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Evaluation of 515 expressed sequence tags obtained from guard cells of *Brassica campestris*

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Abstract. As an attempt to examine the transcripts expressed in a single cell type and to unveil the physiology of guard cells at the molecular level, we generated 515 expressed sequence tags (ESTs) from a directional cDNA library constructed from guard-cell protoplasts of *Brassica campestris* L. ssp. *pekinensis*. A comparative analysis of the guard-cell ESTs against the National Center for Biotechnological Information non-redundant protein database revealed that 133 ESTs (26%) have significant similarity to protein coding sequences in the database. Among them were 35 clones related to genes that have not yet been identified in higher plants. Analysis of RNA gel blots of 14 database-matched clones revealed that five clones harbor the sequences for mRNAs expressed most abundantly in guard cells, one of them detecting an mRNA with highly preferential expression in guard cells. Functional categorization of the putatively identified guard-cell ESTs showed, when compared with maize leaf ESTs, that guard cells expressed a higher proportion of signal transduction components and a lower proportion of structural or photosynthetic genes, as is consistent with the roles of guard cells.

Key words: *Brassica* – Expressed sequence tag – Guard cell

Introduction

Over the last five years, an extensive amount of cDNA sequence information has accumulated in the public databases. Partial cDNA sequences (ESTs; expressed sequence tags) now form the majority of the records in GenBank (Boguski 1995). The EST technique has been applied to several plant species, including rice (Uchimiya et al. 1992; Kurata et al. 1994; Sasaki et al. 1994; Umeda et al. 1994), maize (Keith et al. 1993), *Brassica* (Park et al. 1993; Kwak et al. 1996; Lim et al. 1996) and *Arabidopsis* (Höfte et al. 1993; Newman et al. 1994; Cooke et al. 1996). This has made the repertoire of plant genes quite extensive. Expressed sequence tags can also be useful in searching for genes associated with a particular physiological process. There are two reports of genes expressed under stress and variable sucrose concentrations (Uchimiya et al. 1992; Umeda et al. 1994). The putatively identified ESTs from rice cells cultured under conditions of 2% or 20% sucrose reflected the difference in a number of proteins associated with translation, stress and metabolic pathways, including glycolysis, starch catabolism and amino acid biosynthesis (Uchimiya et al. 1992). The spectrum of expressed genes was further examined under conditions of stress such as high salinity and nitrogen-starvation (Umeda et al. 1994). Genes of the ATP-generating pathway are differentially expressed under nitrogen-starvation, salt, cold stress, and 20% sucrose (Umeda et al. 1994). Very recently, 743 cDNA clones were obtained from developing seeds of castor bean for the purpose of isolating genes involved in lipid biosynthesis, after pre-selection such as differential screening and immuno-screening with antibodies against partially purified endoplasmic reticulum membranes (van de Loo et al. 1995). A large proportion of the clones obtained after pretreatment were homologous to storage proteins, components of the protein synthetic apparatus, or enzymes involved in carbohydrate or lipid metabolism. The results of the above publications successfully demonstrated that a large portion of the partial sequences obtained from single-pass sequencing of randomly selected cDNA clones could be identified by similarity searches of existing databases. It is now widely accepted that even limited sequence information can give an insight into the possible function of a cloned gene. All of these studies, however, only reflect a summation of

Abbreviations: BLAST = basic local alignment search tool; EST = expressed sequence tag; NCBI = National Center for Biotechnological Information

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transcripts expressed in a tissue, an organ, or an organism, which contains many different cells.

Guard cells have been a favorite model system for over a century in studying plant signal transduction and ion-transport mechanisms. They play important roles in growth and survival of plants through stomatal movement, but they also have unique features in development and differentiation. Stomatal movement is regulated by numerous conditions and signals, such as CO_2, humidity, temperature, blue and red light, and a variety of endogenous phytohormones (Assmann 1993; Kearns and Assmann 1993). Ion pumps and channels that direct the flow of ions such as K^+, Ca^{2+}, Cl^-, and H^+ cause the guard cells to swell or shrink and have been intensively studied (Hedrich and Becker 1994; Schroeder 1995; Ward et al. 1995). Recently, a voltage-dependent K^+-channel gene has been cloned from potato guard cells and shown to be expressed in the guard cells by in-situ hybridization (Müller-Röber et al. 1995). Proteins associated with signal transduction, including G-proteins and protein phosphatases, have also been identified in guard cells (Fairley-Grenot and Assmann 1991; Li et al. 1994). In spite of these studies, the function and structure of signal receptors and other signaling components at the molecular level are still largely unknown.

In an effort to find genes that function in a single cell type while investigating the physiology of guard cells at the molecular level, we constructed a cDNA library from guard-cell protoplasts of *Brassica campestris* L. ssp. *pekinensis*. Here we report the results of analyzing 515 ESTs from guard cells.

Materials and methods

Plant materials. Seeds of *Brassica campestris* L. ssp. *pekinensis* and *B. napus* L. were provided by the Han Nong seed company and the Youngnam Agricultural Station, Republic of Korea. Plants were grown on a compound soil mixture of vermiculite:peat moss: perlite: (1:1:1, by vol.) in a temperature-controlled greenhouse with supplementary lighting. The plants were watered with Hoagland solution (Asher and Edwards 1983).

Preparation of guard-cell protoplasts from B. campestris ssp. pekinensis. Guard-cell protoplasts were isolated enzymatically according to the method described by Kruse et al. (1989) with a minor modification. In brief, 500 g of mature leaves of *B. campestris* were blended in a Waring blender in homogenization solution [5 mM $CaCl_2$, 0.5 mM ascorbate, 0.1% polyvinylpyrrolidone (PVP), 10 mM 2-(N-morpholino)ethanesulfonic acid (Mes), pH 6.0] and then washed several times with deionized water. The blended leaf peel was washed in a solution containing 0.25 M mannitol, 1 mM $CaCl_2$, 0.5 mM ascorbate and then incubated in a solution containing 0.7% cellulase (cellulase R-10; Yakult Honsha Co., Tokyo, Japan), 0.1% PVP, 0.25% bovine serum albumin, 0.23 M mannitol, 0.25 M $CaCl_2$, 0.25 mM $MgCl_2$, 0.25 mM ascorbate, 5 μM KH_2PO_4, 2.5 mM Mes, pH 5.5 at 23 °C with shaking at 70 rpm. After the first enzyme treatment, epidermal fragments were rinsed with a solution containing 0.45 M mannitol, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM ascorbate, 10 μM KH_2PO_4, 5 mM Mes, pH 5.5, and then subjected to a second enzyme digestion in a solution containing 2% cellulase, 0.25% bovine serum albumin, 5 mM $CaCl_2$, 0.36 M mannitol, 0.4 mM $MgCl_2$, 0.4 mM ascorbate, 8 μM KH_2PO_4, 4 mM Mes, pH 5.5 at 23 °C for 5 h with shaking at 70 rpm. Guard-cell protoplasts were then concentrated by low-speed centrifugation and stored at –70 °C until use.

Total-RNA isolation. Total RNA was extracted in the presence of 4 M guanidium *iso*thiocyanate from leaves, stamens, petals, roots, stems and flowers of *B. napus* and from guard-cell protoplasts of *B. campestris* L. ssp. *pekinensis* according to the procedure of Cox and Goldberg (1988).

Construction of a guard-cell cDNA library. Polyadenylated RNA was isolated from total RNA of guard-cell protoplasts by three rounds of oligo(dT)-cellulose chromatography (Kwak and Nam 1994). Following the manufacturer's instructions, cDNA was directionally synthesized from 0.5 μg of poly(A^+) RNA using the TimeSaver cDNA synthesis kit (Pharmacia P-L Biochemicals, Uppsala, Sweden) with a *Not*I-oligo(dT) primer. After second-strand synthesis, an *Eco* RI adaptor was ligated to the cDNA, which was followed by a *Not*I digestion. The cDNA was inserted into pT7T3D vector (Pharmacia) predigested with *Eco* RI and *Not*I and then electroporated into *Escherichia coli* DH5α cells using the Gene-Pulser electroporater (Bio-Rad, Hercules, Calif., USA). Approximately 8×10^4 recombinant plasmids were obtained.

Sequencing and sequence analysis of DNA. Sequencing from the 5′ end of the cDNA clones was performed with Sequenase version 2.0 (United States Biochemical, Amersham, Buoks., UK) using the T7 primer. The reaction mixture was separated by conventional 6% polyacrylamide gel electrophoresis. Plasmid DNA for the sequencing reaction was prepared by the standard method (Sambrook et al. 1989). The EST sequences were read automatically and then manually to remove the vector sequences. Comparison analysis of the EST sequences was conducted with BLASTX (basic local alignment search tool, X) e-mail server (Altschul et al. 1990) against the National Center for Biotechnological Information (NCBI) non-redundant protein database. In comparing the EST sequences with sequences in the database, a Poisson P-value of less than 0.01 was considered to indicate significant similarity when using the point acceptable mutation 120 (PAM120) matrix. Similarity between the related sequences was then further examined manually. Redundancy of the EST sequences was also examined using the BLASTN program (Altschul et al. 1990), with a slight modification of the program to allow sequential comparison.

Analysis of RNA gel blots. Twenty micrograms of total RNA prepared from flowers, leaves, stems, roots, stamens, and petals, and from guard-cell protoplasts was fractionated on a denaturing 1.2% formaldehyde-agarose gel and transferred onto a Biotrans nylon membrane (ICN Biomedicals, Irvine, Calif., USA). Prehybridization and hybridization were performed as described by Oh et al. (1996). The membrane was washed in 0.2 × SSC, 0.1% SDS at room temperature for 15 min and then at 42 °C for 15 min (1 × SSC = 15 mM trisodium citrate, 150 mM NaCl). The RNA blots were reprobed with an 18S rRNA gene of *B. napus* (Park et al. 1993) to assess the amount of RNA loaded in each lane.

Results and discussion

Construction of a guard-cell cDNA library and generation of ESTs. To examine transcripts expressed in a single type of cell, we employed the EST approach and chose guard cells as the target, because guard cells are distinctive in their physiology and development (see *Introduction*), and they can be easily separated from other leaf cells. Generating ESTs from a guard-cell cDNA library will provide an insight into the transcriptional activity of guard cells. In addition, the ESTs provide necessary molecular clones that can be used to probe directly into the physiology of guard cells at the

molecular level. The guard-cell protoplasts were isolated from the leaves of *B. campestris* ssp. *pekinensis* by the standard enzymatic method (Kruse et al. 1989). There were few mesophyll cells left among the thousands of guard cells in our preparation, when examined microscopically (data not shown).

We constructed a directionally synthesized cDNA library. The average insert size of the library was 0.85 kb. This cDNA library was used to obtain sequences from the 5′ end of the transcripts, since the 5′ untranslated regions (UTRs) of many plant genes are relatively short (Joshi 1987). Thus, the cDNA clones from this library could give a higher chance of sequencing coding regions, which is very helpful for the identification of the ESTs by database search. In addition, a directionally synthesized cDNA library could yield some full-length cDNA clones which could be used directly for further study.

We generated 515 ESTs from randomly selected guard-cell cDNA clones by single-run partial sequencing from the 5′ end of the cDNAs. The total of the EST sequences added up to 98.6 kb, and the average length of the sequences was 192 nucleotides. Since the comparison of peptide sequence is more effective in database searching (Adams et al. 1991; Park et al. 1993), the EST sequences were compared against the NCBI non-redundant protein database using BLASTX e-mail server which translates a nucleotide sequence into the six-frame translation products. A Poisson P-value of less than 0.01 was considered to indicate significant sequence similarity (Adams et al. 1991). Of the 515 ESTs, 133 (26%) shared substantial similarity with sequences in the NCBI non-redundant protein database (Table 1).

Although we used a directionally synthesized cDNA library, among the 133 putatively identified ESTs, 12 clones (9.0%) matched the sequences in the database when they were translated in a reverse orientation. This indicated that they were reversely inserted into the pT7T3D vector. A similar result was also noted in a previous report (Umeda et al. 1994). The larger proportion of cDNAs, though, was in the proper direction.

We also compared the sequences of 515 clones with one another using the BLASTN program, to examine the prevalence of transcripts in the guard cells. Expressed sequence tag clones with more than 90% identity over a stretch of 50 nucleotide were considered to be overlapping clones. Of the 515 clones examined, 486 clones (90.2%) provided unique ESTs, while 23 clones were represented twice. One clone, DGT1220 (similar to pollen-specific protein), was represented three times and one clone (nonmatched) four times.

Characterization of the guard-cell ESTs by database search. The guard-cell ESTs that shared significant similarity with the previously reported sequences in the database are listed in Table 1. The proportion of the putatively identified ESTs of guard cells was similar to that obtained from rice (28%, Sasaki et al. 1994) but slightly lower than that from *Arabidopsis* (32%, Höfte et al. 1993; Newman et al. 1994). Of the 515 ESTs, 382 clones were not functionally assigned by the comparison analysis against the non-redundant protein database. These unidentified sequences may either be new genes of plants, or may be mostly composed of 5′ UTR sequences. In the latter case, we may not have detected any related sequences in the database, since we performed the comparison analysis with the translated sequences. Examination of 20 unidentified ESTs for the presence of possible open reading frames in their sequence revealed that both new genes and 5′ UTR sequences may have been present. Among the 20 ESTs examined, 12 ESTs contained possible open reading frames but 8 ESTs appeared to consist entirely of non-coding sequences. This result shows that at least of 60% of the unidentified ESTs may represent genes that have not been characterized previously.

Among the putatively identified ESTs, 46 clones (34.6%) matched genes of the Brassicaceae, 52 clones (39.1%) showed similarity to genes of other plants, and 35 clones (26.3%) were similar to non-plant genes. Among the putatively identified 133 ESTs, only two clones (DGT469 and DGT991) had similarity to genes encoded in the chloroplast genome. This result, together with a low proportion of photosynthetic gene in our ESTs (see below), confirms that our guard-cell preparation was almost devoid of other leaf cells such as palisade parenchyma or spongy mesophyll. These cells are rich in chloroplasts and responsible for most photosynthesis. The presence of these cells would have given a higher proportion of chloroplast genes or photosynthetic genes.

As shown in Table 1, we classified the ESTs into three groups; ESTs with high similarity (71–100% identity) to plant genes, ESTs exhibiting less similarity (less than 70% identity) to plant genes, and ESTs with similarity to non-plant genes. The ESTs with high similarity to known plant genes may be *Brassica* counterparts of the matched genes. In contrast, ESTs showing relatively lower similarity (less than 70% identity) to plant genes could be members of a gene family. For example, the horseradish peroxidase gene shared similarities with DGT962 (81% identity), DGT852 (45% identity) and DGT997 (20% identity) at the peptide level, although DGT852 and DGT997 were more similar to the flax peroxidase (49% identity) and the poplar peroxidase gene (51% identity), respectively. This result suggests that DGT962 may be a *Brassica* counterpart of horseradish peroxidase, whereas DGT852 and DGT997 may be newly discovered members of the plant peroxidase gene family.

The putatively identified guard-cell ESTs reveal the transcriptional events in a single cell type. As shown in Fig. 1, we grouped the putatively identified 133 guard-cell ESTs into functional categories in order to examine the spectrum of transcripts expressed in a single type of cell. For comparison analysis, we selected 128 putatively identified maize ESTs that were generated from mature leaf and sheath and are maintained in the maize genome database. These maize ESTs were also grouped into functional categories (Fig. 1). Comparison of the functional categorizations revealed that the guard cells and maize leaf organ as a whole express a quite different

Table 1. Putatively identified guard cell ESTs of *B. campestris*

EST	Putative Identification	Organism	%ID [a]	OL [b]	DB [c]	Acc [d]
(*a*) *ESTs showing high similarity to plant genes*						
DGT7	β-Fructofuranosidase	*Arabidopsis*	97	57	PIR	S37212
DGT15	Glutathione-S-transferase	*Arabidopsis*	89	56	PIR	S38195
DGT44	Calreticulin	Barley	78	47	GP	L27348
DGT77	Multidrug resistance gene	*Arabidopsis*	86	59	GP	Z26467
DGT97	β-Fructofuranosidase	*Arabidopsis*	85	61	PIR	S37212
DGT123	Inorganic pyrophosphatase	Potato	77	55	GP	Z36894
DGT171	ATAF1 protein	*Arabidopsis*	89	50	GP	X74755
DGT209	NADP-dependent malic enzyme	Common bean	82	52	GP	X80051
DGT269	H^+-ATPase proteolipid chain	Maize	86	23	GP	M95063
DGT282	Lipid transfer protein	*Senecio odorus*	77	61	GP	L33792
DGT294	Polygalacturonase inhibitor	Pear	72	37	GP	L09264
DGT299	Pre-hevein-like protein	*Arabidopsis*	75	49	GP	U01880
DGT300	Caffeoyl-CoA 3-O-methyltransferase	Parsley	86	67	GP	M69184
DGT303	Histone H3	Alfafa	100	66	PIR	S04521
DGT312	Chitinase	Oilseed rape	94	62	GP	X61488
DGT332	Phenylalanine ammonia-lyase	*Arabidopsis*	91	34	GP	L33678
DGT337	Glutathione-S-transferase	*Arabidopsis*	95	73	PIR	S39542
DGT357	Glutathione-S-transferase	*Arabidopsis*	94	54	PIR	S38195
DGT381	SPF1 protein	Sweet potato	72	36	GP	D30038
DGT410	Ubiquitin	Soybean	100	39	GP	X13256
DGT467	Plastid ribosome protein CS17	*Arabidopsis*	86	38	GP	Z11151
DGT469	DNA-directed RNA polymerase α chain	Tobacco	95	49	SP	P06269
DGT470	Carboxypeptidase I	Barley	75	28	PIR	B25858
DGT477	UTP-glucose glucosyltransferase	Cassava	76	50	GP	X77460
DGT486	GF14 protein	*Arabidopsis*	92	38	GP	M96855
DGT517	Glutathione-S-transferase	*Arabidopsis*	84	59	GP	D17672
DGT544	40S Ribosomal protein S12	*Arabidopsis*	96	52	GP	Z17759
DGT605	Glutathione-S-transferase	*Arabidopsis*	82	56	PIR	S38195
DGT606	60S ribosomal protein L9 homolog	Garden Pea	91	67	SP	P30707
DGT614	ABI 1 gene product	*Arabidopsis*	93	61	GP	X78886
DGT687	Mitogen-activated protein kinase	Alfafa	93	59	GP	L07042
DGT690	Pyruvate dehydrogenase E1 β subunit	*Arabidopsis*	80	20	GP	U09137
DGT732	Laminin receptor	*Arabidopsis*	80	41	GP	Z18472
DGT770	Aspartate transaminase	Proso millet	89	57	PIR	S22379
DGT845	ATAF2 protein	*Arabidopsis*	72	65	PIR	S37100
DGT856	Chitinase	Oilseed rape	80	25	GP	X61488
DGT865	Photosystem I chain psaN	Barley	76	30	SP	P31093
DGT913	DNA-binding protein	*Petunia*	70	24	PIR	S19159
DGT925	β-Fructofuranosidase	Carrot	77	58	PIR	S23217
DGT962	Peroxidase C1B precursor	Horseradish	82	57	SP	P15232
DGT977	2′-hydroxylsoflavone reductase	Chickpea	72	18	PIR	S15688
DGT982	Malate oxidoreductase	Kidney bean	80	55	SP	P12628
DGT985	Profilin	White birch	78	71	SP	P25816
DGT991	ATP synthase p chain	Spinach	74	35	SP	P00825
DGT1015	β-Ketoacyl-ACP synthase	Barley	94	17	SP	P23902
DGT1022	δ-12 Desaturase	*Arabidopsis*	74	46	GP	L26296
DGT1038	Chitinase	Oilseed rape	82	55	GP	X61488
DGT1060	Elongation factor 1-α	*Arabidopsis*	95	45	GP	Z18461
DGT1062	Glutathione-S-transferase	*Arabidopsis*	78	35	GP	D17672
DGT1079	Malate oxidoreductase	Kidney bean	84	71	SP	P12628
DGT1087	Invertase	Mung bean	83	67	SP	P29001
DGT1215	eIF-4A	Tobacco	100	58	PIR	S22579
DGT1220	Pollen-specific protein precursor	*Arabidopsis*	79	29	GP	Z30916
DGT1293	Sec61 β-subunit homolog	*Arabidopsis*	87	32	GP	Z26753
DGT1302	ADP-ribosylation factor	Rice	80	40	GP	D17760
DGT1308	Phosphoprotein phosphatase	*Arabidopsis*	90	38	PIR	S31162
DGT1310	Auxin-induced protein	Tobacco	72	48	SP	Q03666
DGT1331	Lactoylglutathione lyase	Soybean	81	48	GP	X68819
DGT1373	Mitogen-activated protein kinase	*Arabidopsis*	92	50	PIR	S40470
DGT1411	Ubiquitin precursor	Rice	93	31	PIR	PS0380
DGT1436	Peroxidase	*Arabidopsis*	91	58	PIR	S37495
DGT1451	Receptor-like protein kinase	*Arabidopsis*	88	36	GP	Z37224
DGT1496	GTP-binding protein	*Arabidopsis*	97	44	PIR	S28875
DGT1505	Pyruvate dehydrogenase E1 β subunit	*Arabidopsis*	88	17	GP	U09137

Table 1. (*Contd.*)

EST	Putative Identification	Organism	%ID [a]	OL [b]	DB [c]	Acc [d]
(*b*) *ESTs showing similarity to plant genes*						
DGT62	Homeodomain protein	*Arabidopsis*	33	72	GP	L32873
DGT83	Phosphate translocator	Tobacco	36	62	PIR	S37224
DGT122	SPF1 protein	Sweet Potato	54	24	GP	D30038
DGT174	AWD protein	*Arabidopsis*	57	40	GP	Z18791
DGT221	Amino acid permease	*Arabidopsis*	45	31	PIR	A48187
DGT262	Cytochrome P_{450} protein	Rose periwinkle	46	82	SP	Q05047
DGT298	Polygalacturonase inhibitor	Tomato	66	73	GP	L26529
DGT320	Pyruvate kinase	Tobacco	53	53	PIR	S41379
DGT421	Zinc-finger protein	*Petunia*	62	38	GP	D26086
DGT450	FIL 2 gene product	*Antirrhinum*	68	31	GP	X76995
DGT516	Polygalacturonase inhibitor	Pear	61	21	GP	L09264
DGT566	Cationic peroxidase 2	Peanut	60	53	GP	M37637
DGT584	Tropinone reductase homolog	*Arabidopsis*	64	66	GP	Z29871
DGT600	EDGP precursor	Carrot	55	44	GP	D14550
DGT604	Receptor kinase	*Arabidopsis*	38	52	GP	M80238
DGT617	UTP-glucose glucosyltransferase	Cassava	48	48	PIR	S41953
DGT676	Pollen-specific protein	*Arabidopsis*	56	34	PIR	S36466
DGT685	pPLZ02-like protein	*Arabidopsis*	55	40	PIR	S38450
DGT751	Calmodulin-1	*Arabidopsis*	50	24	PIR	S29415
DGT847	UTP-glucose glucosyltransferase	Cassava	58	46	GP	X77460
DGT852	Peroxidase	Linseed	48	79	GP	L07554
DGT903	B2 protein	Carrot	56	48	PIR	S32124
DGT929	Carbonic anhydrase	*Arabidopsis*	61	49	SP	P27140
DGT931	Chitinase	*Arabidopsis*	53	43	GP	Z25799
DGT968	UTP-glucose glucosyltransferase	Cassava	56	61	GP	X77460
DGT974	Cytochrome P_{450} protein	Rose periwinkle	42	66	SP	Q05047
DGT997	Peroxidase	Poplar	51	37	GP	D13683
DGT1031	Plastocyanin precursor	*Arabidopsis*	61	31	SP	P11490
DGT1074	UTP-glucose glucosyltransferase	Cassava	49	53	GP	X77460
DGT1137	Calnexin homolog	*Arabidopsis*	69	49	SP	P29402
DGT1207	Receptor-like protein kinase	*Arabidopsis*	35	65	PIR	S27755
DGT1214	Strictosidine synthase	Serpentwood	37	51	PIR	S29894
DGT1240	Pollen-specific protein	*Arabidopsis*	65	61	PIR	S36466
DGT1261	Monodehydroascorbate reductase	Pea	68	44	GP	U06461
DGT1290	Hypothetical protein with leucine zipper	Tomato	53	26	PIR	S21495
(*c*) *ESTs showing similarity to non-plant genes*						
DGT4	Long-chain acyl-CoA synthetase	Human	33	54	SP	P33121
DGT109	High Mobility Group 1	Chicken	54	33	SP	P35194
DGT260	EF-G	Rat	53	58	PIR	S40780
DGT283	EF2	*Chlorella*	71	57	SP	P28996
DGT351	RNA polymerase σ-factor	*Bacillus subtilus*	49	72	PIR	B37129
DGT385	tRNA methyltransferase	Yeast	44	45	SP	P15565
DGT399	Membrane-associated protein	*B. subtilus*	43	67	GP	L03216
DGT407	Developmentally regulated protein	Rat	48	31	GP	L20319
DGT432	Ribosomal protein S5	Rat	93	45	SP	P24050
DGT439	Ribosomal protein S10	Rat	64	56	SP	P09900
DGT491	RNA polymerase II largest subunit	Fruit fly	57	28	GP	M19537
DGT511	Histone H3.3 gene	Human	67	30	GP	X05855
DGT704	Hemolytic phospholipase C	*Pseudomonas aeruginosa*	52	25	SP	P06200
DGT776	Phenoxybenzoate dioxygenase	*Pseudomonas pseudoalcaligenes*	59	49	GP	X78823
DGT806	X-linked chronic granulomatous disease protein	Human	38	36	PIR	A25722
DGT850	Kidney EGF	Human	59	27	GP	X04571
DGT909	Mitochondrial transporter	Yeast	52	48	SP	P32332
DGT911	U1 small nuclear ribonucleoprotein	*Xenopus*	57	35	SP	Q03369
DGT948	Protease II	*Escherichia coli*	52	34	SP	P24555
DGT951	Ovo gene product	Fruit fly	46	39	GP	X59772
DGT987	Histone H2A-III gene	*Volvox cateri*	82	34	SP	P16865
DGT1010	Riboflavin synthetase	*Photobacterium leiognathi*	52	59	SP	Q02008
DGT1050	Long chain acyl-CoA synthetase	Human	38	54	SP	P33121
DGT1078	NAD(P)H dehydrogenase	Yeast	45	66	GP	L29279

Table 1. (*Contd.*)

EST	Putative Identification	Organism	%ID [a]	OL [b]	DB [c]	Acc [d]
DGT1081	Mov 34 protein	Mouse	65	44	SP	P26516
DGT1120	PRP16 gene	Yeast	62	53	GP	M31524
DGT1148	Nucleolysin TIA-1	Human	68	16	SP	P31483
DGT1217	Multidrug resistance protein homolog	Human	28	46	PIR	S13428
DGT1243	Follicle stimulating hormone receptor	Rat	31	66	SP	P20395
DGT1254	Tyrosyl tRNA synthetase	*Podospora anserina*	44	36	SP	P28669
DGT1335	Fli I gene product	Fruit fly	55	38	GP	U01182
DGT1405	Fli I gene product	Fruit fly	53	32	GP	U01182
DGT1433	Cytochrome P_{450} 2A3 isoform	Mouse	44	34	PIR	S16068
DGT1453	Protein tyrosine kinase	Rat	39	38	GP	U13396

[a]Percentage identity
[b]Overlap – indicates lengths of identical amino acid residues for peptide matches
[c]Database abbreviations: GP, GenPeptide; PIR, Protein Identification Resource; SP, Swiss-Prot
[d]Accession number of the matched sequences

composition of mRNAs, revealing the differences in the physiological activities between the leaf organ and the guard cells. Proteins involved in defense or stress were much more abundant in guard cells (19.5% of the identified clones) than in maize leaf (10.9% of the identified clones). Proteins associated with signal transduction, including protein kinases and phosphatases, were also more abundant in guard cells (11.3% of the identified clones) than in maize leaf (3.9% of the identified clones). In contrast, structural proteins (9.4% of the identified clones) and proteins associated with photosynthesis (7.8% of the identified clones) were more abundant in the leaf than in guard cells (2.3% and 0.7%, respectively). A higher proportion of genes for signal transduction and a lower proportion of the photosynthetic genes indicate that guard cells devote more cellular activities to processing environmental or endogenous stimuli they encounter than to metabolic activities. This is consistent with the expected roles of guard cells, processing of a diverse signals to regulate stomatal movement. The lower proportion of photosynthetic activity, in particular, is consistent with the previously known physiological data. However, it should be noted that the ESTs generated here may not faithfully indicate the in-planta activities of guard cells, since the guard cells we used in this experiment experienced physical stress during the isolation of protoplasts.

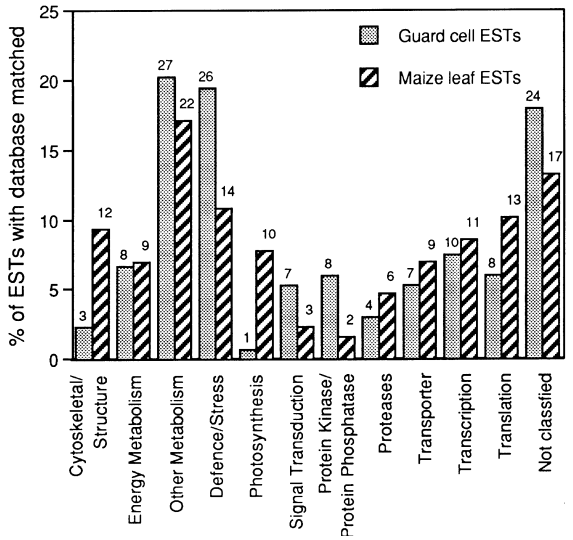

Fig. 1. Functional categories of ESTs from *B. campestris* guard cells and maize leaf. The number of ESTs is indicated above each bar

Some of the ESTs discovered in this study are likely to be related to guard-cell functions such as stomatal movements. For instance, DGT614 matched the *ABI1* gene encoding a protein phosphatase 2C (Leung et al. 1994; Meyer et al. 1994) of *Arabidopsis* with 93% identity at the peptide level. Thus, DGT614 may be the *Brassica* counterpart of the *ABI1* gene product in *Arabidopsis*. The ABI1 protein is a component of the abscisic acid (ABA) signaling pathway and has been shown to regulate stomatal movement (Leung et al. 1994; Meyer et al. 1994). DGT44 shared 78% amino acid sequence identity with a barley calreticulin (Chen et al. 1994), a Ca^{2+}-storage protein in the endoplasmic reticulum. DGT44 could be important in stomatal movements since cytosolic Ca^{2+} from internal stores, such as the vacuole or endoplasmic reticulum, affects the ion channels in guard cells (Gilroy et al. 1990; 1991). DGT732 matched a laminin receptor, a family of cell-surface adhesion receptors (Hynes 1992). DGT985 shared similarity with a profilin, an actin-binding protein that has been known to affect actin polymerization (Babich et al. 1996). Kim et al. (1995) reported that actin filaments in guard cells are involved in stomatal movement, and suggested that actin filaments may regulate ion channels in the plasma membrane of guard cells. Therefore, these two molecules could be involved in regulation of stomatal movements, playing a role for the cytoskeleton. DGT687 and DGT1373 showed a similarity to mitogen-activated protein kinases. Recently, it has been reported that two mitogen-activated protein kinases are involved in signal transduction of water stress in plants (Jonak et al. 1996; Mizoguchi et al. 1996). These EST clones, thus, may also participate in the signal transduction of water stress in guard cells, one of the most important functions of guard cells.

Interestingly, as shown in Table 1, some of the ESTs matched proteins unexpected in plants. The matched genes include a developmentally regulated protein gene of rat (GenBank accession number L20319), the human X-linked chronic granulomatous disease gene (Teahan et al. 1987), the mouse *Mov 34* gene (Soriano et al. 1987; Gridley et al. 1991), the *Drosophila fliI* gene (Campbell et al. 1993), and the *Drosophila ovo* gene (Mevel-Ninio et al. 1991). At present, the possible function of these EST clones cannot be easily assigned in plants, although their presence in guard cells suggests that they are somehow involved in guard-cell functions. However, DGT1335 with a similarity to the *Drosophila fliI* gene could be involved in regulation of the cytoskeleton since the *fliI* gene exhibits sequence similarity to the actin-binding protein gelsolin (Campbell et al. 1993). The human X-linked chronic granulomatous disease gene encodes the β-chain of the very unusual cytochrome b_{-245}, and disruption of the gene causes loss of a microbicidal oxidase system in phagocytes (Teahan et al. 1987). It is possible that DGT806 with a similarity to the β-chain of the cytochrome b_{-245} could be involved in a defense system of guard cells.

Expression patterns of the putatively identified guard-cell ESTs. We carried out RNA blot analyses with 14 database-matched EST clones to examine the expression patterns of these ESTs in guard cells and in several plant organs. We chose the ESTs that are expected to be involved in guard-cell functions or development as well as a few random ESTs. The 14 ESTs included DGT77 (a multidrug-resistance gene homolog), DGT1335 (a *fliI* gene homolog), DGT751 (a calmodulin-1 gene homolog), DGT985 (a profilin gene homolog), and DGT1217 (a multidrug-resistance gene homolog) that may function in stomatal regulation and the clones DGT913 (a *Petunia* DNA-binding protein gene homolog) and DGT1290 (similar to the hypothetical protein with a leucine zipper gene) that may function in guard-cell development. The data in Fig. 2 show that the expression patterns of the clones were highly variable. However, the expression pattern of the mRNAs for five clones (DGT77, DGT1010, DGT1290, DGT1335 and DGT771) were distinctive. The mRNAs for these clones, including a clone (DGT771) with a highly preferential expression in guard cells, were most abundantly expressed in guard cells. This expression pattern suggests that the clones have important or characteristic functions in guard cells. DGT77 is a homolog of the multidrug-resistance gene which is an ABC-type transporter. Thus, this clone is expected to function mostly in guard-cell transport functions. The guard-cell preferential expression of DGT1290, a homolog of a leucine zipper gene, is particularly interesting. Many of the leucine zipper-containing proteins are involved in gene regulation and, thus, the gene for this clone may be involved in regulating the genes preferentially expressed in guard cells or in controlling guard-cell development. The high-level expression of a riboflavin synthetase homolog was unexpected. The data indicate that the enzyme for riboflavin synthesis is much more active in guard cells than in the other organs. The higher-level expression of DGT1335, a *fliI* gene homolog, may be expected from its possible involvement in the regulation of the cytoskeleton in guard cells (see above). The function of DGT771 will be described in detail elsewhere. The expression pattern of DGT1217, another multidrug-resistance gene homolog, is in contrast to that of the DGT77 clone which is more prevalent in guard cells. However, the expression pattern shows that the gene for DGT1217 is utilized in guard cells at a comparable level to that in other organs. The expression pattern of DGT985, a profilin gene homolog, also contrasts with that of DGT1335, a homolog to a mammalian actin-binding protein gene: DGT985 is not expressed preferentially in guard cells. This result shows that, while the actin-binding protein encoded by DGT985 has a rather ubiquitous function, the possible actin-binding protein encoded by DGT1335 functions more preferentially in guard cells. Utilization of a different actin-binding protein is to be expected, considering that guard cells have a much higher need for a

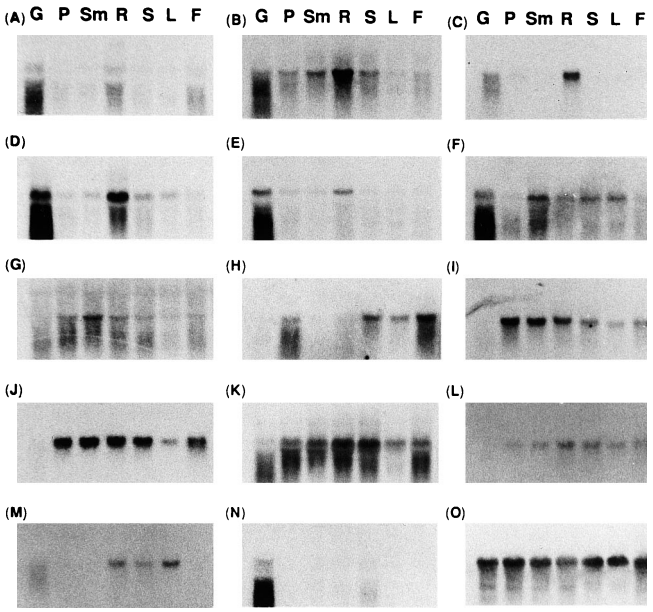

Fig. 2. RNA gel blot analysis of 14 database-matched ESTs. Total RNA (20 μg) from guard-cell protoplasts (*G*) of *B. campestris*, and petals (*P*), stamens (*Sm*), roots (*R*), stems (*S*), leaves (*L*) and flowers (*F*) of *B. napus* were separated on 1.2% denaturing agarose gels. After blotting, the blots were probed with the EST clone DGT77 (a multidrug-resistance gene homolog, **A**), DGT850 (a kidney epidermal growth factor homolog, **B**), DGT913 (a *Petunia* DNA-binding protein homolog, **C**), DGT1010 (a bacterial riboflavin synthetase homolog, **D**), DGT1290 (a homolog to a hypothetical protein with a leucine zipper, **E**), DGT1335 (a *FliI* gene homolog, **F**), DGT407 (a homolog to the developmentally regulated protein of rat, **G**), DGT450 (a *FIL2* gene homolog, **H**), DGT751 (a calmodulin-1 homolog, **I**), DGT174 (an AWD protein gene homolog, **J**), DGT985 (a profilin gene homolog, **K**), DGT1148 (a nucleolysin TIA-1 homolog, **L**), DGT1217 (a multidrug resistance protein gene homolog, **M**), DGT771 **N**), and with the 18S rDNA **O**). The sizes of the mRNAs detected by these clones are approx. 4.7 and 2.0 (**A**), 1.6 (**B**), 1.4 (**C**), 1.8 (**D**), 2.5 (**E**), 2.4 (**F**), 1.7 (**G**), 1.7 (**H**), 0.9 (**I**), 1.4 (**J**), 0.8 (**K**), 1.8 (**L**), 1.2 (**M**) and 2.7 (**N**) kb

continual rearrangement of actin filaments. DGT913, a homolog of a petunia DNA-binding-protein gene, was preferentially expressed in guard cells and roots. This clone may be involved in gene regulation common to guard cells and root cells. Four clones, DGT174, DGT450, DGT751, and DGT1148 showed a much lower expression level in guard cells than in the other organs. The reduced expression of DGT751, a homolog of *Arabidopsis* calmodulin-1 gene, is interesting. Guard cells appear to utilize this type of calmodulin less than the other organs for signal transduction. These data, overall, show that the expression level of most of the ESTs in guard cells is different from that in the other organs, although the proportion of guard-cell-specific transcripts is limited. Together with the functional categorization of the ESTs, the gel-blot data indicate that guard cells have transcriptional activities distinctive from leaf cells as a whole, consistent with their unique roles.

In conclusion, 515 guard-cell ESTs were generated from guard-cell protoplasts, 133 of which were putatively identified. We have discussed some possible correlation between guard-cell physiology and the identity and expression pattern of the ESTs. These EST clones, together with identification of their putative functions and the data obtained from RNA gel-blot analysis of 14 database-matched EST clones should provide important materials and/or clues to enhance our understanding of guard-cell functions and development at the molecular level.

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