CHILE
PÓTENCIA ALIMENTARIA Y FORESTAL

UNIVERSIDAD DE CHILE
FAC. DE CS. AGRONÓMICAS

GOBIERNO DE CHILE
MINISTERIO DE AGRICULTURA
FIA

Día de Campo

"COSECHA MECANIZADA DE HABAS *BABY* EN CHILE"

Este día de campo se enmarca dentro de las actividades de Difusión programadas y comprometidas en el desarrollo de la Gira Tecnológica **"Recopilación de información y evaluaciones en terreno sobre la maduración, cosecha mecanizada e industrialización de haba tipo baby"** que fue financiado por la Fundación para la Innovación Agraria (FIA) y apoyada por la empresa de Alimentos y Frutos S.A. (Alifrut).

INTRODUCCIÓN

En Chile, la superficie sembrada de haba se ha mantenido en un promedio de 2.500 ha, de las cuales aproximadamente un 30% se destina a la industria del congelado. La región con mayor superficie de siembra es la R. Metropolitana (40% de la superficie) en tanto que el 60% restante se distribuye entre las regiones IV, VI, VII y VIII.

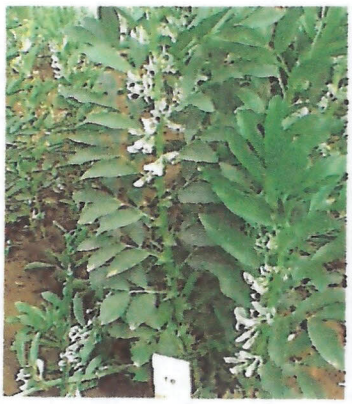
Los cultivares sembrados tradicionalmente en el país, pertenecen a la variedad botánica *major* y se caracterizan por producir granos de forma aplanada, anchos y de calibre grande. A nivel tecnológico y de manejo, existe algunas características comunes entre lo cultivares sembrados en Chile, entre las cuales se pueden destacar:

- *Son de hábito de crecimiento indeterminado*. Son plantas que a partir de los 6 a 7 nudos vegetativos comienzan a producir sucesivamente hacia arriba nudos reproductivos, cuyo número depende del cultivar y de las condiciones ambientales y de manejo a las cuales la planta está expuesta. Cabe destacar que, tanto el eje central como el de las ramas siguen el mismo esquema de crecimiento reproductivo. Este hecho conlleva a que el desarrollo de las vainas así como el de los granos se produzca en forma diferida en la planta y por ende una madurez bastante desuniforme. Además hay que indicar que un porcentaje no menor de las vainas se sitúan cerca del nivel del suelo, llegando incluso a tener contacto con éste.

- *Su cosecha se realiza en forma manual*. Esto ocurre, debido a las características de hábito de crecimiento indeterminado descrito anteriormente, tanto en cultivos destinados para consumo en fresco como para la industria. Para ambos mercados y dado el alto costo de la mano de obra, la cosecha se realiza de una sola vez, lo que implica que en la medida que existan más factores que incidan en la heterogeneidad en la madurez de las vainas, la calidad de los granos se verá más deteriorada, existiendo por un lado, granos sobre maduros, que presentan una textura harinosa y sin dulzor; y granos inmaduros que pasan a formar parte del desecho y por ende pérdida de rendimiento y rentabilidad del cultivo.

- *La siembra se realiza en forma manual o semi mecanizada*. Este hecho obedece al gran tamaño de la semilla de los cultivares utilizados en Chile, motivo por el cual no se pueden utilizar máquinas sembradoras neumáticas como ocurre con otras especies leguminosas para consumo en fresco como lo son arveja y poroto. Por ello la siembra se lleva a cabo en forma manual o utilizando sembradoras que no son de precisión, lo que significa desuniformidad en la distribución y profundidad de la semilla

Además, estos cultivares presentan una baja estabilidad en su rendimiento debido, por un lado, al alto porcentaje de polinización cruzada que presentan (35 a 50%), lo que los hace muy dependientes de la presencia de abejas o abejorros, que pueden o no estar presentes en función de las condiciones climáticas imperantes. Además hay que destacar que la mayor parte de los cultivares son introducidos en el país, no siendo desarrollados específicamente para las condiciones edafoclimáticas chilenas.



Crecimiento indeterminado

Vainas que topan el suelo

Cosecha manual

En base a lo anteriormente indicado, se pensó en la introducción a Chile, de cultivares que pudieran uniformar más su producción, y cuyas vainas fueran formadas en la parte superior de la planta, con el objeto de intentar su cosecha mecanizada. Esta idea quedó plasmada en la ejecución del proyecto antes mencionado, y cuyo principal objetivo ha consistido en la introducción y evaluación en Chile de cultivares de haba tipo "*baby*" provenientes de España (IFAPA Centro Alameda del Obispo de Cordoba). Los genotipos introducidos corresponden a los cultivares **Retaca, Alargá** y **Verde Bonita,** que fueron liberados en el año 2001 y han sido desarrollados específicamente para la industria del congelado y el enlatado como habas *baby* debido al pequeño tamaño de sus granos. En este tipo de habas es altamente demandado, especialmente en España.

La introducción de estos cultivares no tiene por objetivo desplazar a los que ya existen, sino por el contrario, el desarrollo de esta innovación permitirá ampliar y diversificar la oferta exportable de haba, así como incrementar la cantidad de hectáreas y productores dedicados a su cultivo en el país.
Algunas características generales de estos cultivares se presentan a continuación:

- Son de hábito de crecimiento determinado, es decir que todas sus estructuras vegetativas (tallos y ramas) culminan en una inflorescencia, motivo por el cual las vainas se producen en el tercio superior de las plantas. Estas vainas son erectas y se desarrollan en pocos nudos reproductivos, lo que significa que todas ellas se desarrollan en un período de tiempo bastante corto, uniformando de este modo la madurez de sus vainas y granos; este hecho, permite obtener un producto de mejor calidad.

  El carácter determinado de estos cultivares podría ser una excelente alternativa para realizar la cosecha mecanizada de las vainas, sin embargo aún no se cuenta con máquinas adaptadas para ello.


Floración y formación de vainas en el tercio superior de las plantas

- Son de baja altura, por lo que no presentan problemas de tendedura, como ocurre con la mayor parte de los cultivares indeterminados.

- Su siembra puede realizarse mecanizadamente con sembradoras neumáticas disponibles en el país, ya que los granos son de tamaño pequeño, lo que permite una distribución más uniforme de ellos en el suelo.

- Su producción fluctúa entre un 25 a un 50% de la que es posible obtener en los cultivares de hábito indeterminado; esto, debido a la menor cantidad de vainas por planta y al menor tamaño de los granos; sin embargo, debido a que son plantas de reducido crecimiento se pueden establecer a altas densidades, compensando, en parte, este menor rendimiento individual.

- Tienen una alta estabilidad en su rendimiento, debido a que las plantas presentan un alto nivel de autofertilidad, es decir que para el proceso de fecundación de las flores no se requiere estrictamente de la presencia de abejas o insectos polinizadores.

En Chile, estos cultivares se han estado evaluando desde el año 2006, en la Región Metropolitana, VI y VII. Durante este período se han determinado algunas características generales, así como recomendaciones en cuento a densidad y fechas de siembra.

Principales características de los cultivares evaluados en Chile.

Son cultivares cuyo ciclo de desarrollo fluctúa entre 130 y 155 días desde siembra a cosecha en verde, dependiendo del cultivar, fecha de siembra y condiciones climáticas imperantes. Las plantas presentan una altura que no supera los 70 cm, siendo el cultivar Retaca el de menor tamaño. El tallo principal está provisto de 5 o 6 nudos vegetativos y entre 4 a 5 nudos reproductivos, cada uno de los cuales produce entre una a dos vainas. En total las plantas presentan en promedio 8 a 11 vainas las que miden entre 10 y 12 cm de largo, presentando entre 3 a 4 granos por vaina, con un tamaño de grano entre 1,3 a 1,6 cm de largo. En los Cuadros 1 y 2 se presenta un detalle de los principales parámetros evaluados para cada uno de los cultivares.

Cuadro 1. Características de cultivares de crecimiento determinado del tipo *baby*, evaluados en la zona Central de Chile

Parámetro evaluado	Cultivares		
	Retaca	Alarga	Verde Bonita
Altura de planta (cm)	56	63	62
Altura inserción de primera vaina (cm)	21	31	28
Nudos vegetativos eje principal (N°)	5	5	6
Nudos reproductivos eje principal (N°)	3,3	3,5	3,6
Número de ramas totales (% comercial)	5,7 (40)	5,4 (42)	5,2 (44)
Largo vaina (ancho vaina) (cm)	9 (1,2)	10 (1,3)	11 (1,2)
Largo pomedio de grano (ancho promedio de grano) (cm)	1,3 (0,9)	1,35 (1,0)	1,45 (1,0)

Altura media 56-63 cm

Durante la temporada 2006, se evaluaron diferentes densidad de plantas (entre 140.000 a 286.000 plantas/ha), determinándose que la más alta densidad evaluada fue la que generó el mayor rendimiento tanto de vainas como de granos. Esta densidad correspondió a plantas establecidas a una distancia entre hileras de 35 cm y 10 plantas/metro. En el Cuadro 2, se presentan los resultados de las evaluaciones realizadas a esta densidad.

Cuadro 2. Componentes de rendimiento y rendimiento de grano y vaina de cultivares tipo *baby*, de crecimiento determinado establecidos a una densidad de 287.000 plantas/ha

Parámetro evaluado	Cultivares		
	Retaca	Alarga	Verde Bonita
Vainas/planta (N°)	9	5	7
Granos/vaina (N°)	3,3	3,5	3,5
Peso/vaina (g)	5,1	5,5	6,1
Peso de 100 granos verdes (g)	48	50	53
Rendimiento vainas/ha (ton)	12,5	11,5	14,2
Rendimiento granos/ha (ton)	3,2	2,9	3,0
Rendimiento industrial (%)	26	25	21

MANEJO DE LA SIEMBRA COMERCIAL "ESCUELA AGRÍCOLA LAS GARZAS"

- Cultivares : Retaca y Verde Bonita
- Fecha de siembra : 4 de julio
- Siembra : Máquina sembradora tipo Gaspardo
- Dosis de semilla : 370 kg/ha para el cultivar Verde Bonita
  270 kg/ha para el cultivar Retaca
- Densidad de plantación : 286.000 plantas/ha. (35 cm entre hileras y 10 plantas/metro)
- Fertilización : 65 unidades de N/ha (40 a la siembra y 25 a inicios de floración) (total Urea: 141 kg /ha)

  : 60 unidades de P_2O_5/ha (130 kg/ha de Súper fosfato triple)

  : 90 unidades de K_2O/ha (150 kg/ha de Muriato de potasio)
- Control de malezas : Linuron + Herbadox en pre emergencia (dosis 1 + 3 lt/ha)
- Control de *Botrytis* : Dos aplicaciones durante la temporada:
  Una de Rovral (1,7 kg/ha)
  Una de Benlate: Captan (500 g/ha : 2 kg/ha)
- Control de minador de hoja (*Lyrionisa)* : Una aplicación de Trigard en dosis de 125 g/ha
- Cosecha : 13 de noviembre (132 días desde siembra a cosecha)

GRACIAS

Santiago, noviembre de 2008

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Este seminario se enmarca en el proyecto "Introducción de nuevas alternativas varietales para la producción de habas tipo "baby" y bases de mejoramiento del haba en Chile", financiado por la Fundación para la Innovación Agraria (FIA).

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INSCRIPCIONES

La asistencia a este Seminario es gratuita, previa inscripción.

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UNIVERSIDAD DE CHILE
FACULTAD DE CIENCIAS AGRONÓMICAS

SEMINARIO

EL CULTIVO DE HABA EN CHILE Y NUEVAS POSIBILIDADES INDUSTRIALES A TRAVÉS DE LA INTRODUCCIÓN DE HABAS BABY

Santiago, 3 de julio de 2008

EXPOSITORES

José Ignacio Cubero Salmerón
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PROGRAMA

8:45 - 9:25	Inscripciones
9:25 - 9:30	Palabras de bienvenida Fernando Santibáñez (Vice-Decano)
9:30 - 10:30	Situación mundial de las leguminosas de grano (consumo hortícola y consumo en seco) José Ignacio Cubero
10:30 - 11:30	Manejo y producción del cultivo de haba en Chile Hugo Faiguenbaum
11:30 - 12:00	Café
12:00 - 13:00	Fijación simbiótica y uso de inoculantes en especies leguminosas Cecilia Baginsky
13:00 - 14:30	Almuerzo
14:30 - 15:15	Resultados de investigación obtenidos en el desarrollo del cultivo de haba *baby* en Chile Cecilia Baginsky
15:15 -15:45	Aspectos de producción de haba *baby* en España Hugo Faiguenbaum - Ian Homer
15:45 - 16:15	Café
16:15 - 17:15	Mejoramiento genético en especies leguminosas para consumo humano. Presente y futuro José Ignacio Cubero

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ORIGINAL PAPER

Development of a new diagnostic marker for growth habit selection in faba bean (*Vicia faba* L.) breeding

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Abstract Faba bean varieties with determinacy of the apical meristem are relevant to green production. A diagnostic CAPS (cleavage amplification polymorphic sequence) marker for determinate growth habit (*ti*) in faba bean was previously developed by Avila et al. (Mol Breed 17:185–190, 2006) but was effective only on a limited range of cultivars or genotypes. In this study, we studied the reasons for this limited application and developed a new marker useful for most faba bean-breeding programs. By designing a new set of primers, the complete genomic *Vf_TFL1* sequences from different genotypes contrasting for the character were obtained and additional base changes associated with the *ti* phenotype were identified. The comparison among faba bean sequences showed that the previous CAPS marker was based on a SNP (single nucleotide polymorphism) at position 469 in the intron 2–3, a silent mutation. On the contrary, a SNP at position 26 that distinguishes determinate and indeterminate growth habit genotypes lead to an amino acid change (Leu-9 to Arg) in the determinate growth habit genotypes that could account for the *ti* phenotype. A dCAPS marker based on this SNP that creates a *Taq*I site in the *ti* allele was developed. The marker was 100% successful in predicting *ti* phenotypes in a broad range of faba bean germplasm representing all major cultivars historically grown in Europe. The outcome confirms the utility of the new dCAPS in worldwide marker-assisted selection programs.

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Introduction

The development of determinate growth habit faba bean (*Vicia faba* L.) varieties has been the objective of different breeding programs around the world (reviewed by Robertson and Filipetti 1991; reviewed by Huyghe 1998; Nadal et al. 2005). A determinate plant type is characterized by a terminal inflorescence where the stem growth abruptly terminates after four to five flowering nodes. This results in a considerable reduction of plant height and lodging and promotes better partition of assimilates between vegetative and reproductive growth and consequently, an increase in harvest index. Moreover, pod ripening is concentrated in time and space, which facilitates crop management and mechanical harvesting.

This trait is relevant to the fresh market (canning or freezing) that traditionally uses major faba bean types with indeterminacy of the apical meristem resulting in high cost for manual harvesting and logistic constraints at harvesting time (Nadal et al. 2005).

Several faba bean mutants with determinate growth habit have been described (Sjodin 1971; Filipetti 1986; Steuckardt et al. 1982). A single gene is responsible for the character (Sjodin 1971; Filipetti 1986) and the recessive allele conferring determinate growth habit was named *ti* (standing for terminal inflorescence). Recently, Avila et al. (2006) demonstrated that an ortholog of *CEN/TFL1*-like

genes is responsible for the character in *V. faba*. *CEN/TFL1*-like genes are extensively recognized as a group of homologous genes responsible for growth habit in different plant species. The *CENTRORADIALIS* (*CEN*) was first cloned in *Anthirrhinum* (Bradley et al. 1996). Next, several orthologs have been described in other plant species such as *Arabidopsis*, *TERMINAL FLOWER* 1 (*TFL1*) (Bradley et al. 1997), tomato, *SELF PRUNING* (*SP*) (Pnueli et al. 1998), tobacco (*CET*) (Amaya et al. 1999), *Lolium perenne* (*LpTFL1*) (Jensen et al. 2001), pea (*PsTFL1-a*) (Foucher et al. 2003), and more recently in citrus (*CsTFL1*) (Pillitteri et al. 2004) and rice (*RCN*) (Zhang et al. 2005).

Using a candidate gene approach, we developed a diagnostic CAPS marker useful for selecting determinate growth plants among the parental lines involved in our faba bean-breeding program (Avila et al. 2006). Diagnostic markers have the significant advantage of being completely linked with the trait being selected. Since they are designed on the gene sequence, no recombination between the marker and the trait is possible for greatly improving the selection efficiency. Nevertheless, they may represent natural polymorphism that does not affect gene function. For this reason, diagnostic markers still require independent validation in the new lines to be used in a breeding program. Actually, in our routine analysis with this marker several false positives were detected when the CAPS marker was assayed in additional genetic backgrounds.

The aim of the present study was to assess the usefulness of the CAPS marker described by Avila et al. (2006) and further develop new molecular markers linked to *Ti* with a more extensive application. For this purpose, we obtained the entire genomic *Ti* sequences from different faba bean genotypes contrasting for the character and identified additional bases changes, such as single nucleotide polymorphisms (SNPs), which might be accounting for the *ti* phenotype in order to develop more efficient markers for selection. The relationship between the *Ti* sequence (*Vf_TFL1*) and others *CEN/TFL1*-like genes obtained from the databases was also studied to provide an insight into the conservation and diversification of the gene.

Materials and methods

Plant material

In order to study the effectiveness of the CAPS marker developed by Avila et al. (2006) to predict the growth habit phenotypes, 36 new inbred lines and varieties from the germplasm collection at INRA-URLEG (Dijon, France), kindly provided by Dr. Gerard Duc, were analyzed.

To obtain the whole genomic sequence of the gene determining the growth habit in faba bean (*Ti*), we analyzed four determinate growth habit genotypes (*ti*): the Spanish cultivars "Alargá", "Verde Bonita" and "Retaca" developed and released by IFAPA-Centro "Alameda del Obispo" and the inbred line Vf2 from the faba bean collection at IFAPA-Centro "Alameda del Obispo". As wild-type genotypes (*Ti*) we used three inbred lines: 2N52, described as rust resistant (Sillero et al. 2000; Avila et al. 2003); line 29H ascochyta blight resistant (Sillero et al. 2001, Avila et al. 2004; reviewed Tivoli et al. 2006), and Vf6 from the faba bean collection at IFAPA.

CAPS analysis

The analysis of the CAPS marker was performed as described by Avila et al. (2006).

Primer design

The complete genomic sequence of the gene *Ti* was amplified using two primer pairs named hereafter primer set 1 and primer set 2. Primer set 1, previously designed by Avila et al. (2006), allows amplification of a DNA region from exon 2 to the end of the exon 4, including introns 2–3 and 3–4. Primer set 2 was designed based on conserved domains identified after the alignment of published nucleotide sequences *CEN/TFL1* homologs using the *PsTFL1a* sequence as a template since this was the most faba bean related sequence retrieved from genebank databases. The forward TFL1-C primer (5′-ATGGCAAGAATGGCTCA AGAAC-3′) and the reverse TFL1-D (5′-CCTGGAA TATCTGTCACAATC-3′ amplified exons 1 and 2 and the beginning of the exon 3 (Fig. 1).

DNA extraction, PCR conditions, cloning and sequencing

Genomic DNA was extracted according to the protocol by Torres et al. (1993). PCR amplification was performed with 50 ng of genomic DNA as template in a volume of 25 µl using the AmpliTaq Gold with Gene Amp kit and the recommendations of the suppliers (Applied Biosystems, manufactured by Roche, Brachburg, NJ, USA). PCR conditions for primer set 1 were as described by Avila et al. (2006) while for primer set 2, the thermal profile was an initial denaturation at 94°C for 10 min followed by 30 cycles of 94°C for 45 s, 58°C for 1 min, and 72°C for 45 s with a final extension of 7 min at 72°C.

The amplification products were ligated and transformed with the pGEM-T Easy Vector System I (Promega Corporation, USA), and nucleotide sequence was determined

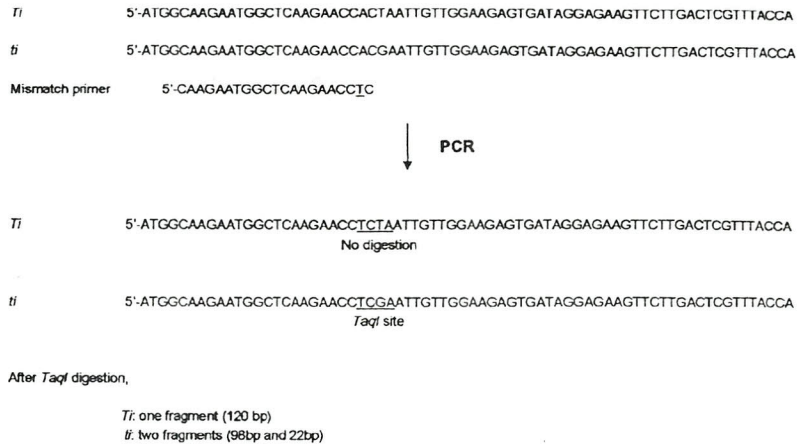

Fig. 1 Derived cleaved amplified polymorphic sequence (dCAPS) marker for detection of the one-base substitution at the first exon of the *Ti* gene

using a BigDye terminator cycle sequencing v 3.1 kit (PE Biosystems, Foster City, CA) on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Servicio de Secuenciación Automática de DNA, SCAI (University of Córdoba, Spain).

Sequence analysis

The complete sequence of the faba bean *TFL1* gene for seven genotypes (four determinate growth habit genotypes and three wild type lines) was obtained. These sequences were aligned using the program CLUSTAL X (Thompson et al. 1997) to investigate the presence of extended base changes or SNPs. *PsTFL1a*, a *TFL1* homolog in pea, was also included in the alignment to investigate the sequence conservation between *TFL1* in faba bean and pea. Next, the predicted amino acid sequences were aligned to identify any amino acid changes caused by the SNP previously detected.

Finally, a phylogenetic tree of *TFL1*-related proteins was constructed using the NJ method with the program CLUSTAL X and the following sequences: *TSF* (AB027506); *FT* (AB027504); *CiFT* (AB027456); *MFT* (AF147721); *BFT* (NM125597); *PsTFL1a* (AY340579); *Ps_TFL1c-LF* (AY343326); *Bn TFL1-1* (AB017525); *TFL1* (U77674); *CET4* (AF145261); *CET2* (AF145260); *CET1* (AF145259); *SP* (U84140); *CEN* (S811193); *ATC* (AB024715); *RCN3* (AF159883); *RCN1* (AF159882) and the wild type line 2N52.

dCAPS analysis

To detect a one-base substitution by derived cleaved amplified polymorphic sequence (dCAPS) analysis (Neff et al. 1998), a mismatch primer *Ti*-dCAPS Fw (5′-CAAGAATGGCTCAAGAACCTC-3′) that generates a *Taq*I site specifically in the *ti* allele was constructed (Fig. 1). PCR amplification using the primer pair *Ti*-dCAPS Fw and *Ti*-dCAPS Rev (5′-TGGTGTTGATAGTGGAAGGGA) was performed with 50 ng of genomic DNA as template in a volume of 25 µl using the AmpliTaq Gold with Gene Amp kit and the recommendations of the suppliers (Applied Biosystems, manufactured by Roche, Brachburg, NJ, USA). Cycling conditions consisted of an initial denaturation step at 94°C for 10 min followed by 30 cycles of 94°C for 35 s, 58°C for 30 s, and 72°C for 20 s that ended at 72°C for 7 min. Ten microliters of each PCR product were digested with *Taq*I in a total volume of 25 µl at 65°C for 2 h. After digestion, each sample was electrophoresed in a 2.5% agarose gel. The applicability of the new markers was studied in the INRA-URLEG collection.

Accession numbers

The sequence data described herein have been submitted to NCBI/GenBank data libraries with accession numbers EF193847 (2N52), EF193848 (Alargá), EF193849 (Verde Bonita), EF193850 (Vf2), EF193951 (Vf6) and EF193952 (29H).

Results

Testing the CAPS marker for growth habit in faba bean

The CAPS marker previously developed on the *Ti* gene to select growth habit in faba bean was useful for the parental lines involved in our breeding program (Avila et al. 2006). Nevertheless, after testing its effectiveness in predicting phenotypes using new parental lines, several genotypes did not show the expected restriction patterns. Actually, the

wild type line Vf6, used in several mapping studies by our group, revealed the restriction pattern corresponding to a *ti* genotype. This result emphasizes the need to perform marker validation to asses the efficiency of any marker in different genetic backgrounds.

For this purpose we assayed the CAPS marker in a faba bean collection kindly provided by Dr. Gerard Duc from INRA-URLEG (Dijon, France). The collection included 35 indeterminate growth habit and 1 determinate growth habit genotypes. Line TICOL 1191 (the only one with terminal inflorescence), revealed the expected restriction pattern that unexpectedly shared 15 indeterminate growth habit plants (Table 1).

Obtaining the sequence of *Vf_TFL1*

To isolate the complete genomic *TFL1*-related sequence in faba bean, we used the primers reported by Avila et al. (2006), referred as primers set 1 to amplify the sequence coding from the amino acid 75 to 174 and the corresponding introns. The amplification product comprises

Table 1 Validation test of the available diagnostic markers for growth habit selection in faba bean (*Vicia faba* L.) using a European inbred line collection

European inbred lines	Growth habit observed	Expected phenotype[a]	
		CAPS	dCAPS
AD23 MAINTENEUR 2300	Indeterminate	D	I
G58 MAINTENEUR 2302	Indeterminate	D	I
GLORIA 2308	Indeterminate	NA	I
19 TB ØT 2316	Indeterminate	I	I
DIVINE 44.2 2391	Indeterminate	I	I
MELODIE M 2393	Indeterminate	I	I
LADY 2401	Indeterminate	D	I
19 TB T 2317	Indeterminate	I	I
FABIOLA ØT 2318	Indeterminate	I	I
FABIOLA T 2319	Indeterminate	D	I
POUILLY 2327	Indeterminate	I	I
DIVA 2366	Indeterminate	I	I
DISCO 2390	Indeterminate	D	I
MAXIME 196	Indeterminate	I	I
AQUITAINE 267	Indeterminate	NA	I
MARAIS POITEVIN 276	Indeterminate	NA	I
GERS 277	Indeterminate	D	I
LORRAINE 279	Indeterminate	D	I
PICARDIE 437	Indeterminate	NA	I
DIANA 455	Indeterminate	D	I
WIERBOON 704	Indeterminate	D	I
MIKKO 1179	Indeterminate	D	I
TICOL 1191	Determinate	D	D
STRUBE 1216	Indeterminate	D	I
OPTICA 1482	Indeterminate	NA	I
BOURDON 1505	Indeterminate	D	I
WEBO 1508	Indeterminate	I	I
TROY 1579	Indeterminate	NA	I
CÔTE D'OR 1626	Indeterminate	I	I
HG115 1757	Indeterminate	I	I
ASCOTT 1777	Indeterminate	I	I
TALO 1795	Indeterminate	NA	I
SORAVI 2070	Indeterminate	NA	I
BLANDINE 2073	Indeterminate	D	I
POLLEN 2074	Indeterminate	D	I
MAYA 2077	Indeterminate	NA	I

I indeterminate growth habit, *D* determinate growth habit, *NA* no amplification

[a] Expected phenotype attending the restriction pattern obtained using the CAPS marker developed by Avila et al. (2006) and the dCAPS developed in the present study

exons 3, 4, part of exon 2 and the introns 2–3 and 3–4. Besides, a new primer pair (primer set 2) corresponding to conserved domains identified from the alignment of published TFL1/CEN homologs were designed to obtain the amplification of exons 1, 2 and the beginning of the third exon (from amino acid 1 to 95). Primer set 2 was designed using the *PsTFL1a* sequence as a template since this was the most faba bean related sequence retrieved from genebank databases. The new TFL1 faba bean sequence will be referred as *Vf_TFL1* hereafter.

Using primer set 1, a single amplification product of 540 bp was obtained (Avila et al. 2006). Primer set 2 generated a band of 550 bp. Both amplicons were excised from the gel, cloned and sequenced in three wild type accessions (2N52, Vf6 and 29H) and four lines showing determinate growth habit ("Retaca", "Alargá", Vf2 and "Verde Bonita"). No differences between "Retaca" and "Alargá" sequences were found since they share the determinate growth habit donor. Therefore, we will only refer to "Alargá" in future.

The exon/intron boundaries were predicted on the basis of the *PsTFL1a* sequence (AY340579). *Vf_TFL1* complete sequences were obtained by consensus alignment of the sequences obtained from the amplification of primer set 1 and 2. Both amplicons share a fragment covering the region between amino acid 75 and 95 in exons 2 and 3 including intron 2–3 that allows inferring the entire *Vf_TFL1*sequence.

According to EuGene'Hom software (Foissac et al. 2003), *Vf_TFL1* is predicted to encode a protein of 174 amino acids. Faba bean wild type plants show 96% amino acid identity (167/174) with *PsTFL1a*, considering the six faba bean genotypes (Fig. 2). Out of the seven amino acid changes detected, three were identical among faba bean accessions (positions 128, 135 and 147) but different to *PsTFL1a*. Besides, the amino acid changes found at positions 14, 24 and 27 were found in a single faba bean variety: Vf2, 2N52 and "Verde Bonita", respectively (Fig. 2). Only the amino acid change at position 9 (Leu-9 to Arg) differentiates between determinate growth habit and wild type genotypes, the latter being identical to *PsTFL1a*, which is characterized by indeterminacy of the apical meristem. For this reason, the SNP leading to this amino acid change was used to design a new dCAPS marker.

Studying the homology of the *Vf_TFL1* sequence with other *CEN/TFL1*-like genes

The *Vf_TFL1* sequence corresponding to the wild type faba bean line 2N52 was aligned with *TFL1/CEN* related sequences obtained from the databases. The phylogenic tree based on amino acid similarity revealed two clear groups corresponding to "*CEN* like" and "*TFL1* like" sequences (Fig. 3). These results are in agreement with those reported by Foucher et al. (2003). Clustering of *Vf_TFL1* pointed out that the sequence is not a member of the "*CEN* like" group but a *TFL1* like sequence, forming a clade with *PsTFL1a* (AY340579) and *PsTFL1c* (AY340579) together with the remaining *TFL1* like sequences: *TFL1* (U77674) and *Bn TFL1-1*(AB017525). In addition to this, other members of the *Arabidopsis TFL1* family, such as *FT*, *TSF*, and *BFT* (Mimida et al. 2001) are more distant (Fig. 3).

SNPs analysis

The 15 SNPs detected among faba bean lines are shown in Table 2. According to the intron/exon boundaries predicted and considering the complete sequence of *Vf_TFL1* and the restriction pattern reported by Avila et al. (2006), the SNP leading to the CAPS marker previously reported is located in a non-coding region (Intron 2–3) at position 469 (Table 2). Therefore, this substitution seems to result from natural variation with no effect on the determinacy of the apical meristem because it was also found in the inbred line Vf6 (indeterminate genotype) and shares sequence with the determinate genotypes used in the study.

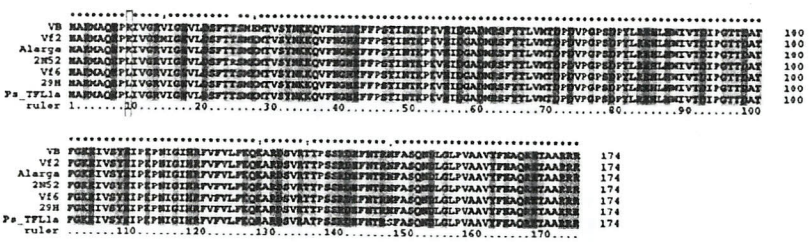

Fig. 2 Alignment of the deduced amino acid sequences of *Vf_TFL1* sequences with determinate growth habit ("Verde Bonita", Vf2, "Alargá"), indeterminate growth habit (2N52, Vf6, 29H) and *PsTFL1a* from *Pisum* (Foucher et al. 2003). Identical amino acids are indicated by an *asterisk*. The amino acid change differentiating between determinate and indeterminate growth habit is indicated by a *box*. The multiple alignments were generated with CLUSTAL X software

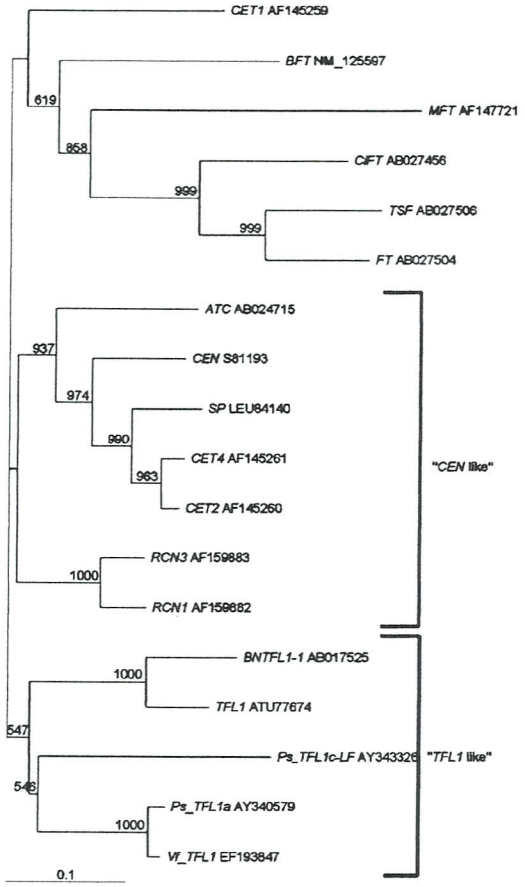

Fig. 3 Phylogenetic tree of *TFL1*-related proteins constructed using the NJ method with the program CLUSTAL X. Bootstrapping values higher than 500 are shown. The sequences used are indicated by the gene name and the NCBI number: *TSF* (AB027506); *FT* (AB027504); *CiFT* (AB027456); *MFT* (AF147721); *BFT* (NM_125597); *PsTFL1a* (AY340579); *PsTFL1c-LF* (AY343326); *BnTFL1-1* (AB017525); *TFL1* (ATU77674); *CET4* (AF145261); *CET2* (AF145260); *CET1* (AF 145259); *SP* (LEU84140); *CEN* (S811193); *ATC* (AB024715); *RCN3* (AF159883); *RCN1* (AF159882) and the *ti* mutant *Vf_TFL1* (EF193847) from 2N52

The unique SNP that allows distinguishing between determinate and indeterminate genotype was found at position 26, near to the 3′ end of the sequence and was used to design a dCAPS marker as explained in the "Materials and methods". Figure 4 shows the restriction pattern corresponding to the new marker (*Ti*-dCAPS) which is able to unambiguously differentiate between mutants and wild type genotypes.

As for the CAPS marker, the new diagnostic marker was assayed in a distantly related germplasm collection (Table 1) showing 100% efficiency in selection. In both cases, genomic amplification with the dCAPS primers produced a single band of 120 bp. After restriction of this PCR fragment with *Taq*I, determinate genotypes revealed a smaller fragment (98 pb) while indeterminate individuals remained uncut (Fig. 4, Table 1).

Discussion

Diagnostic markers are those directly associated with the gene that influences the trait (Gale 2005). Since they are designed on the sequence of the gene responsible for the character being selected, it is expected that they do not require independent validation for each parental line used in a breeding program (Gale 2005). Our results have, however, shown that the development of diagnostic markers does not warrant their worldwide application unless the marker is designed in sequence changes that account for the target phenotype.

In this study, we assayed a diagnostic CAPS marker for the determination of growth habit in faba bean, reported by Avila et al (2006), in new accessions but several genotypes do not present the expected restriction patterns. Further screening of a faba bean collection from INRA-URLEG (Dijon, France) displayed numerous false positives, since the restriction pattern expected for determinate growth habit genotypes was found in several wild type individuals. These results revealed that the effectiveness of this CAPS marker is limited and further studies were necessary to develop a new diagnostic marker useful on a range of cultivars or genotypes. Besides, the outcome highlights the fact that even the molecular markers based on the gene responsible for the trait should be validated for routine screening for MAS since there is no guarantee that they will be useful in different backgrounds.

Avila et al. (2006) showed that an ortholog of *CEN/TFL1*-like genes was responsible for the determinate growth habit in faba bean but did not investigate the sequence of this gene. Although *CEN* and *TFL1* display a strong similarity in the amino acid sequence, both genes are not particularly closely related (Mimida et al. 2001). In fact, they have slightly different functions although they are derived from the same ancestral gene. *TFL1* controls flowering time and maintains the fate of inflorescence meristem while *CEN* is involved only in the inflorescence maintenance (Bradley et al. 1996, 1997). Our results pointed out that the *Vf_TFL1* sequence obtained in this study does not belong to the "*CEN* like" group. Thus, *Vf_TFL1* is a *TFL1*-like gene that formed a clade with the *TFL1*-like sequences included in the phylogenetic tree (Fig. 3). Consequently, *Vf_TFL1* plays an essential role in determining the inflorescence architecture (indeterminate or determinate), an important trait for valuable green production in faba bean. Besides, it may also regulate flower transition since *tfl1* mutants in *Arabidopsis* display early

Table 2 Sequence analysis of *Vf_TFL1* in the selected genotypes

Position	Location	Nucleotide substitution[a]	Mutant genotypes	Major effect[b]
26	E_1	G/T	Vf2, 'Alargá', 'Verde Bonita'	Leu-9 to Arg
40	E_1	G/A	Vf2	Val-14 to Met
60	E_1	C/T	29H	Silent
71	E_1	C/G	2N52	Thr-24 to Arg
79	E_1	A/G	'Verde Bonita'	Lys-27 to Glu
215	I_{12}	C/T	2N52, 29H	
236	I_{12}	G/C	2N52	
283–321	I_{12}	Deletion	29H	
357	I_{12}	G/T	29H	
376	E_2	C/T	Vf2	Silent
463	I_{23}	A/G	Vf2	
469	I_{23}	C/G	2N52, 29H	
511	I_{23}	T/A	2N52, 29H	
523	I_{23}	Deletion	2N52, 29H	
691	E_4	C/A	2N52, 29H	Silent

E exon, *I* intron

[a] The first nucleotide corresponds to the wild type while the second one appears in the mutant(s) genotype

[b] "Silent" indicates that the substitution has no effect at the amino acid sequence of the protein

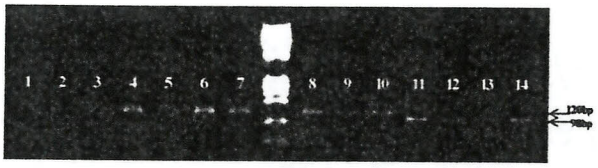

Fig. 4 Visualization of growth habit specific dCAPS marker in faba bean lines. *Lines 1–7* correspond to the PCR amplification of the dCAPS primers without enzymatic digestion in lines 29H, 2N52, Vf6 (*Ti* genotypes) and Vf2, "Retaca", "Verde Bonita" and "Alargá" (*ti* genotypes). *Lines 8–14* correspond to the respective restriction pattern obtained after *Taq*I digestion in the same lines. Molecular weights are marked with *arrows*

flowering (Shannon and Meeks-Wargner 1991; Bradley et al. 1997).

The analysis of the complete *Vf_TFL1* sequence determined that the CAPS marker previously reported was derived from a SNP located at position 469, within intron 2–3. Consequently, this substitution resulted in a silent mutation with no effect on the determinacy of the apical meristem.

Similar results were reported by Foucher et al. (2003) studying three homologs of the TFL1/CEN family (*PsTFL1a*, b and c). Sequencing of *PsTFL1a* in three independent *det* lines revealed mutations in the introns and exons regions, when compared with the wild type. Some of the SNPs were silent mutations but others significantly modify the *PsTFL1a* structure leading to a non-functional protein responsible for the determinate growth habit in the *det* mutants.

From the 15 SNPs detected among the faba bean lines, only one could discriminate between determinate and indeterminate genotypes. In this case, the mutation was found in exon 1 and involves a change at the amino acid sequence (Leu-9 to Arg), a residue notably conserved across the *CEN/TFL1*-like genes. The Leu is conserved in all *CEN/TFL1* sequences used in this study, including the most distantly related proteins such as *FT* and *TSF* from *Arabidopsis*. The high degree of conservation suggests that mutation of this Leu-9 might likely affect the *TFL1* function and could account for the *ti* phenotype.

Amino acid changes may affect the enzyme function and therefore the phenotype. Foucher et al. (2003) reported that single amino acid changes originate the determinate growth habit phenotype in two different pea mutants. The same findings have been reported in *Arabidopsis* (Oshima et al. 1997) and tomato (Pnueli et al. 1998). Furthermore, occasionally a single amino acid substitution promotes a change in the enzymatic function even between the homologous genes. For instance, Hanzawa et al. (2005) studied two 60% identical *Arabidopsis CEN/TFL1*-like genes homologs involved in clear and opposite functions showing that swapping a single amino acid is sufficient to convert a repressor to an activator of flowering and vice versa.

Other mutations at conserved positions are preserved among mutants for determinate growth habit in several species. This is the case of the replacement of Thr-6 by Ile in the *det-3* mutant in pea (Foucher et al. 2003), which is conserved in the *tfl1-14* mutant of *Arabidopsis* (Oshima et al. 1997). To our knowledge, the change Leu-9 to Arg described in this study has not been previously reported in any other species.

We have used a candidate gene approach based on the sequence of *PsTFL1a*, a *TFL1* homolog in pea, to develop a new dCAPS for efficient discrimination selection of

growth habit in faba bean. The methodology has proven successful due to the high conservation found between the pea and faba bean genes. Therefore, it is expected that the same methodology is applicable to other traits of interest for faba bean breeding.

The new diagnostic marker is expected to facilitate efficient detection of determinate growth habit genotypes in faba bean-breeding populations. Moreover it will play an important role during selection in pyramiding additional suitable genes to develop new cultivars for green production.

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