Monoclonal antibodies specific for the different floral organs of the garden pea (Pisum sativum L.) were raised using two different types of immunization procedures. These antibodies were powerful tools to study the functionality of floral organ identity genes in pea homeotic mutants. The mAbs were used extensively as developmental markers for the immunohistochemical characterization of two pea floral homeotic mutants (stp-1 and stp-2) to elucidate the different degree of transformation present in the mutated organs. Mutations in the Stamina pistilloida (Stp) locus, the pea ortholog of Fim (Antirrhinum) and UFO (Arabidopsis), produce floral phenotypes resembling those of loss-of-B-function. The initial screening of the antibodies obtained against protein extracts from complete flower buds or individual floral organs allowed the isolation of a mAb (S1) that was used to identify sepal tissues in flower sections and to visualize the complete transformation of petals into sepals in stp-2 or to identify partial homeotic transformations (sepaloid sectors) in the keel of stp-1. A second mAb (P1), which recognized an antigen present in petals, was used to identify the abnormal sepaloid features of the two petals forming the keel in the stp-1 mutant. However, using this immunization procedure we did not obtain any antibody specific for carpel or stamen tissues. To overcome this problem we developed an improved immunosubtractive strategy and several mAbs specific for these floral organs were obtained. mAbs OV1, OV4, and OV5 recognized antigens mainly present in different carpel tissues and mAb A1 a protein present only in anther tissues (epidermis, connective, endothecium). These mAbs were used as markers to identify carpelloid or stamenoid tissues present in the stp-1 and stp-2 floral homeotic mutants.

Keywords Pea (Pisum sativum L.) · Stamina pistilloida · Floral homeotic mutants · Monoclonal antibodies

Introduction

The pea flower is organized into four whorls and shows a pentameric arrangement of sepals and petals, ten stamens and a central carpel. The ontogeny of the pea flower is different from those of Antirrhinum majus and Arabidopsis thaliana (Tucker 1989; Beltrán et al. 1996; Ferrándiz et al. 1999). The more remarkable features of pea flower ontogeny are the existence of four common primordia to petals and stamens, the early carpel primordium initiation and the abaxial-adaxial unidirectional initiation of organ primordia within each different floral whorl, in contrast to the centripetal and sequential floral ontogeny in other plants. Organ differentiation within each of these common primordia appears to be a complex process that plays a central role in the ontogeny of pea flowers.

The genetic analysis and molecular characterization of floral homeotic mutants has brought to light basic principles of the regulation of flower development. Many genes controlling processes such as meristem identity, floral organ identity, genes that regulate the spatial expression of these and other genes controlling tissue differentiation within floral organs have been isolated from different plant systems (Sessions et al. 1998; Theissen et al. 2000). In higher eudicotyledonous plants, the identity of the different floral organs is specified by five classes of homeotic genes (A, B, C, E and D), with A specifying sepals, A+B+E petals, B+C+E stamens, C+E carpels and D ovules (Weigel and Meyerowitz 1994; Pelaz et al. 2000; Theissen et al. 2000). The homeotic transformations observed in pea floral mutants (Ferrándiz et al. 1999) suggest that the identity and developmental pattern of the four organ types in pea flowers is governed by at least the same developmental functions proposed for the plant model systems Antirrhinum majus and Arabidopsis thaliana.
Mutations in the *Stamina pistilloida* (*Stp*) locus cause phenotypes resembling those of loss-of-B-function. This fact suggests that *Stp* is required for the normal development of petals and stamens in the pea flower. A *stamina pistilloida* mutant (*stp*) was characterized by Monti and Devreux (1969). Another *stp* mutant was isolated by Gottschalk (1963) and was originally named *n192*. Mutant allelism testing (Beltrán et al. 1996; Ferrándiz et al. 1999) showed that *n192* is allelic to *stamina pistilloida* (Monti and Devreux 1969); both behave as single recessive characters. Therefore, we named the *stamina pistilloida* mutant *stp*-1 and the *n192*, *stp*-2. The *stp*-1 allele shows homeotic transformations restricted positionally to the adaxial antesepal stamens and to the petals (green sepaloid sectors), suggesting the complexity of the spatial and temporal regulation of the B-function in pea. Flowers from plants homozygous for the *stp*-2 allele show a more complete transformation of petals into sepals and stamens into carpels, typical of loss-of-B-function mutations. In addition, *stp*-2 mutant flowers also produce ectopic flowers, a phenotype inconsistent with a strictly homeotic role, leading us to postulate (Ferrándiz et al. 1999) that *Stp* may play an important role in the control of common primordia identity and determinacy in the pea flower. Molecular analysis revealed that *Stp* is the pea ortholog of *Fiml/UFO* (Taylor et al. 2001).

Scanning electron microscopy (SEM) has been extensively used for the identification of floral homeotic mutants in different plant species, and to obtain information on the cell types present in the mutated floral organs. Such a technique only provides information about the different cells present in the epidermis of a mutated organ, hence the identification of cell or tissue mixtures (mosaics) is often based on cellular shape, external ornamentation or size criteria. Frequently, the altered floral organs from pea homeotic mutants also show cell patches of the original floral organ in their tissues. However, the characterization of these mosaic organs with incomplete homeotic transformations is still difficult due to the absence of appropriate floral organ or tissue markers allowing floral tissue mixtures to be distinguished. The use of monoclonal antibodies (mAbs) as developmental markers permits a large variety of molecules useful in the characterization of cellular and tissue differentiation at the biochemical level to be detected. Immunolocalization techniques can also have extraordinary resolution, allowing detection of the antigen at specific locations within a cell, as well as between cells (Evans et al. 1988), facilitating the study of the spatial and temporal expression of a gene by detecting the gene product even at very early stages of floral organ development. The isolation and characterization of cell- and tissue-specific genes through differential hybridization of nucleic acids is certainly an important step towards increasing our understanding of plant differentiation and development. Molecules of fundamental importance in these processes may be detected through hybridoma technology (Bracale et al. 1991). However, this technique may not detect compounds of low abundance, low antigenicity or those masked by the immunodominance of others (Anderson et al. 1984).

The main difficulty in producing mAbs highly specific for the different flower parts is probably due to the presence of the large amount of proteins that all floral organs have in common. In 1790, Goethe proposed that floral organs are modified vegetative leaves (Goethe 1790). This hypothesis was corroborated by the analysis of floral homeotic mutants and recently by the finding that a small number of MADS domain transcription factors is sufficient to convert vegetative leaves into floral organs (Honma and Goto 2001; Pelaz et al. 2001). Several strategies have been proposed to overcome both this problem and the immunodominance of some glycosylated molecules present in crude floral extracts (Cañas and Malmberg 1992; Vermeersch Williams et al. 1992). The isolation of mAbs specific for antigens present only in reproductive organs (stamens and carpels) has been proposed previously as an alternative approach for isolating genes involved in male and female differentiation. The availability of such antibodies could allow screening of an expression library for clones producing the proteins of interest (Bracale et al. 1991). In this paper we describe a new immunosubtractive method to increase the production of mAbs specific for a particular floral organ or tissue; the antibodies produced were used to identify tissue mixtures in the floral organs of the two pea floral homeotic mutants *stp*-1 and *stp*-2 at different stages of development, including the early stages in which petals and stamens differentiate from the common primordia. Thus, we have isolated a set of mAbs specific for antigens of the different floral tissues, allowing us to identify the different degree of transformation present in a determinate floral organ and to elucidate the temporal and spatial distribution of the antigens recognized by the mAbs.

### Materials and methods

#### Genetic stocks and plant growth

Wild-type pea seeds (*Pisum sativum* L. cv. Alaska n° 7) and two lines of *stamina pistilloida* bearing recessive mutations, *stp*-1 (*stp*, cv. Parvus line WL6014, Monti and Devreux 1969) and *stp*-2 (*n192*, cv. Dippo Gelbe Viktoria line 192, Gottschalk 1963) were obtained from different collections (IBMCP, KL MU and Dr. W. Gottschalk, respectively). Pea seeds from the different genetic stocks were surface-sterilized with 1% sodium hypochlorite for 20 min, followed by three rinses with sterile water. The sterilized seeds were then placed individually in 16-cm-diameter pots containing vermiculite as substratum and irrigated with Hoagland No. 1 solution supplemented with oligoelements (Hewitt 1966). The plants were grown in the greenhouse at 22°C (day) and 18°C (night) supplemented with additional lighting [400 W Philips HDK/400 HPI (R) N] during the winter in order to maintain a 16-h light photoperiod.

#### Pollen viability and germination tests

Pollen viability tests were performed in vitro using the solidified medium of Bino et al. (1987), containing 3 mM H$_3$BO$_3$, 1.7 mM