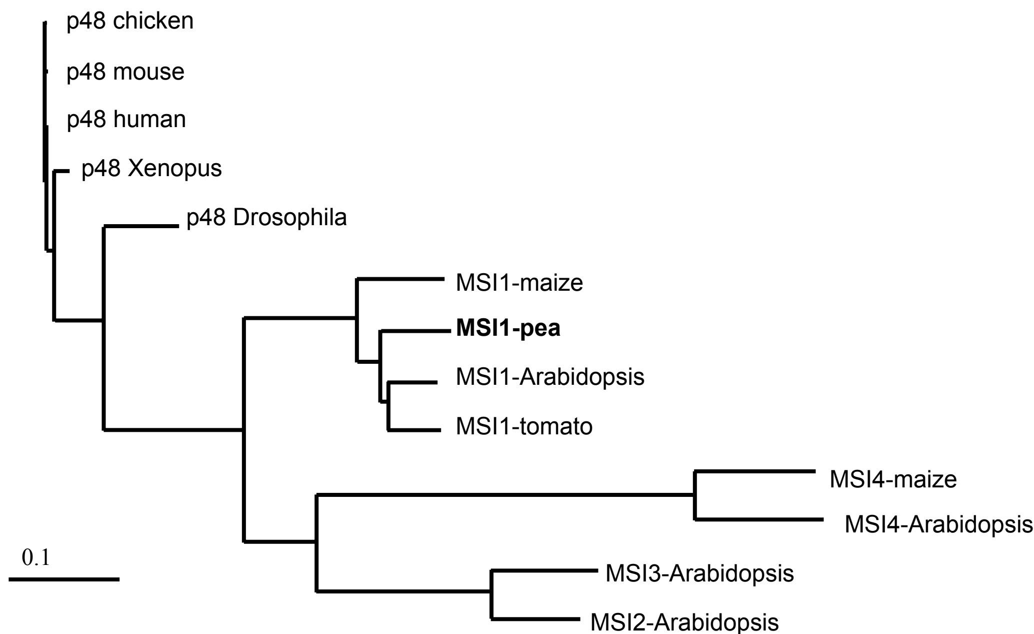

Figure 8. A phylogenetic tree indicating the relative closeness of the genetic relationship between different homologues of MSI1 based on deduced protein sequences.

are present on linkage group VI and there appears to be a lot of duplicated sequence between the regions where these loci are located.

3.10.3 Expression analysis of PsMSI1

Using northern analysis, a single 1.35 kb transcript was detected in all tissues studied. A comparison between an ancestral ecotype (JI64; a non-fasciated pea) and the fasciated line (JI820), revealed clear differences in the levels of expression of *PsMSI1*. As shown in Figure 10, the strongest expression was observed in the apical meristem of the non-fasciated genotype, followed by the expression seen in young roots of the same line. Expression in the stem was considerably reduced. Although no particular differences were observed between apical meristems, roots or stems of the fasciated genotype, overall expression levels were reduced when compared with the non-fasciated line.

3.10.4 Cloning FAS1 *in pea*

Degenerate primers were designed to anneal to regions showing sequence similarity between FAS1 proteins from *A. thaliana*, *Glycine max*, *Medicago truncculata* and *Oryza sativa*. A PCR fragment of approximately

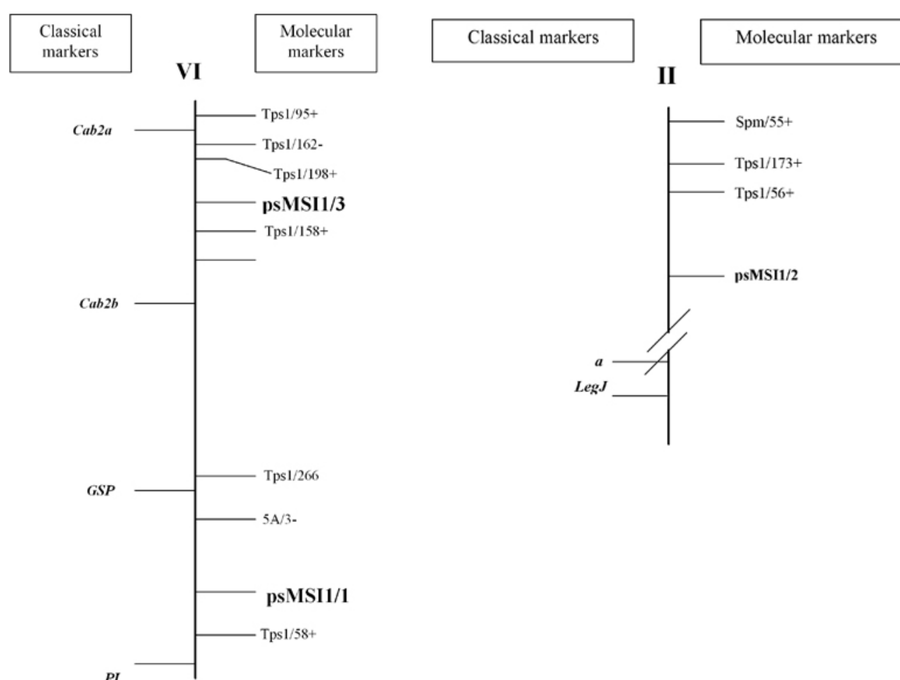

Figure 9. Abbreviated map showing the relative position of *PsMS1* bands on linkage groups VI and II

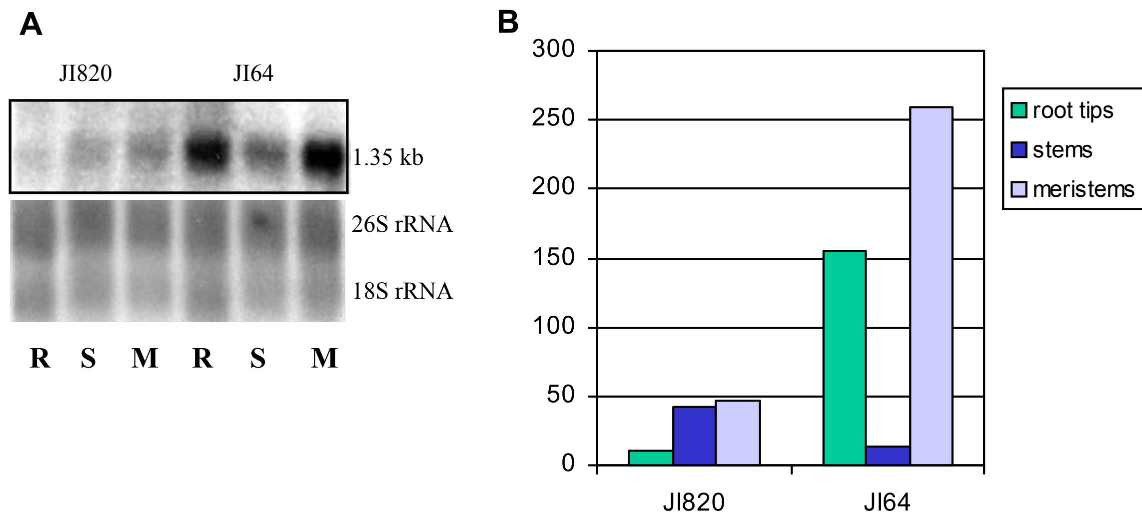

Figure 10. A **-** Northern analysis to determine the levels of expression of *PsMSI1* in the roots (R), shoots (S) and meristems of fascinated (JI820) and non-fasciated (JI64) lines. B **-** A graph showing the relative levels of expression of *PsMSI1* in each tissue.

200bp in size corresponding to a coding region of the *FAS1* gene was generated using Promega's Access reverse transcriptase-PCR with the designed forward and reverse degenerate primers. The primers used are shown below:

FORWARD 5' TGTGAGGAAAGATGAAGAGGAATGTC 3'
REVERSE 5' ATTCCTCAGTCTTCTTATGTCATCC 3'

Once assembled correctly, sequence homology to genes from other species was determined using the NCBI-database blast search facility and, according to the level of homology, the sequence was identified as the *P. sativum* homologue of *FAS1* (*Ps FAS1*). A 5'- and 3' RACE strategy was then used to try and determine the remainder of the *PsFAS1* sequence, but was unsuccessful probably due to the low abundance of message for this gene. Screening a cDNA library of pea also failed to identify clones bearing *FAS1* sequence supporting this hypothesis. There are very few closely related sequences for this gene in the databases and therefore constructing a phylogenetic tree would not be valuable.

3.10.5 Mapping FAS1 in pea

In order to map *PsFAS1*, differences in the parental lines of the mapping populations available at the JIC had to be determined. This was done by screening a number of primer combinations specific to the cloned *PsFAS1* fragment against the parental lines.

No size difference was found between the parentals using the following primer combination:

FORWARD 5' TGTGAGGAAAGATGAAGAGGAATGTC 3'
REVERSE 5' ATTCCTCAGTCTTCTTATGTCATCC 3'

However, when the sequences were analysed, single nucleotide polymorphisms (SNPs) were found between JI284 and JI399, and the other parental lines (Figure 11). This formed the basis for determining a mapping position for *PsFAS1* using the JI15 X JI399 mapping population. The first 24 individuals within the JI15 X JI399 population were enough to give a mapping position associated with a high level of statistical significance. Figure 12 shows a schematic indicating the position of *PsFAS1* on linkage groupV. Again, this position does not correspond to the *fa* locus.

3.10.6 Cloning FAS2 in pea

An identical strategy to that used for the cloning of *PsFAS1* (see previous section) was adopted for cloning *PsFAS2*. Degenerate primers for the cloning of *PsFAS2* are shown below:

FORWARD: 5' GCCGGCGCCGAT
REVERSE: 5' CCTTTGTTAACATCCCATATAATGCAA

A 320bp fragment was cloned and sequenced. After correct sequence assembly, the level of homology with genes from other species was determined using the NCBI-database blast search facility and according to this level, the sequence was identified as the *P. sativum* homologue of *FAS2* (*PsFAS2*). A 5'- and 3' RACE strategy was then used to try and determine the remainder of the *PsFAS2* sequence, but again was unsuccessful for the same reasons as mentioned for *PsFAS1*. Likewise the construction of a phylogenetic tree would be unhelpful.

3.10.7 Mapping of FAS2 in pea

To map *PsFAS2*, differences in the parental lines of the mapping populations available at the JIC was determined. This was done by screening a number of primer combinations specific to the cloned *PsFAS2* fragment against the DNA of parental lines. Using the following primer combination:

FORWARD: 5' GTTACTTATCTCAACAGCCTCTCTTACC
REVERSE: 5' GCTTCCATATTAACAGTTCACCTCC

A size difference was found between PCR-fragments derived from JI813 and the others examined. This difference amounted to 26bp of sequence consisting of 24 bp of insertion (red) and 2bp altered (yellow

```
        201                                              250
   BC1  TGAGGTAGAG GCTTGATGTA TTTTACTGTA ACAAGTTTTC TTTTATATTT
  JI15  TGAGGTAGAG GCTTGATGTA TTTTACTGTA ACAAGTTTTC TTTTATATTT
 JI284  TGAGGTAGAG GCTTGATGTT TTTTACTGTA ACAAGTTTTC TTTTATATTT
 JI399  TGAGGTAGAG GCTTGATGTT TTTTACTGTA ACAAGTTTTC TTTTATATTT
 JI813  TGAGGTAGAG GCTTGATGTA TTTTACTGTA ACAAGTTTTC TTTTATATTT
JI1194  TGAGGTAGAG GCTTGATGTA TTTTACTGTA ACAAGTTTTC TTTTATATTT
JI1204  TGAGGTAGAG GCTTGATGTA TTTTACTGTA ACAAGTTTTC TTTTATATTT
 clone  TGAGGTAGAG GCTTGATGTA TTTTACTGTA ACAAGTTTTC TTTTATATTT

        251                                              300
   BC1  TACAATTTTA TGATTTTAG. GAATAATGTT AACCTTTTGG TGTCTTGAAA
  JI15  TACAATTTTA TGATTTTAG. GAATAATGTT AACCTTTTGG TGTCTTGAAA
 JI284  TACAATTTTA TGATTTTAGT GAATAATGTT AACCTTTTGG TGTCTTGAAA
 JI399  TACAATTTTA TGATTTTAGT GAATAATGTT AACCTTTTGG TGTCTTGAAA
 JI813  TACAATTTTA TGATTTTAG. GAATAATGTT AACCTTTTGG TGTCTTGAAA
JI1194  TACAATTTTA TGATTTTAG. GAATAATGTT AACCTTTTGG TGTCTTGAAA
JI1204  TACAATTTTA TGATTTTAG. GAATAATGTT AACCTTTTGG TGTCTTGAAA
 clone  TACAATTTTA TGATTTTAG. GAATAATGTT AACCTTTTGG TGTCTTGAAA

        301                                              350
   BC1  TTTGTACATG TTATGTTTAT TTTTATTTAA TGTTTTATAA TCTAATGTCA
  JI15  TTTGTACATG TTATGTTTAT TTTTATTTAA TGTTTTATAA TCTAATGTCA
 JI284  TTTGTACACG TTATGTTTAT TTTTATTTGA TGTTTTATAA TCTAATGTCA
 JI399  TTTGTACACG TTATGTTTAT TTTTATTTGA TGTTTTATAA TCTAATGTCA
 JI813  TTTGTACATG TTATGTTTAT TTTTATTTAA TGTTTTATAA TCTAATGTCA
JI1194  TTTGTACATG TTATGTTTAT TTTTATTTAA TGTTTTATAA TCTAATGTCA
JI1204  TTTGTACATG TTATGTTTAT TTTTATTTAA TGTTTTATAA TCTAATGTCA
 clone  TTTGTACATG TTATGTTTAT TTTTATTTAA TGTTTTATAA TCTAATGTCA

        351                                              400
   BC1  AGCTGTGGCA CACGTGGTAT CAAATGTCCG GAGATGTCTT TGTTGAGCAC
  JI15  AGCTGTGGCA CACGTGGTAT CAAATGTCCG GAGATGTCTT TGTTGAGCAC
 JI284  AGCTGTGGCA CATGTGGTAT CAAATGTCTG GAGATGTCTT TGTTGAGCAC
 JI399  AGCTGTGGCA CATGTGGTAT CAAATGTCTG GAGATGTCTT TGTTGAGCAC
 JI813  AGCTGTGGCA CACGTGGTAT CAAATGTCCG GAGATGTCTT TGTTGAGCAC
JI1194  AGCTGTGGCA CACGTGGTAT CAAATGTCCG GAGATGTCTT TGTTGAGCAC
JI1204  AGCTGTGGCA CACGTGGTAT CAAATGTCCG GAGATGTCTT TGTTGAGCAC
 clone  AGCTGTGGCA CACGTGGTAT CAAATGTCCG GAGATGTCTT TGTTGAGCAC
```

Figure 11. A section of the genomic sequence of *PsFAS1* from different accession lines of pea in the John Innes collection, the lines JI15, JI284, JI399, JI813, JI1194 and JI1204 are all parental lines used in the production of the JI mapping populations. SNPs are indicated in bold at positions 220bp, 270bp and 329bp.

highlight; see Figure 13). The JI813 X JI1201 population was scored for *PsFAS2* according to this difference in band size. A mapping position, therefore, was determined on the basis of these scores. There was some ambiguity in the data gathered in this experiment. It is likely that *PsFAS2* can be positioned on linkage group VII. However, these data may also suggest a position on linkage group VI.5, a theoretical linkage

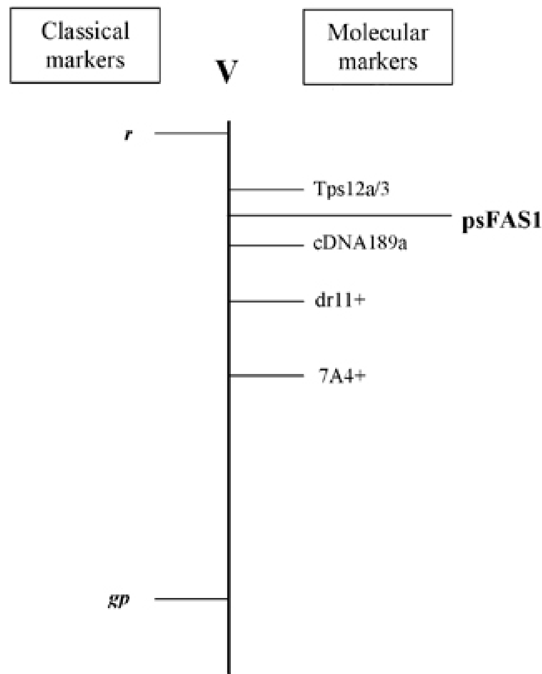

Figure 12. Abbreviated map showing the relative position of *PsFAS1*

group that exists for those markers with mapping data placing them between groups VI and VII (Figure 14; N. Ellis, JIC, pers. comm.), neither of which correspond to the *fa* locus.

From the mapping positions on chromosomes II, V and VI of all the genes cloned, it is clear that none of them correspond to the position of Mendel's fasciation locus, *fa*, which is located on chromosome IV nor to *blb* which is on the other side of the classical marker *a* to MSI/2 on chromosome II. It still remains possible, however, that they may map to the *bif* or *fa2* loci.

4. GENERAL SUMMARY AND CONCLUSIONS

We have identified some basic parameters that should be considered when pea engineering varieties for stem strength. We have also developed laboratory a method of mechanical testing which may help identify these features and potential candidate genes in the future. We selected for examination, potential phenotypes for modifying stem tissue structure and cloned and mapped three pea genes through their equivalents involved in stem architecture in *Arabidopsis*. These genes, however, are not responsible for the corresponding phenotypes in pea. Moreover, we have shown that 5 loci appear to be involved in determining fasciation in its widest context in pea stems. More recently, additional

```
         1                                                 50
   BC1   ~~~~~~~~~~ ~~~~TTACTT ACTTACATCA ATTCAATCTA AAAAAAA..C
  JI15   ~~~~~~~~~~ ~~~~~~ACTT ACTTACATCA ATTCAATCTA AAAAAAA..C
JI1201   CCGATTAAGA AACATTACTT ACTTACATCA ATTCAATCTA AAAAAAA..C
 JI813   ~~TTCCGATT AAGAAACATT ACTTACATCA ATTCAATCTA AAAAAAAACT

         51                                               100
   BC1   TCAAAAACAT TCGACTGTAG GGATCAAGAC AGTACCATCA GAACCAGAAG
  JI15   TCAAAAACAT TCGACTGTAG GGATCAAGAC AGTACCATCA GAACCAGAAG
JI1201   TCAAAAACAT TCGACTGTAG GGATCAAGAC AGTACCATCA GAACCAGAAG
 JI813   TCAACAACAT TCAACTGTGG GGATCAAGAC AGTACCATCA GAACCAGAAG

         101                                              150
   BC1   CCAATAGTTC CCCTGAAGCA TAACAAATTA AAATTATTAC TTACAGAAAA
  JI15   CCAATAGTTC CCCTGAAGCA TAACAAATTA AAATTATTAC TTACAGAAAA
JI1201   CCAATAGTTC CCCTGAAGCA TAACAAATTA AAATTATTAC TTACAGAAAA
 JI813   CCAATAGTTC CCCTGAAGCA TAACAAATTA AAATTATTAC TTACAGAAAA

         151                                              200
   BC1   TATAACTTCA TTCTCTTACT AAAACAACAA ATTACTCAAA AAAAACTGAA
  JI15   TATAACTTCA TTCTCTTACT AAAACAACAA ATTACTCAAA AAAAACTGAA
JI1201   TATAACTTCA TTCTCTTACT AAAACAACAA ATTACTCAAA AAAAACTGAA
 JI813   CATAACTTCA TTCTCTTACT .......... .......CAA AAAAACTGAA

         201                                              250
   BC1   ATTTCACTCA TTGAGATCTA TCCTTATCCT AATCCTAAAA AACGAAAATC
  JI15   ATTTCACTCA TTGAGATCTA TCCTTATCCT AATCCTAAAA AACGAGAATC
JI1201   ATTTCACTCA TTGAGATCTA TCCTTATCCT AATCCTAAAA AACGAGAATC
 JI813   ATTTCACTCA TTGAGATCTA TCCTAATCCT A.....AAAA AACGAGAATC

         251                                              300
   BC1   GGTAATGCGA TGTGATGTGA TGTTACCGGA AGGAGAGAAT CGAATAACAT
  JI15   GGTAATGCGA TGTGATGTGA TGTTACCGGA AGGAGAGAAT CGAATAACAT
JI1201   GGTAATGCGA TGTGATGTGA TGTTACCGGA AGGAGAGAAT CGAATAACAT
 JI813   GTTAATGCGA TGTGATGTGA TGTTACCGGA AGGAGAGAAT CGAATAACAT

         301
   BC1   TAACTGCCGA
  JI15   TAACTGCCGA
JI1201   TAACTGCCGA
 JI813   TAACTGCTGA
```

Figure 13. Comparison of genomic sequence between *fas2* genes of BC1, JI15, JI1201 and JI813. A 26bp difference is shown between parental lines in the mapping population JI1201 X JI813.

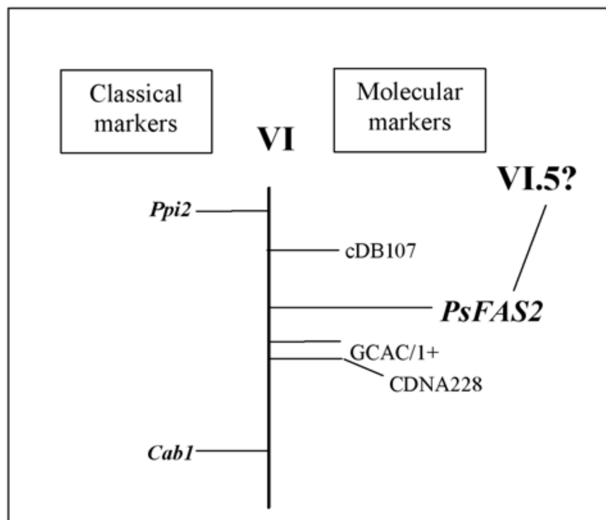

Figure 14. Abbreviated map showing the relative positions of *PsFAS2*.

genes affecting vascular development have been identified in *Arabidopsis* (e.g. Parker *et al*., 2003) and these, together with other meristem genes such as the *clavata* family mentioned earlier, may present opportunities for future targets in pea.

In the field, however, different circumstances prevail and it is clear that in order to exploit such lines other approaches need to be taken in addition to those used here. QTL analysis for standing ability, such as that being carried out by Canadian groups (Tar'an *et al*., 2003), is one such approach and it would both complement and be informed by our studies.

Information could also be gained from model species such as *Lotus japonicus* and *Medicago truncatula*. We have a number of target genes remaining to be isolated from pea that could potentially help in the improvement of standing ability. Although the pea has been an important plant model for genetics, the cloning of genes, as is transformation, is problematic. Technologies for plant genetics and breeding, however, are improving all the time. We have developed in another programme novel methods of mutant isolation using a more amenable model legume, *Lotus japonicus* in collaboration with other researchers at the Centre. In particular, we have developed TILLING methodologies for *L. japonicus* (http://www.lotusjaponicus.org/tillingpages/homepage.htm). TILLING permits mutants to be obtained for genes of known sequence. Originally it was envisaged that natural variation alone would be used in this project or that genes would need to be modified by genetic manipulation. However, TILLING permits a non-GM solution to the introduction of novel variation into a plant and is especially appropriate for crops with under-developed genomics resources and for an in-breeder like pea. Coupled with marker-assisted breeder, such variation can be introduced into elite varieties rapidly. We consider that using model legumes for rapid identification of genes and linking this to TILLING in peas will be the way forward for introducing the variation we have identified and for improving the pea crop in the future. TILLING is currently being developed in France for direct access to pea mutants for known genes.

In addition to the work here, we have isolated a line of *L. japonicus* in another project which has a particularly erect and stiff stem (Figure 15). It is inherited as a single gene recessive and is currently being mapped as part of map-based cloning approach. We have also cloned separately the equivalent *Lotus japonicus* genes to those isolated here, so that mutants could be isolated via TILLING (Perry *et al*., 2002) to discover their phenotype and for functional analysis.

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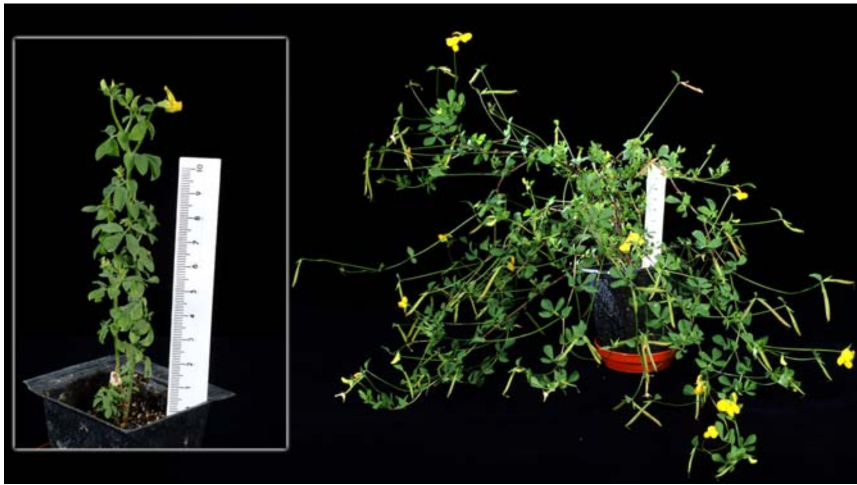

Figure 15. A young plant of an erect mutant of *Lotus japonicus* (inset) in comparison with the WT scrambling phenotype (main picture)

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