Development and Gene Expression in Saffron Corms

Manuel Álvarez-Ortí, Lourdes Gómez-Gómez, Ángela Rubio, Julio Escribano, Julia Pardo, Felicitas Jiménez and José-Antonio Fernández*
Laboratorio de Biotecnología. Instituto de Desarrollo Regional,
Universidad de Castilla-La Mancha
Campus Universitario s/n. Albacete E-02071
Spain

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Abstract

The corm occupies a central position in saffron life cycle, as it is the vegetative organ that accumulates reserves for sprouting after dormancy periods. Until now, not much is known about the physiology of the corm and its gene expression pattern. In an attempt to obtain information about this organ, six developmental stages were defined in the corm and analysed two of them, generating 650 partial complementary DNA sequences (expressed sequence tags, ESTs) from two cDNA libraries constructed from corms. In the first one, at a stage characterized by storage accumulation and corm growth (C3), a remarkable amount of sequences with similarity to genes related to cell growth; protein synthesis, folding and degradation; transcription factors; and proteins related to the formation and maintenance of cell wall and other cellular structures were found. Furthermore, many sequences with similarity to genes involved in defence and stress responses, and to carbohydrate metabolism were detected. In the second library, from dormant corms (C4), a greater percentage of sequences with similarity to genes related to transport of nutrients and metabolites was present. In both libraries, a high percentage of sequences with no similarity in the public databases was found (36 % in C3 and 50 % in C4) remarking the lack of knowledge about gene expression in the corm.

INTRODUCTION

Saffron is the most expensive spice in the world. This spice is made from the dried stigmas of *Crocus sativus* L., a triploid sterile plant that is vegetatively propagated by means of corms. However, little is known about the physiology of the plant, especially of the corm, as many researches have focused their efforts on its biochemical composition (Chrungo et al., 1983; Farooq et al., 1985; Oda and Tatsumi, 1993; Jirage et al., 1994). The corm is a subterraneous stem that accumulates reserves for sprouting after dormancy periods. In spite of its crucial function as the propagative organ, there is a lack of studies related to gene expression and genetic mechanisms involved in storage accumulation, sprouting, or dormancy.

A rapid way to establish an inventory of expressed genes in a specific tissue and developmental stage is by partial sequencing of cDNA called expressed sequence taggs (ESTs). Single-pass sequences are determined from one or both ends of randomly chosen cDNA clones, in a sufficiently accurate way to unambiguously identify the corresponding genes in most cases (Bouchez and Höfte, 1998). This has been made with different organs at various developmental stages of plants like *Arabidopsis* (White et al., 2000), *Brassica* species (Park et al., 1993; Lim et al., 2000), the forest tree *Cryptomeria japonica* (Ujino-Ihara, 2000), citrus fruits (Moriguchi et al., 1998), or in the model legume *Lotus japonicus* (Asamizu et al., 2000a; Endo et al., 2000). Collection of ESTs from metabolically active tissues can provide quantitative estimates of gene expression levels to unravel plant metabolic and regulatory networks (Ohlrogge and Benning, 2000), as it has been made, for example, to study xylem formation in pine (Allona et al., 1998), wood-forming tissues of poplar (Sterky et al., 1998) or lipid biosynthesis (White et al., 2000). Further, by comparison with non-redundant public databases, it facilitates new

gene discovery (Asamizu et al., 2000b), and soon we will have extensive information about which genes respond to changes in developmental processes such as germination and flowering (Somerville and Somerville, 1999).

Actually, there are more than 9.5 million entries in the public database of Expressed Sequence Tags (dbEST), being the more represented model plants like *Glycine max*, tomato, maize, Arabidopsis or rice, all of them with more than 100,000 entries (www.ncbi.nlm.nih.gov). However, in these databases little sequences are from corms or related organs. Early work on the sequence complexity of RNA pools in various organs in tobacco indicated that individual organs contain more than 6,000 mRNAs not present in other organs of the same plant (Kamalay and Goldberg, 1980). Sequencing of corm cDNAs at different developmental stages would increase our knowledge about the physiological processes occurring in this organ.

In addition, the long-term objective of this work is to provide a set of new targets for the improvement of this crop. Due to sterility of saffron, there is no way to improve this crop with methods of classic genetics. Here we study the corm of saffron to find new targets that can be used in genetic engineering to improve this crop.

MATERIALS AND METHODS

Plant Material

The corms used for this study were grown in an outdoors small field located in Tarazona de la Mancha (Albacete, Spain), reproducing crop conditions. We analysed gene expression during storage accumulation that is in stage C3 and dormancy, during stage C4. In the first case, we observed that the maximum enlargement of the corm is produced during February, so we collected the daughter corms at 15 days intervals during this month. In the second case, we collected corms during July. Both types of corms were selected according to their sanitary state and immediately frozen and maintained at -80 ºC until processing.

cDNA Library Construction

Poly(A^+)RNA was purified from total RNA by oligo(dT)-cellulose chromatography. The synthesis of cDNA, size selection, addition of linkers, insertional ligation, and packaging into λ vector (λZAP Express, Stratagene, La Jolla, CA) followed the manufacturer's instructions and did not deviate significantly from standard methods (Sambrook and Russell, 2001). The total primary titter of each library in recombinant plaque forming units was 1×10^6. After amplification, samples of each of the cDNA libraries were used to subclone inserts by mass excision for conversion of λ to phagemid vector. The resulting phagemid libraries were plated at low density on Luria Bertani agar plates containing kanamycin (25 mg l^{-1}).

Sequencing

650 clones were random chosen (449 from C3 library and 201 from C4 library), and plasmid DNA purified by means of alkaline lysis (Sambrook and Russsell, 2001). Sequencing of insert was made using oligo T7 as primer and dicloroRhodamine terminators (DNA Sequencing Kit, dRhodamine Terminator Cycle Sequencing Ready Reaction P/N 403402, PE Biosystems, Warrington, England), in a PCR based sequencing reaction. It was carried out in 10 µl containing 4 µl of sequencing kit, 3.2 pmol of oligo T7 and 0.5 µg of plasmid DNA, in 25 cycles of 96 ºC, 10 sec; 50 ºC, 5 sec; 60 ºC, 4 min, with a previous denaturation step of 96 ºC, 3 min. Sequencing products were resolved by means of capillary electrophoresis in an automated sequencer AbiPrism 310 (Applied Biosystems, Foster City, CA).

Analysis of Sequences

In order to remove vector sequences and poor quality data, computer-processed sequences were checked manually, compared with electropherograms, and further edited

to improve the quality and reliability of the data. Sequences shorter than 100 bases were discarded. The nucleotide sequences and the six deduced aminoacid translations of the partial cDNA sequences were searched against non redundant databases available through the NCBI using the BLASTN 2.1.1 and BLASTX 2.1.1 (Altschul et al., 1997). Searches with nucleotide sequences by using BLASTN were also performed with EST databases. E-values < 10e-5 were considered as significant and the top scoring genes were used to group the transcripts by their putative function.

RESULTS

Corm Development

The corm is the vegetative organ of saffron. After flowering, the base of the stem enlarges, producing a daughter corm that propagates the plant (Figure 1A). As a previous step for gene expression analysis in saffron corm, we divided its development into six stages (C1 to C6), according to morphological and physiological characters observed in the corm (Figure 1B). Similar stages have been proposed by de Castro et al. (1992) for corms of taro (*Colocasia esculenta*), although they only consider five stages because they do not include the senescent corm that we call stage C6. Corms at stage C1 appear as latent buds attached to the surface of older corms, and protected by scale like leaves. Stage C2 begins with floral stem sprouting and lasts until the end of flowering. It is a stage of active tissue formation, when the roots, flowers and green leaves appear. An enlargement characterizes C3 corms. During the period in which this stage is present, the plant has green leaves, and the corm act as a sink organ for carbohydrates originated in photosynthesis. Anyway, the corm remains attached to the mother corm, which still maintains its growth. Once the leaves are dried up, the corm advances to stage C4. It has reached its definitively size, and begins a dormant period to overcome the arid conditions of summer. During this period it grows independently because the mother corm is senescent. Corms at stage C5 maintain the sprouting and growth of daughter corms at stages C1-C3. Finally, corms at stage C6 are senescent, when the daughter corms advance to stage C4 and become independent.

Single-pass Sequencing and Classification of ESTs

To initiate a functional genomic analysis of corm metabolism, we constructed and analysed two cDNA libraries from corms collected in February (stage C3), the moment of the major enlargement, and in July (stage C4), the moment of dormancy previous to sprouting. Our general aim is to identify characteristic proteins of these developmental phases, which will help understanding the molecular and biochemical processes underlying growth and dormancy. In addition, these specific sequences might help characterizing growth and corm formation and optimising corm saffron production and treatments to break dormancy. The libraries were prepared from parenchyma tissue extracted from the inner part of the corm. 449 random clones were partially sequenced from the library of corms at stage C3 and 201 from the library of corms at stage C4, with an average length of the sequences of 407 and 342 bp respectively. Analysis of ESTs resulted in 351 and 191 unique sequences from stages C3 and C4. Qualitative information was obtained from the search with BLASTN and BLASTX against non-redundant database of GenBank. The top scoring genes were annotated, and classified into ten functional classes according to their putative identification (Figure 2). Functional classes were as follow:

1.- Transposable Elements. In this class we included ESTs that showed similarity to class I transposons, which replicate through an RNA intermediate (retrotransposons) and class II transposons which move directly through a DNA form. 15 ESTs from C3 library (3.3 %) and 5 from C4 (2.4 %) were found similar to several classes of retrotransposons: LTR retrotransposons, *copia*-like, and other sequences related to retroviruses.

2.- Protein Metabolism. ESTs with similarity to proteins involved in protein synthesis, folding, sorting, and degradation were included in this class. It was the functional class in

which the highest number of ESTs was included in C3 library (16 %). In this stage, we found 32 ESTs for ribosomal proteins, 6 translation factors, 12 involved in protein folding, 6 in protein sorting, and 13 in protein degradation (Table 2).

3.- Transport. In this category we included proteins related to selective movement and redistribution of ions and small molecules through the cell and organelle membranes. This is an essential process for cell growth and homeostasis. In addition, we included proteins related to transport of vesicles, which direct greater molecules to their proper organelle or outside the cell. We found opposite results in this category, since a greater percentage was obtained in stage C4 (6 % in C4; 2.4 % in C3). Transporters found in stage C4 included membrane transporters like aquaporins, inorganic pyrophosphatase, or cation transporters. We included in this category other proteins related with vesicle trafficking like an oxisterol binding protein or a synaptovrebin, an integral membrane protein of the v-SNARE family, which may participate in the synthesis of storage vacuoles (Sanderfoot and Raikhel, 1999)

4.- Cell Organization. In this category we included proteins related to creation and maintenance of cell wall and other cellular structures like cytoskeleton or nuclear lamina, that allow the correct cell development. In both stages we found expression of proline rich proteins, the major components of the protein matrix of cell wall. In addition, in C3 we found several ESTs with similarity to proteins involved in cell growth, like those with cell wall loosening activity: expansin and xyloglucan endotransglycosilase; or profilin and lamins, which mediate in the reorganization of cytoskeleton and nuclear lamina respectively after cell division. On the other hand, it was interesting the appearance in C4 of one EST with similarity to laccase, a protein involved in lignification.

5.- Development and Gene Regulation. Similar percentages of ESTs were found in this class (4.9 % in C3; 4.5 % in C4). However, ESTs showed similarity to proteins related to different developmental processes in both stages. In stage C3, we found expression of transcription factors related to cell growth and tissue organization, like the transcription factor SCARECROW, that has been found to be expressed in meristematic cells and involved in radial organization during embryogenesis and postembryonic period (Di Laurenzio et al., 1996; Wysocka-Diller et al., 2000); a translationally controlled tumour protein (TCTP) which has been found associated to cell growth (Pay et al., 1992); and protein B12D, which expression has been found in seed maturation and germination (Aalen et al., 2001). In stage C3 we also found ESTs with similarity to proteins involved in other developmental processes like apoptosis, that is an essential process in growth and development of many eucaryotic organisms; other auxin or ethylene regulated proteins involved in different developmental processes, and a tousled kinase, that has been proposed to participate in floral development (Roe et al., 1997), although in the corm at stage C3 may be involved in other processes because flowering has occurred months ago. It has been proposed that may have an important role in DNA replication during S phase of cellular cycle (Silljé et al., 1999). In addition we found expression of other proteins which function is unknown, but are expressed during particular moments of development. It is the case of an SRG1-like protein that is expressed in senescent organs (Callard et al., 1996).

6.- Signal Perception and Transduction. Two of the principal elements in the signal transduction pathways are intracellular Ca^{2+} and protein kinases. We found ESTs with similarity to proteins participating in both ways being expressed in stage C3. In the first one we found 5 ESTs with similarity to calmodulin that has been functionally identified as a primary Ca^{2+} receptor, because the Ca^{2+}-calmodulin complex can activate many other enzymes. Calmodulin participates in many cellular processes including one related to storage accumulation like potato tuberization (Reddy et al., 2002) that could be similar to enlargement of the corm. In addition we found one EST with similarity to an inositol kinase that may be involved in formation of Ca^{2+} channels. On the other way of signal transduction we found several sequences with similarity to protein kinases, including receptor-like protein kinases. We found other sequences with similarity to proteins that regulate signal responses, like 14-3-3 protein, a family of proteins involved in many

critical pathways regulated by phosphorilation, and that may act as a second messenger in auxin mediated processes (DeLille et al., 2001). In the stage C4, only one sequence in this category, a receptor-like protein kinase was found.

7.- Defence Against Pathogens and Stress. A great difference between stages was found in the category of defence against pathogens and stress response. 12 % of the ESTs sequenced in C3 library were included in this category, while only 4 % of ESTs from C4 library were included. ESTs with similarity to antimicrobial peptides (thionines and snakines), pathogenesis related proteins (chitinases, thaumatin, and polygalacturonase inhibitor), other proteins involved in virus resistance (RNAases and a protein related to post translational gene silencing), other proteins related to abiotic stress (late embryogenesis abundant protein and other stress induced proteins), and oxidative stress (catalase and dehydroascorbate reductase) were found and included in this category (Figure 3).

8.- Metabolism. Carbohydrate metabolism must be a principal process during storage accumulation that is in stage C3. In the library of this stage, we found sequences with similarity to proteins involved in carbohydrate metabolism, like phosphoglucomutase, sucrose synthase, starch phosphorilase, and UDP-glucose pirophosphorilase. All of them are involved in mobilization of sucrose for biosynthesis or energy production. In addition we found an EST with similarity to trehalose-6 phosphate synthase, which participates in the formation of trehalose, a metabolite that is involved in the regulation of carbohydrate metabolism in several ways (Goddijn and van Dun, 1999; Rolland et al., 2002). In the library of stage C4 we did not find any sequence related to carbohydrate metabolism.

9.- Others. Sequences not included in the rest of classes were grouped in this category, like ESTs related to chloroplast genome.

10.- Unknown. In both stages, a high number of sequences did not show significant similarity to known proteins (40.53 % in C3 and 60.20 % in C4). In this category, the highest percentage corresponded to ESTs that did not show significant similarity (E value > 10e-5) to known sequences when compared to public databases (35.63 % in C3 and 50.25 % in C4) while the rest were ESTs which show similarity to unknown proteins (Table 2).

None of the ESTs obtained showed similarity to fungal sequences, indicating that no contamination of the samples was produced despite the fact that corm is a subterraneous organ and is subjected to multiple pathogen attack.

Differences in percentage of ESTs were observed, being remarkable in the categories of protein metabolism, signal perception and transduction and defence, where a higher percentage was found in corms at stage C3, during enlargement. On the other hand, the percentage of ESTs included in the category of transport was higher in corms at stage C4. Only 11 sequences appeared on both developmental stages, including one ribosomal protein, two proteins with chaperonin activity, one thiol protease, one hypothetical protein from *Arabidopsis* with similarity to a cation transport protein and other putative trasporter protein, one chitinase, one methalotionein and three with no similarity to known proteins.

DISCUSSION

In this paper we consider 6 developmental stages in order to make easier further studies on the corm. These stages correspond to different physiological processes: C1 are buds in a latent stage; C2 are sprouting and flowering corms; C3 stage is characterized by enlargement and storage accumulation; C4 by dormancy; C5 are corms that support the growth of daughter corms on its surface; and finally, C6 are senescent corms. We have analysed cDNA libraries prepared from two of them. In first place we sequenced ESTs from corms during enlargement, as the corm is a storage organ, and reserves accumulation is a critical process in order to propagate the plant. The second stage that we analysed were dormant corms, as this is the material that growers use as seed, and an interesting material in order to find genes involved in resistance against arid conditions or related to dormancy.

Higher levels of cell activity were found in the corm at stage C3, as it is suggested by the presence of a greater number of sequences related to protein metabolism, signal perception and transduction, proteins involved in cell growth, or defence against pathogens and stress response. This is an active stage, when the corm is enlarging and accumulating reserves, principally carbohydrates. In this aspect, we found expression of enzymes involved in carbohydrate metabolism during C3 stage. On the other hand, in the corm at stage C4 we found the expression of the major storage protein, a mannan-binding lectin (Escribano et al., 2000). These data suggest that during enlargement, the corm acts as a sink organ for carbohydrates produced in photosynthesis, while during dormancy, the corm produces storage proteins which serve as an amino acid source for sprouting.

Furthermore, during enlargement, the corm produces a set of defensive proteins. One of these defence mechanisms is the synthesis of pathogenesis related proteins (PR). They include chitinases, enzymes that degrade structural polysaccharides of fungal cell walls reducing fungal growth. The expression of chitinases and other defence proteins like glucanases, is induced by the transduction of the signal of oligogalacturonides released from the cell wall when a polygalacturonase (PG) secreted by a fungal pathogen attacks the plant cell. Another PR protein present in the cell wall, polygalacturonase inhibitor (PGIP), contributes to defence retarding the activity of PG and increasing abundance of oligogalacturonides. In the corm of saffron in stage C3, we found expression of chitinases, thaumatin and PGIP. In addition, we also found expression of antimicrobial peptides, like thionins or snakins, which are small cysteine-rich peptides that plants synthesize as a defence barrier against bacterial or fungal pathogens, inhibiting protein synthesis or by means of direct interaction with membrane proteins (Florack and Stiekema, 1994). These proteins have been suggested that form constituent barriers, especially in storage and reproductive organs (Segura et al., 1999). Other defence mechanism, against viruses, is the postranscriptional gene silencing (PTGS), a mechanism based in sequence-specific RNA degradation induced by viral infection. We found one sequence with similarity to gene SGS3 from *Arabidopsis*, which has been found related to PTGS in plants (Mourrain et al., 2000).

We also found sequences with similarity to proteins involved in diverse stress tolerance in the library of stage C3. One of them showed similarity to a late embryogenesis abundant protein (LEA). This proteins were first identified as genes induced in seeds during maturation and dehydration (Ingram and Bartels, 1996), but later studies have found them associated to diverse stress tolerance: hydric and salt (Oztur et. Al, 2002) or cold (Ndong et al., 2002), and their potential as molecular tools for genetic improvement of crops has been suggested (Xu et al., 1996). In addition in stage C3 we found other 2 ESTs with similarity to proteins related to abscisic stress, and a low temperature and salt responsive protein. In the stage C4, in corms collected during the summer, we found a sequence with similarity to a protein induced by dehydration. This is in concordance with the ESTs with similarity to aquaporins, more present in stage C4, because these proteins participate in the uptake and transport of nutrients, facilitating it in dry conditions.

In the category of transport we found a greater percentage of ESTs in stage C4 related to stage C3. Transporters found in stage C4 included membrane transporters like aquaporins, inorganic pyrophosphatase, or cation transporters. The presence of aquaporins is necessary to facilitate an intense flow of water under stress or nutrient deficiency (Javot and Maurel, 2002). During the summer, the saffron corm overcomes high hydric stress conditions, so it is not striking the presence of ESTs with similarity to aquaporins during stage C4. Inorganic pyrophosphates is an exclusive transport system of plant vacuolar membranes, in which free energy of hydrolysis of inorganic pyrophosphate rather than ATP is used to pump H^+. In addition, the appearance in the library of C4 of proteins involved in vesicle trafficking and formation of storage vacuoles is in concordance with the presence of ESTs with similarity to the major storage protein of the corm. This protein is accumulated in storage vacuoles until it serves as a source for amino acids.

Finally, a great number of ESTs did not show significant similarity when they were compared to the public databases, emphasizing the lack of studies about gene expression in the corm. Future work will include the generation of a greater number of ESTs from cDNA libraries from the rest of developmental stages of the corm in order to further characterize gene expression patterns in the corm.

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Tables

Table 1. Number of clones (n) and putative identification of ESTs included in protein metabolism category. Differences between stages C3 and C4 are shadowed.

C3		C4	
n	**Putative Id.**	**n**	**Putative Id**
	Protein synthesis		
32	Ribosomal proteins	2	Ribosomal proteins
6	Translation factors	1	RNA polymerase
2	Other	2	PolyA polymerase
		2	Splicing
		1	Other
	Folding		
8	Chaperonins	1	Chaperonin
1	Nascent polypeptide associated complex	2	Nascent polypeptide associated complex
1	Disulfide isomerase protein		
2	Ciclophilin		
	Sorting		
1	Transport to nucleus		
4	Transport to endoplasmic reticulum		
1	Transport to lytic vacuoles		
	Degradation		
9	Proteases	2	Proteases
3	Ubiquitin		
1	Ubiquitin conjugated protein	1	Ubiquitin conjugated protein

Table 2. Number of ESTs (n) and percentage (%) assigned to unknown class.

	C3		C4	
	n	%	n	%
Similarity to unknown proteins	22	4.90	20	9.95
No significant similarity	160	35.63	101	50.25
Total	182	40.53	121	60.20

Figures

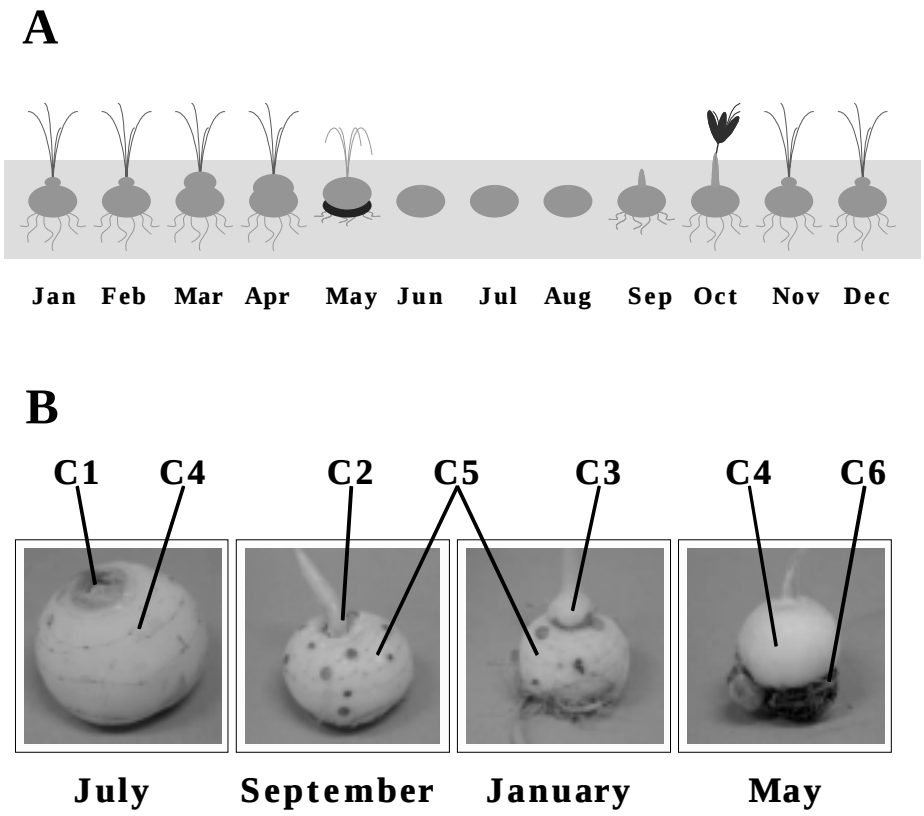

Fig. 1. A) Schematic representation of corm development. B) Corm developmental stages.

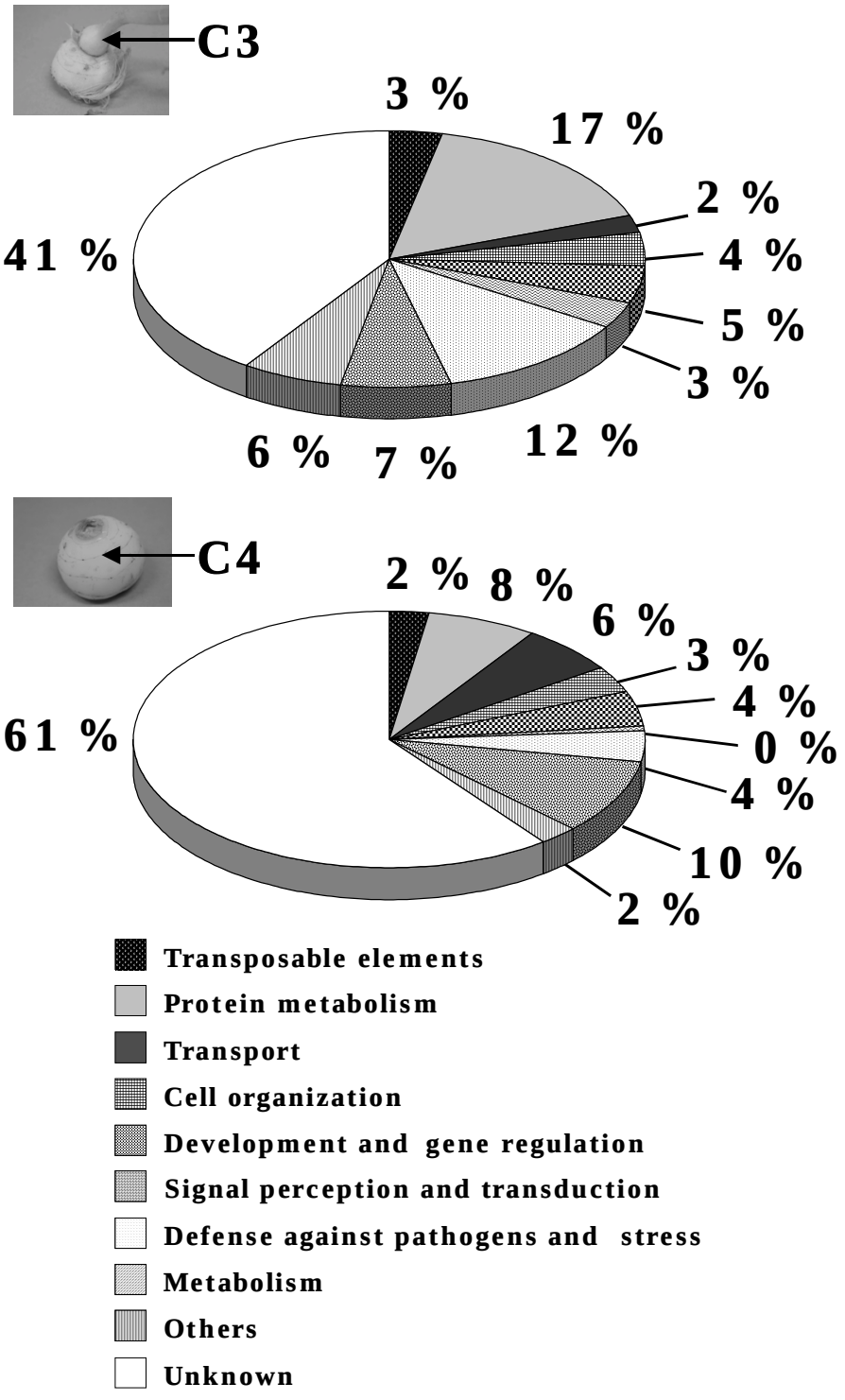

Fig. 2. Percentages of clones assigned to each category. Pictures refer to the material from which cDNA libraries were prepared. C3 are daughter corms collected in February, during enlargement. C4 are dormant corms collected in July.

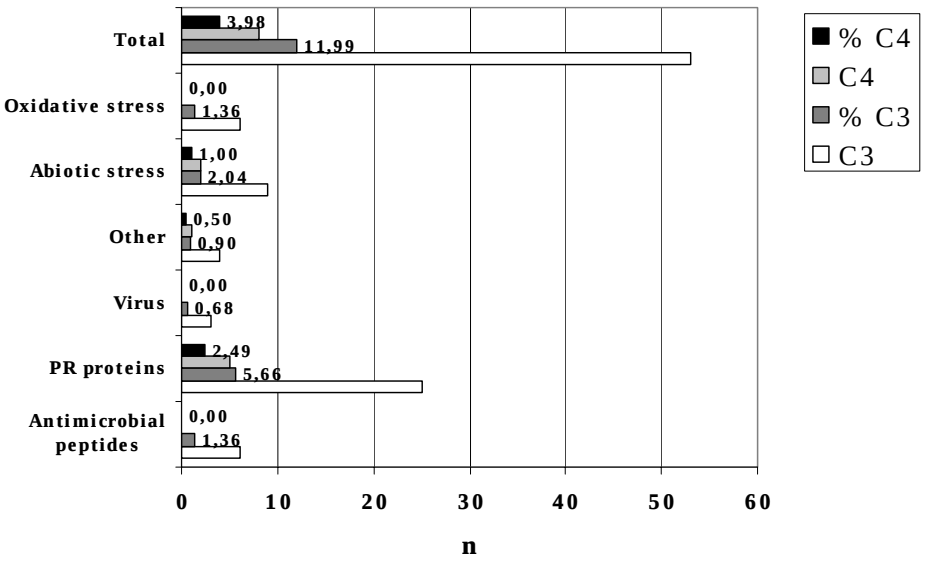

Fig. 3. Number of clones (n) and percentage of sequences (%) assigned to defence category