Rapid Isolation of Monoclonal Antibodies. Monitoring Enzymes in the Phytochelatin Synthesis Pathway[1]

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Genomics projects have identified thousands of interesting new genes whose protein products need to be examined at the tissue, subcellular, and molecular levels. Furthermore, modern metabolic engineering requires accurate control of expression levels of multiple enzymes in complex pathways. The lack of specific immune reagents for characterization and monitoring of these numerous proteins limits all proteomic and metabolic engineering projects. We describe a rapid method of isolating monoclonal antibodies that required only sequence information from GenBank. We show that large synthetic peptides were highly immunogenic in mice and crude protein extracts were effective sources of antigen, thus eliminating the time-consuming step of purifying the target proteins for antibody production. A case study was made of the three-enzyme pathway for the synthesis of phytochelatins. Enzyme-linked immunosorbent assays and western blots with the recombinant proteins in crude extracts demonstrated that the monoclonal antibodies produced to synthetic peptides were highly specific for the different target proteins, gamma-glutamyl cysteine synthetase, glutathione synthetase, and phytochelatin synthase. Moreover, immunofluorescence localization studies with antibacterial γ-glutamyl cysteine synthetase and antiglutathione synthetase antibodies demonstrated that these immune reagents reacted strongly with their respective target proteins in chemically fixed cells from transgenic plants. This approach enables research to progress rapidly from the genomic sequence of poorly characterized target genes, to protein-specific antibodies, to functional studies.

Tens of thousands of new genes and their predicted protein products have been identified in genome sequencing projects on bacteria (Blattner et al., 1997; McClelland and Wilson, 1998), protists (Morio et al., 1998), fungi (Galibert et al., 1996; Kowalczuk et al., 1999), animals (Hattori et al., 2000; Pennisi, 2000), and plants (Sato et al., 2000a, 2000b). General information about molecular function can be inferred from the sequences of at least 40% of these proteins based on their homology to better characterized proteins. However, the scientific community is essentially ignorant of the spatial and temporal regulation of and the specific functions for the vast majority of these new proteins. The rapid production of highly specific immune reagents would greatly enhance the functional analysis of novel proteins identified by genomics.

Metabolic engineering projects also need numerous protein-specific immune reagents to monitor the coordinate synthesis of plant enzymes in complex biochemical pathways (Chartrain et al., 2000; Christensen and Nielsen, 2000). For example, the most efficient phytodetoxification of methylmercury, the most biohazardous form of mercury, requires the synthesis in plants of two bacterial enzymes, MerB and MerA. These enzymes catalyze a coupled reaction to form metallic mercury, the least toxic form of mercury (Bizily et al., 2000). Quantitative immunological monitoring of the levels of these two proteins in plants demonstrated that multiple kinetic factors restricted the efficiency of the coupled reaction. Manipulating much more complex plant pathways, such as carbohydrate metabolism (Lerchl et al., 1995; Geigenberger et al., 1998; Siedow and Stitt, 1998) or synthesizing pharmaceutical precursors for codeine (Chou and Kutchan, 1998; Huang and Kutchan, 2000), will require monitoring the levels of dozens of proteins expressed simultaneously.

An ideal case study can be made for the rapid production of immune reagents with the metabolic pathway required for synthesizing phytochelatins (PCs) in plants and fission yeast (*Schizosaccharomyces pombe*). The reactions catalyzed by the three enzymes required for PC synthesis, gamma-glutamyl Cys synthetase (γ-ECS), glutathione synthetase (GS), and PC synthase (PS), are described in Figure 1A. PCs form ligand complexes with thio-reactive toxic metals, such as Cd(II), Hg(II), and AsO_3^{-3}, and aid in their transport into vacuoles (Salt and Rauser, 1995), where they are sequestered. Arabidopsis mutants defective in the synthesis of PCs or their precursor tripeptide, glutathione, are hypersensitive to Cd(II) and other toxic metals (Howden et al., 1995a, 1995b), demonstrating the role of PCs in protecting plants from toxic metals. Further proof for this role came from overexpression of a bacterial γ-ECS in transgenic *Brassica juncea* (Zhu et al., 1999). These plants have higher GSH and PC levels and an increase in metal ion tolerance and accumulation over controls. Plant and fungal genes encoding PS recently have

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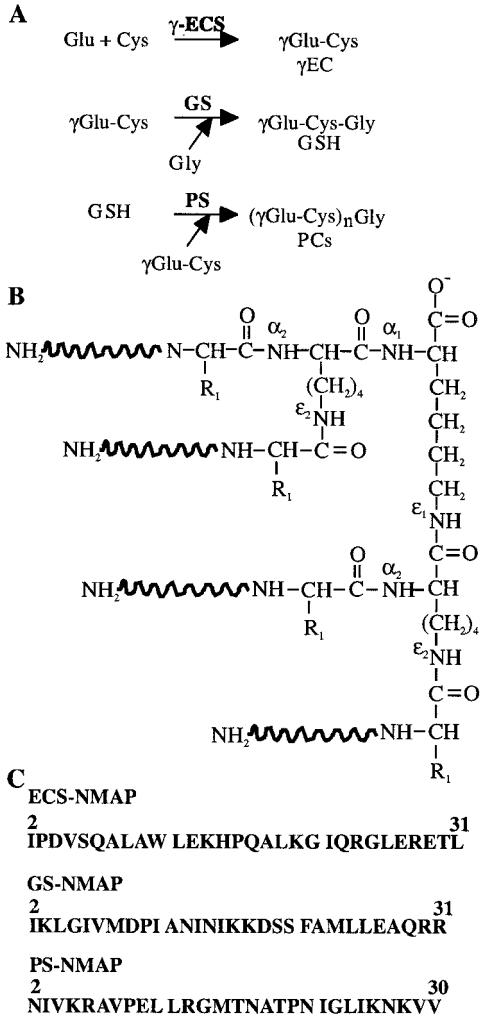

Figure 1. PC-synthesizing enzymes and multiple antigenic peptides (MAPs). A, Three enzymes are required for PC synthesis. The PCs are non-ribosomally synthesized peptides made with the structure (γ-Glu-Cys)$_n$Gly, where *n* is generally 2 to 11. γ-ECS catalyzes the condensation of Glu and Cys to form γGlu-Cys, which contains an unconventional peptide bond between the γ-carboxyl group of Glu and the α-aminio group of Cys. GS catalyzes the formation of a peptide bond between Gly and the carboxyl group of Cys in γGlu-Cys, resulting in the synthesis of the tripeptide glutathione (GSH). GS is not highly specific and other amino acids, commonly Ala (β-Ala) or Ser, can be substituted for Gly in this reaction. PS catalyzes the addition of multiple γGlu-Cys units to GSH or its relatives, resulting in the synthesis of PCs. B, Diagram of an MAP. MAPs were synthesized for all three PC-synthesizing enzymes. The 4-fold redundant MAP structure shown is built from a core of three lysines. The first Lys residue, which is attached to the support resin during synthesis, becomes the C-terminal residue in the MAP. The two lysines added during the second round of amino acid additions provide the four amino groups on which the four redundant peptides are built. The amino groups of the core lysines added in the first (α_1 and ϵ_1) and second (α_2 and ϵ_2) addition cycles are indicated. The first residue of the redundant peptide is drawn with its amino acid side chain indicated as R_1. The remaining N-terminal portion of the redundant peptide is shown with a squiggly line. C, Three N-terminal peptides synthesized as MAPs in this study. The peptides for γ-ECS (ECS-NMAP [N-terminal MAP]) and GS (GS-NMAP) were 30 amino acids long, whereas that for PS (PS-NMAP) was 29 residues long. Numbers indicate residue locations in the sequence relative to the first encoded residue, the initiator methioneine, which was omitted from each sequence.

been characterized (Clemens et al., 1999; Cobbett, 1999; Vatamaniuk et al., 1999). Plant PS overexpression in yeast increases tolerance to and accumulation of Cd(II). It is reasonable to propose that the coordinate overexpression of all three PC-synthesizing enzymes in the appropriate tissues may result in even higher levels of metal resistance and perhaps hyperaccumulation. However, engineering their coordinate synthesis and adjusting the relative enzyme levels for maximum efficiency in plants will require an accurate quantitative measurement of all three enzymes in numerous transformed plants and their genetic segregants. These assays would be performed best with specific immune reagents.

We describe a novel procedure to rapidly generate highly specific monoclonal antibodies (mAbs) that combines the use of synthetic MAP immunogens and crude extracts expressing full-length recombinant protein antigens. In a few months from identifying sequences in GenBank, monoclonal reagents to γ-ECS, GS, and PS were generated with only moderate effort. The advantages and implications of this streamlined method for mAb production are discussed briefly.

RESULTS

Synthetic Peptide Immunogens

Our goal was to generate specific mAbs for functional studies as rapidly as we cloned genes of interest and expressed them in transgenic Arabidopsis plants. Synthetic peptide immunogens were designed from the amino acid sequences of the three PC synthesizing enzymes, γ-ECS, GS, and PS, obtained from GenBank (Fig. 1A). The first two were derived from *Escherichia coli* sequences and PS from the fission yeast sequence. They were synthesized as MAPs (Fig. 1B), with 29 to 30 N-terminal amino acid residues (Fig. 1C), and designated ECS-NMAP, GS-NMAP, and PS-NMAP, respectively. Each MAP immunogen was injected three or more times into each of three mice (see "Materials and Methods").

The titers of antibodies in whole mouse serum were assayed against the three full-length proteins expressed from high copy vectors in *E. coli* (see "Materials and Methods"). At least two of the three mice injected with each of the three immunogens (ECS-NMAP, GS-NMAP, and PS-NMAP) gave significant titers to the bacterial extracts expressing the parent proteins, as shown in Table I. Although background was observed with the control bacterial extracts with the empty vector plasmid, the extracts expressing the corresponding antigen gave titer values 3.5 to 13-fold higher than background. The moderate background titers for the γ-ECS and GS control extracts are to be

Table I. *Titers of mouse[a] serum against three PC-synthesizing enzymes*

Mouse No.	Antigen						
	γ-ECS extract	GS extract	PS extract	Control extract	Ratio[b] extracts	PS[c] NMAP	BSA[c] control
ECS-NMAP no. 1	1.89	–	–	0.144	13.1	–	–
ECS-NMAP no. 2	1.83	–	–	0.159	11.5	–	–
GS-NMAP no. 1	–	2.20	–	0.229	9.6	–	–
GS-NMAP no. 2	–	2.12	–	0.335	6.3	–	–
PS-NMAP no. 1	–	–	0.28	0.048	5.8	1.50	.021
PS-NMAP no. 2	–	–	0.24	0.070	3.5	2.28	.030

[a] Data are for the two mice with the best activities that were used as the sources of splenic B cells for hybridoma fusions. Titer values are given as optical density units taken at A_{405} minus the background at A_{485}. They are typical of several independent measurements that were made on a 1:3,000 dilution of serum. [b] Ratio of the titer values for the recombinant protein extracts relative to control extracts. [c] Titers against PS-NMAP are compared with controls where the ELISA wells were treated with BSA.

expected because these *E. coli* enzymes are expressed at low levels in wild-type *E. coli* cells. Even the best titers to whole fission yeast PS proteins for the mice injected with PS-NMAP were low, presumably due to low levels of PS protein expressed in the bacterial extracts. However, the background to the PS protein in control bacterial extracts was also lower, making the titers reproducible and easily interpreted. Mouse sera consistently gave very high titers to their corresponding immunogenic MAPs as shown for two mice immunized with PS-NMAP (Table I).

Screening and Purifying Hybridoma Cell Lines

The culture supernatants from hybridoma cells derived from mice with the highest titers were screened using the bacterial extracts expressing the three enzymes required for the synthesis of PCS as the source of antigen in ELISAs (Materials and Methods). Several hybridoma pools secreting mAbs elicited with ECS-NMAP and GS-NMAP were easily identified as reacting with the over-expressed γ-ECS and GS proteins in bacterial extracts. Data for four such hybridoma pools for each protein are presented in Figure 2, A and B. Cell supernatants from these eight pools produced ELISA titers 10–30-fold over the background in control bacterial extracts. Several of these cell lines were further purified to homogeneity by limit dilution (Kohler and Milstein, 1975) using the same ELISA to identify positive hybridoma clones. The hybridomas making mAbs that were the most active against extracts expressing γ-ECS and GS were selected for further analysis and designated mAbECS1, 2, and 3 and mAbGS1, 2, 3, and 4, respectively.

Putative hybridoma cells producing PS antibodies were also identified using the PS-NMAP and PS-containing bacterial extracts as antigens, as indicated in Figure 2, C and D, respectively. From the 1,082 hybridoma cell pools assayed in the ELISAs, 17 cell pools gave significant reactivity with PS-NMAP as antigen. Among these 17, four lines gave significant reactivity with bacterial PS extracts, mAbPS1, 2, 3, and 4 (Fig. 2D) and low background values with the control bacterial extract lacking PS. Two pools containing mAbPS5 and mAbPS10 had significant but low ratios of activity between PS and control extracts and were not examined further. The improved quantification of ELISAs with PS-NMAP as antigen was essential during purification of the mAb-producing cell lines because sensitive quantitative judgement is needed to distinguish pure cell lines from mixtures containing other undesired cell lines during limit dilution screening. The initial screening of hybridoma cell extracts with the NMAP antigen had been performed as a precaution due to concerns that the low titers obtained with PS protein extracts might result in our missing active pools. As shown in Figure 2, C and D, this concern for the reliability of the protein ELISAs proved to be unfounded.

Western Analysis of mAb Specificity

The reactivity of the mAbs from purified cell lines were each assayed on western blots of extracts from bacteria expressing γ-ECS, GS, and PS as shown in Figure 3, A through C, respectively. In each case, strong bands were observed in lanes with the high expression vector, which expressed the correct protein, and very little if any signal was observed in the control bacterial extract lane on short exposures of the autoradiograph. Each band migrated at the expected molecular mass position relative to standards (Bio-Rad, Hercules, CA). Because the γ-ECS and GS genes cloned encode native *E. coli* proteins, relatively weak bands were observed in the correct molecular mass position in the control extracts on long exposures of the film, but the levels in the overexpression cells were significantly greater.

The reactivity of the mAbs from purified cell lines were also assayed on western blots of extracts from plants. The three PC-synthesizing enzymes were expressed in transgenic plants under the control of a constitutive Arabidopsis actin ACT2 promoter (An et

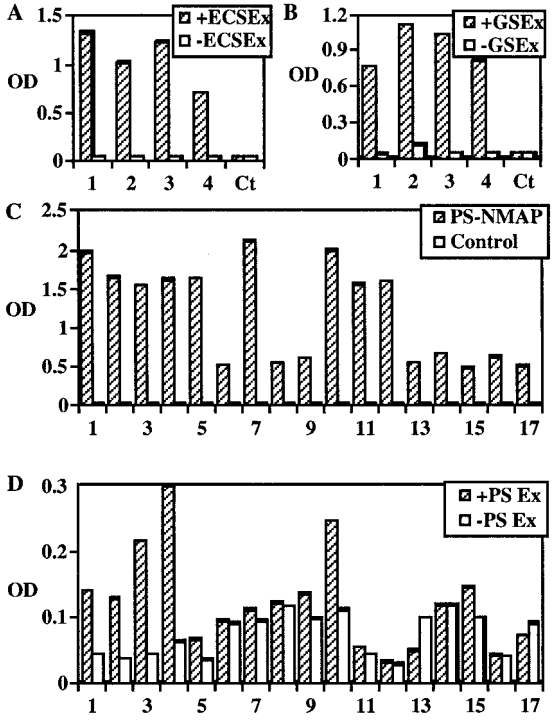

Figure 2. ELISA assays with peptide and crude extracts as antigens. A, Secreted antibodies from four hybridomas were reactive with γ-ECS protein in crude bacterial extracts. The ELISA reactivity of the four best mAbs with bacterial extracts expressing γ-ECS from pET15b vector (+ECSEx) as compared with bacterial extracts with the empty vector (−ECSEx). B, Secreted antibodies from four hybridomas were reactive with GS protein expressed from a pBluescript (+GSEx) in contrast to crude bacterial extracts from strains with an empty vector (−GSEx). C, Secreted antibody from 17 hybridomas were reactive with microtiter plate wells containing 10 ng PS-NMAP (synthetic peptide, PS-NMAP) and blocked with bovine serum albumin (BSA) in contrast to sample wells only blocked with BSA (Control). D, Subset of the secreted antibody from the same 17 hybridomas shown in 2C were reactive with PS protein in crude bacterial extracts expressed from pET15b (+PSEx) in contrast to empty vector controls (−PSEx). Optical density titer values in A through D are given for 25 μL of the hybridoma cell supernatent.

al., 1996). The mAbs to γ-ECS and GS reacted strongly to proteins of the expected molecular weights in extracts of transgenic plants expressing these proteins as shown in Figure 3, D and E. However, very weak bands were detected with mAbs to PS expressed in transgenic plants (not shown). The low level of PS protein detected may also result from the fact that many yeast genes are poorly expressed in plants due to cryptic polyadenylation sites within their coding regions (Grec et al., 2000).

Detection of Protein in Chemically Fixed Cells

Immunolabeling studies on paraformaldehyde-fixed cells revealed cytoplasmic localization of γ-ECS and GS proteins as shown in Figure 4. MAbs to both γ-ECS (Fig. 4A) and GS (Fig. 4, B and C) reacted strongly to their respective antigens expressed in transgenic leaf cells. The fluorescence staining with mAbGS1 and mAbGS2 was uniform throughout the cytoplasm, suggesting the expected distribution of soluble GS protein. In contrast, mAbECS1 exhibited punctate staining. The subcellular localization of ECS suggests that the cytoplasmically expressed bacterial protein may be associated with small organelles or vesicles after synthesis. The mAbGS1 antibodies did not show any staining in the wild-type control plant cells (Fig. 4D). The mAbGS2 or mAbECS1 antibodies

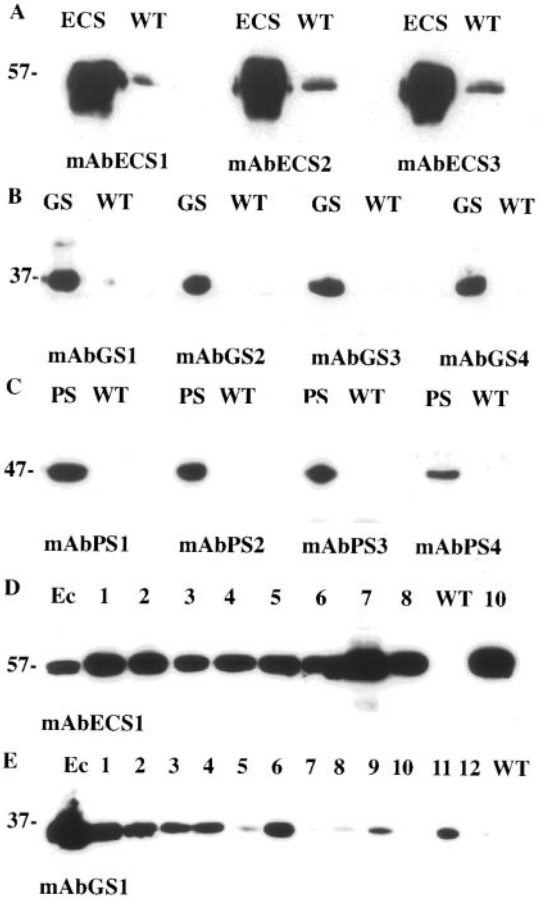

Figure 3. Western analysis of mAbs reactive with PC-synthesizing enzymes. A through C. Western analysis of several monoclonal antisera to γ-ECS, GS, and PS crude protein extracts from *E. coli* are presented in A through C, respectively. The cell supernatants or purified monoclonal antiserum from each of the best three or four cell lines reacting with each protein were used as the source of antibody. The reactivity of crude bacterial protein extracts from bacteria overexpressing the protein of interest were compared with control bacterial extracts from strains with an empty vector. The background observed in all control wells for γ-ECS and some control wells for GS antibody result from endogenous expression of these *E. coli* proteins. D and E, Western analysis of monoclonal antisera mAbECS1 and mAbGS1 to γ-ECS and GS expressed in transgenic Arabidopsis plant extracts are shown in D and F, respectively. Extracts from the different sets of independent recombinant plants are labeled numerically. *E. coli* extracts expressing protein and wild-type plant extracts lacking protein are labeled Ec and WT, respectively. The molecular mass in kilodaltons of the native *E. coli* proteins is listed in the left margin.

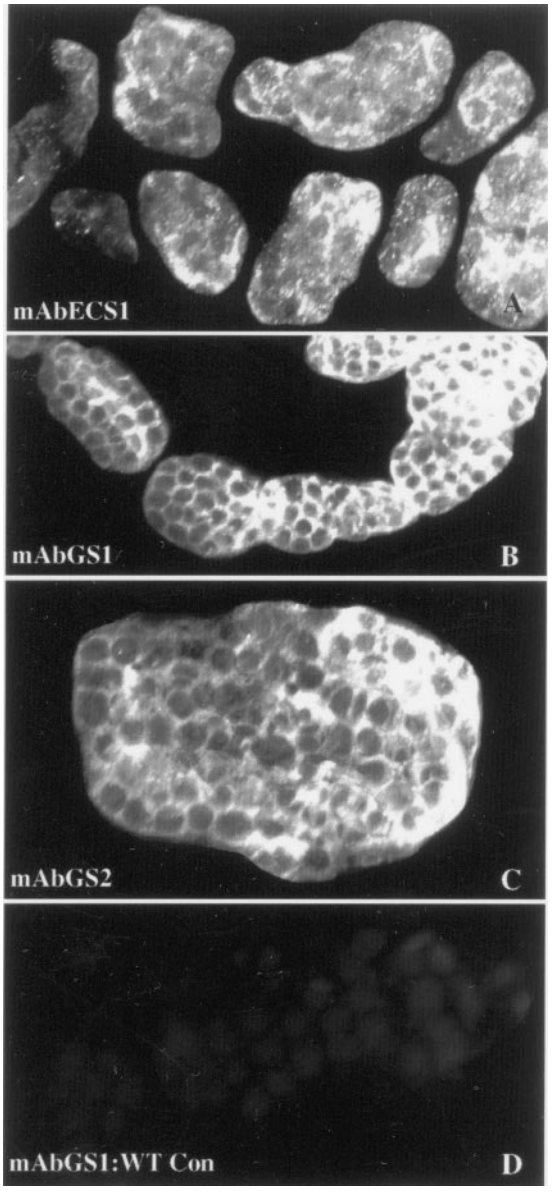

Figure 4. Immunofluorescence localization of ECS and GS proteins in transgenic Arabidopsis. A, Leaf cells from transgenic plants expressing bacterial γ-ECS labeled with mAbECS1. B and C, Leaf cells from transgenic plants expressing bacterial GS labeled with mAbGS1 (B) and mAbGS2 (C). D, Wild-type plant cells reacted with mAbGS1.

also showed no staining of plant cells (not shown). In a similar manner, the serum from nonimmunized mice also showed no staining on transgenic leaf cells (not shown).

DISCUSSION

Several factors limit the ability to rapidly generate specific immune reagents. First, it is generally desirable to use a highly purified protein as immunogen to stimulate production of a specific antibody. Highly pure immunogens lower the chance of obtaining false positives on western blots or misleading activities during screening of the hybridomas. Synthetic peptides can partially solve the requirement for pure protein immunogen because they do not contain contaminating cellular antigens and can be rapidly synthesized at relatively low cost. They often stimulate the production of excellent polyclonal immune reagents (McLean et al., 1990). However, there are a few disadvantages to synthetic peptide immunogens. They must be coupled (haptenized) to carrier proteins, such as keyhole limpet hemocyanin or BSA, to fully stimulate the immune system. The need for chemical coupling to carriers has been partially circumvented by the use of MAPs first proposed for use in developing synthetic vaccines (Posnett et al., 1988; Tam, 1988). MAPs typically contain four (Fig. 1B) or eight redundant copies of the peptide of interest. MAPs are highly immunogenic and in our laboratory nearly always give high titers of anti-MAP antibodies. We have nearly always obtained high quality antiprotein polyclonal antisera with MAPs as immunogens and with peptides conjugated to carrier protein as the immunogens (Condit et al., 1990; McLean et al., 1990; Kandasamy and Meagher, 1999; Palanivelu et al., 2000), when these peptides were designed from mobile terminal regions of proteins (Tainer et al., 1984).

The use of synthetic peptides to produce antibodies, however, has other shortcomings that we appear to have overcome in this study. First, polyclonal antisera are comprised of antibodies to many target proteins and hence are inherently not as precise as mAbs. Laboratory animals eat commercial feed prepared from dried plants and undoubtedly make antibodies to some plant proteins in this material as they do to other plant-based, oral vaccines (Dalsgaard et al., 1997). We believe it is for this reason that many control animals already make antibodies to plant proteins before they are immunized (data not shown), creating a common background problem with plant extracts. Using mAbs eliminates the high background problems of polyclonal sera. Second, synthetic peptides are not constrained by the rest of the native protein structure and fold into many novel conformations not found in the native protein. Hence, they stimulate the production of many antibodies that do not react with the whole protein and frequently fail to generate any antibody that reacts with the parent protein (McLean et al., 1990). This makes peptides less than ideal for generating and screening mAbs that react with native proteins. This is well illustrated by comparing Figure 2, C with D. We believe that using N terminally derived MAPs resembling the native terminal sequences circumvents this problem by giving a higher percentage of antiprotein antibodies. Third, short synthetic peptides containing B cell epitopes and presented as MAPs may stimulate a good primary response, but they often do not contain T cell epitopes necessary to stimulate a broader secondary response and produc-

Table II. *Oligonucleotide primers used to amplify the γ-ECS, GS, and PS genes by PCR*
Sequences are listed 5′ to 3′. The underlined segments contain restriction sites and clamps.

Gene	Sense Primer	Antisense Primer
ECS	CACAGCCTCGAGTAAGG AGGAGGAGCCACCATGG CAATCCCGGACGTATCA CAGGCGCT	GAGTCGGGATCCAAGCTTTCA GGCGTGTTTTTCCAGCCACAC CGCAA
GS	CGAGCCCTCGAGTAAGGA GCCACCATGGCAATCAAG CTCGGCATCGTGATGGACC CCAT	ACGTGCGGATCCAAGCTTATT ACTGCTGCTGTAAACGTGCTT CGAT
PS	CGAGCGCTCGAGTAAGGA GGAGCCACCATGGCAACC AATGCAACACCAAATATC GGT	CGAGCGGGATCCGAGCTCTCA CGTATTTTTACAGCAGCTTGA ACTA

tion of antiprotein antibodies (Tam and Lu, 1989). This is another reason why synthetic peptide immnogens are often coupled to carrier proteins, because the carriers contain T-cell epitopes. We presume that the relatively long peptides (29–30 amino acids) used in this study included both B cell and T helper cell stimulating epitopes and lead to the successful production of antiprotein antibodies. If necessary, specific T cell epitopes can be included in the peptide design (Cruz et al., 2000).

In this study of the PC-synthesizing enzymes, we explored one rapid method to circumvent the need for purified protein immunogens or purified protein antigens during screening of mAbs. We have shown that when MAPs comprised of long amino-terminal sequences are used as immunogens, antibodies to whole protein are seen in mouse sera and a reasonable portion of the hybridoma cell population produce antibodies to the whole protein. However, a large complement of cellular and secreted proteins are N terminally processed as they pass into the lumen of the endoplasmic reticulum or are transported into mitochondria or chloroplasts. In Arabidopsis, 26% to 43% of the 26,000 genes are predicted to encode such processed proteins (Arabidopsis Genome Initiative, 2000). The new N termini generated as these proteins are processed are moderately predictable from sequence and this processing should be taken into account in peptide immunogen design (Murphy et al., 2000). However, some chemical modifications such as glycosylation in the endoplasmic reticulum may mask native epitopes, thus rendering N-terminal MAP immunogens ineffective at generating protein antibodies. In addition, we have shown that crude bacterial extracts containing the protein of interest can be used as the source of antigen during screening to identify those hybridomas producing specific antiprotein antibodies. Although these assays with antigens in crude extracts have always worked in our laboratory with crude serum (Table I), they work best and generally have lower background when used with monoclonal antisera (Fig. 2). Using synthetic peptide immunogens and crude protein extracts expressing antigens circumvents the need for purified protein at any stage in the process of producing mAbs. Thus, it is anticipated that for most proteins, particularly those that are not drastically modified after translation, this approach will authenticate immune reactions with plant proteins that have not been purified or characterized previously.

CONCLUSION

Specific mAb reagents were rapidly generated to all three enzymes required for PC synthesis. The antibodies reacted specifically with the proteins of interest on western blots of crude extracts from both *E. coli* and transgenic Arabidopsis plants. The mAbs tested were effective histochemical reagents reacting strongly with paraformaldhyde-fixed proteins expressed in the transgenic plant cells. The combination of synthetic peptides as immunogens and recombinant proteins in crude extracts as antigens eliminated the need to purify any of the three proteins. This represents a substantial saving in time and labor in the production of mAb reagents. This streamlined method should be of general benefit to wide variety of fields including cell biology, molecular genetics, metabolic engineering, and proteomics.

MATERIALS AND METHODS

Production of mAbs

mAbs were prepared essentially as described by Kohler and Milstein (1975) with the following modifications. MAPs were synthesized in an MPS350 peptide synthesizer (Advanced Chemtech, Louisville, KY). Three 6-week-old female mice were given multiple injections of MAP immunogens (Tam, 1988) at 3-week intervals. The first injection (100 μg) was given intraperitoneally and subcutaneously with the immunogen emulsified with complete Freund's adjuvant. Three to five booster injections of the MAP (100 μg) were given intraperitoneally mixed with incomplete Freund's adjuvant. Seven days after the third and subsequent injections, a test bleed was performed and serum

antibody levels were evaluated by ELISA (see below). Mice showing the highest antibody titers were selected and again injected intraperitoneally with 50 μg of the MAP in phosphate-buffered saline (PBS), 3 d before fusion. Splenocytes were isolated from these mice, fused with the myeloma cell line SP2/O, and the resulting hybridoma cells were plated over macrophages in 96-well plates. Ten days after fusion, media from wells showing cell growth were tested by ELISA. Monoclonal cell lines producing antibodies were identified and then expanded to flasks to prepare large quantities of hybridoma supernatant. Antibodies from the supernatant were then isolated by ammonium sulfate precipitation and purified by using the Affi-Gel Protein A kit (Bio-Rad) following the manufacturer's instructions. This work was carried out at University of Georgia's Monoclonal Facility (Athens).

Cloning γ-ECS, GS, and PS for Bacterial Expression

The *γ-ECS* (accession no. X03954) and *GS* (accession no. 28377) genes were amplified by PCR, using synthetic primers listed in Table II, from genomic DNA of *Escherichia coli* SK1592 provided by Dr. Sidney Kushner's laboratory (University of Georgia). The fission yeast (*Schizosaccharomyces pombe*) *PS* gene (accession no. Z68144) was amplified from a plasmid SpPCS in pYES2 vector clone kindly provided by Dr. Julian I. Schroeder (University of California, San Diego). The two oligonucleotide primers for each gene added synthetic flanking sequences necessary for cloning and bacterial expression. The sense primers contained restriction endonuclease cloning sites *Xho*I and *Nco*I, a TAA stop codon, and bacterial translation signals (Rugh et al., 1996). The antisense primers contained cloning sites *Bam*HI and *Hin*dIII. The PCR products encoding all three genes were cloned first into the *Xho*I/*Bam*HI replacement region of pBluescript KS(II) (Stratagene, La Jolla, CA) and electroporated into *E. coli* strain Top10F (Invitrogen, Carlsbad, CA). Sequencing confirmed the fidelity of the amplified coding sequences. To express higher levels of protein, the three genes were subcloned into the *Nde*I (blunt end)/*Bam*HI replacement region of the expression vector pET15b (Novagen, Madison, WI) using post-ligation digestion with *Xho*I to select against the parent pET15b vector. These plasmids were expressed in *E. coli* strain BL121 (Novagen) as per the manufacturer's instructions.

Protein Extraction

E. coli strains containing recombinant pBluescript plasmids were cultured overnight at 37°C with 0.4 mM isopropylthio-β-galactoside and 200 mg L^{-1} ampicillin, whereas *E. coli* strain BL121 with recombinant pET15b plasmids was cultured overnight at 28°C with 1 mM isopropylthio-β-galactoside and 300 mg L^{-1} ampicillin. The overnight cultures were centrifuged at 4°C. To prepare protein extracts for ELISA the cold pellet was washed with borate saline buffer (BSB; 100 mM boric acid, 25 mM $Na_2B_40_7$, and 75 mM NaCl, pH = 8.5), resuspended in 2 volumes of BSB plus 5 mg mL^{-1} EDTA-free protease inhibitor tablet (Roche, Mannheim, Germany), and immediately sonicated in ice to break the cells. After centrifugation at 10,000*g* for 10 min at 4°C, the supernatant (approximately 1.5 μg protein μL^{-1}) was divided into aliquots and kept at −70°C for further use as antigen. The quantification of protein in bacterial extracts was performed using Nano Orange TM Protein Quantitation Kit (Molecular Probes Inc., Eugene, OR). Protein samples for western blots were prepared by mixing the BSB extract with equal volume of 2× SDS sample buffer (Laemmli, 1970).

ELISA Measurement of Antibody Titers

For protein ELISAs, equal amounts of total bacterial protein extracts with and without the protein antigen (500–1,000 ng $well^{-1}$ in 50 μL BSB) were adsorbed overnight to each well in paired wells of ELISA plates. For peptide ELISAs, wells were coated with MAP peptides at 20 ng $well^{-1}$ in 50 μL BSB. The remaining steps for ELISAs were carried out as described previously (Tam, 1988).

Expression of γ-ECS, GS, and PS in Arabidopsis

The three cDNAs encoding *γ-ECS, GS,* and *PS* were cloned under control of the constitutive *ACT2* promoter described previously (An et al., 1996). Arabidopsis plants were transformed by vacuum infiltration (Bariola et al., 1999). T_1 seeds were sterilized and germinated on solid Murashige and Skoog medium supplied with 0.8% (w/v) phytoargar, kanamycin (50 mg L^{-1}), and timentin (300 mg L^{-1}). Seedlings that survived the selection were transplanted into soil, and the leaf tissues were collected and kept at −70°C. Wild-type and transgenic plant protein extracts were prepared as described earlier (Kandasamy et al., 1999).

SDS-PAGE and Western Blotting

The bacterial and plant protein extracts were separated on 10% (w/v) SDS-PAGE gels (Laemmli, 1970) and western membrane imprints were prepared as described by Bizily et al. (2000). The membrane was blocked for 2 h in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% [v/v] Tween 20) containing 5% (w/v) dry milk and 20% (v/v) goat serum and probed with the primary antibody in the blocking buffer for 2 h at room temperature. Either the hybridoma cell supernatant was used directly at 0.1 to 0.2 mL mL^{-1} or the purified mAbs were used at 1 μg mL^{-1} final concentration (approximately 1:2,000 dilution). After washing (3 × 5 min), the blot was incubated with horseradish peroxidase-conjugated, anti-mouse, secondary antibody (Amersham, Piscataway, NJ) at 1:2,000 dilution in blocking buffer for 30 min. After washing again in TBST (3 × 8 min), the blots were treated with the enhanced chemiluminescence detection solution (Amersham), and exposed for 1 to 5 min to x-ray film (Hyperfilm, Amersham).

After resolution of the crude bacterial or plant protein extracts by SDS-PAGE, approximate protein expression

levels in the gels were compared by Coomassie staining to make sure protein levels were equivalent for ELISAs or western blots. The levels of bacterially expressed γ-ECS from a pET15b vector and GS expressed from a pBluescript KS(II) vector were detectable over and above the complement of resident bacterial proteins (not shown). Bands of PS protein were not visible over the complement of *E. coli* proteins when the *PS* gene was expressed from either vector suggesting that PS synthesis was very inefficient. Regardless of these low levels, crude extracts with PS synthesized from the pET15b vector was used in subsequent studies. ELISAs and western blots later confirmed the presence of PS protein.

Immunofluorescence Microscopy

To analyze the subcellular localization of the PC-synthesizing enzymes, and the reactivity of the antibodies against chemically fixed proteins, we performed immunofluorescence microscopy of 2-week-old transgenic Arabidopsis seedlings expressing these enzymes. Samples were fixed in 4% (w/v) paraformaldehyde in 50 mM PIPES buffer (1,4-piperazinediethanesulfonic acid) containing 5 mM EGTA, 1 mM $MgSO_4$, and 0.5% (w/v) casein protease inhibitor cocktail (Roche) for 1 h at room temperature and processed for immunolabeling as described previously (Kandasamy et al., 1999). Tissues on slides were blocked for 1 h in TBST-BSA-GS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% [v/v] Tween 20, 5% [w/v] BSA, and 10% [v/v] goat serum) and then incubated in the primary antibody diluted (5–10 $\mu g\ mL^{-1}$) in TBST-BSA-GS overnight. After thorough rinsing in PBS, the slides were incubated for 2 to 3 h with fluorescein isothiocyanate (Sigma, St. Louis) conjugated anti-mouse IgG at 1:100 dilution. The slides were mounted in 80% (v/v) glycerol in PBS containing 1 mg mL^{-1} p-phenylenediamine (an inhibitor of photo bleaching, Sigma) and observed with a Bio-Rad MRC-600 confocal-scanning microscope.

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LITERATURE CITED

An Y-Q, McDowell JM, Huang S, McKinney EC, Chambliss S, Meagher RB (1996) Strong, constitutive expression of the *Arabidopsis ACT2/ACT8* actin subclass in vegetative tissues. Plant J **10:** 107–121

Arabidopsis Genome Initiative T (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408:** 796–815

Bariola PA, MacIntosh GC, Green PJ (1999) Regulation of S-like ribonuclease levels in *Arabidopsis*: antisense inhibition of RNS1 or RNS2 elevates anthocyanin accumulation. Plant Physiol **119:** 331–342

Bizily S, Rugh CL, Meagher RB (2000) Phytodetoxification of hazardous organomercurials by genetically engineered plants. Nat Biotechnol **18:** 213–217

Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF et al. (1997) The complete genome sequence of *Escherichia coli* K-12. Science **277:** 1453–1474

Chartrain M, Salmon PM, Robinson DK, Buckland BC (2000) Metabolic engineering and directed evolution for the production of pharmaceuticals. Curr Opin Biotechnol **11:** 209–214

Chou WM, Kutchan TM (1998) Enzymatic oxidations in the biosynthesis of complex alkaloids. Plant J **15:** 289–300

Christensen B, Nielsen J (2000) Metabolic network analysis: a powerful tool in metabolic engineering. Adv Biochem Eng Biotechnol **66:** 209–231

Clemens S, Kim EJ, Neumann D, Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin syntheases from plants and yeast. EMBO J **18:** 3325–3333

Cobbett CS (1999) A family of phytochelatin synthase genes from plant, fungal and animal species. Trends Plant Sci **4:** 335–337

Condit CM, McLean BG, Meagher RB (1990) Characterization of the expression of the petunia glycine-rich protein-1 gene product. Plant Physiol **93:** 596–602

Cruz LJ, Quintana D, Iglesias E, Garcia Y, Huerta V, Garay HE, Duarte C, Reyes O (2000) Immunogenicity comparison of a multi-antigenic peptide bearing V3 sequences of the human immunodeficiency virus type 1 with TAB9 protein in mice. J Pept Sci **6:** 217–224

Dalsgaard K, Uttenthal A, Jones TD, Xu F, Merryweather A, Hamilton WD, Langeveld JP, Boshuizen RS, Kamstrup S, Lomonossoff GP et al. (1997) Plant-derived vaccine protects target animals against a viral disease. Nat Biotechnol **15:** 248–252

Galibert F, Alexandraki D, Baur A, Boles E, Chalwatzis N, Chuat JC, Coster F, Cziepluch C, De Haan M, Domdey H et al. (1996) Complete nucleotide sequence of *Saccharomyces cerevisiae* chromosome X. EMBO J **15:** 2031–2049

Geigenberger P, Hajirezaei M, Geiger M, Deiting U, Sonnewald U, Stitt M (1998) Overexpression of pyrophosphatase leads to increased sucrose degradation and starch synthesis, increased activities of enzymes for sucrose-starch interconversions, and increased levels of nucleotides in growing potato tubers. Planta **205:** 428–437

Grec S, Wang Y, Le Guen L, Negrouk V, Boutry M (2000) Cryptic polyadenylation sites within the coding sequence of three yeast genes expressed in tobacco. Gene **242:** 87–95

Hattori M, Fujiyama A, Taylor TD, Watanabe H, Yada T, Park HS, Toyoda A, Ishii K, Totoki Y, Choi DK et al.

(2000) The DNA sequence of human chromosome 21: the chromosome 21 mapping and sequencing consortium. Nature **405:** 311–319

Howden R, Andersen CR, Goldsbrough PB, Cobbett CS (1995a) A cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana.* Plant Physiol **107:** 1067–1073

Howden R, Goldsbrough PB, Andersen CR, Cobbett CS (1995b) Cadmium-sensitive *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. Plant Physiol **107:** 1059–1066

Huang FC, Kutchan TM (2000) Distribution of morphinan and benzo[c]phenanthridine alkaloid gene transcript accumulation in *Papaver somninferum*. Phytochemistry **53:** 555–364

Kandasamy MK, McKinney E, Meagher RB (1999) The late pollen specific actins in angiosperms. Plant J **18:** 681–691

Kandasamy MK, Meagher RB (1999) Actin-organelle interactions: association with chloroplast in *Arabidopsis* leaf mesophyll cells. Cell Motil Cytoskeleton **44:** 110–118

Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature **256:** 495

Kowalczuk M, Mackiewicz P, Gierlik A, Dudek MR, Cebrat S (1999) Total number of coding open reading frames in the yeast genome. Yeast **15:** 1031–1034

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lerchl J, Geigenberger P, Stitt M, Sonnewald U (1995) Impaired photoassimilate partitioning caused by phloem-specific removal of pyrophosphate can be complemented by a phloem-specific cytosolic yeast-derived invertase in transgenic plants. Plant Cell **7:** 259–270

McClelland M, Wilson RK (1998) Comparison of sample sequences of the *Salmonella typhi* genome to the sequence of the complete *Escherichia coli* K-12 genome. Infect Immunol **66:** 4305–4312

McLean BG, Eubanks S, Meagher RB (1990) Tissue specific expression of divergent actins in soybean root. Plant Cell **2:** 335–344

Morio T, Urushihara H, Saito T, Ugawa Y, Mizuno H, Yoshida M, Yoshino R, Mitra BN, Pi M, Sato T et al. (1998) The *Dictyostelium* developmental cDNA project: generation and analysis of expressed sequence tags from the first-finger stage of development. DNA Res **5:** 335–340

Murphy RF, Boland MV, Velliste M (2000) Towards a systematics for protein subcelluar location: quantitative description of protein localization patterns and automated analysis of fluorescence microscope images. Proc Int Conf Intel Syst Mol Biol **8:** 251–259

Palanivelu R, Belostotsky DA, Meagher RB (2000) Conserved Expression of *Arabidopsis thaliana* poly (A) binding protein 2 (PAB2), in distinct vegetative and reproductive tissues. Plant J **22:** 199–210

Pennisi E (2000) Human Genome Project: chromosome 21 done, phase two begun. Science **288:** 939

Posnett DN, McGRath H, Tam TP (1988) A novel method for producing anti-peptide antibodies. J Biol Chem **263:** 1719–1725

Rugh CL, Wilde D, Stack NM, Thompson DM, Summers AO, Meagher RB (1996) Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial *merA* gene. Proc Natl Acad Sci USA **93:** 3182–3187

Salt DE, Rauser WE (1995) MgATP-Dependent transport of phytochelatins across the tonoplast of oat roots. Plant Physiol **107:** 1293–1301

Sato S, Nakamura Y, Kaneko T, Katoh T, Asamizu E, Kotani H, Tabata S (2000a) Structural analysis of *Arabidopsis thaliana* chromosome 5: X. Sequence features of the regions of 3,076,755 bp covered by sixty P1 and TAC clones. DNA Res **7:** 31–63

Sato S, Nakamura Y, Kaneko T, Katoh T, Asamizu E, Tabata S (2000b) Structural analysis of *Arabidopsis thaliana* chromosome 3: I. Sequence features of the regions of 4,504,864 bp covered by sixty P1 and TAC clones. DNA Res **7:** 131–135

Siedow JN, Stitt M (1998) Plant metabolism: where are all those pathways leading us? Curr Opin Plant Biol **1:** 197–200

Tainer JA, Getzoff ED, Alexander H, Houghten RA, Olsen AJ, Lerner RA, Hendrickson WA (1984) The reactivity of anti-peptide antibodies is a function of the atomic mobility of sites in a protein. Nature **312:** 127–134

Tam JP (1988) Synthetic peptide vaccine design: Synthesis and properties of a high-density multiple antigenic peptide system. Proc Natl Acad Sci USA **85:** 5409–5413

Tam JP, Lu YA (1989) Vaccine engineering: enhancement of immunogenicity of synthetic peptide vaccines related to hepatitis in chemically defined models consisting of T- and B-cell epitopes. Proc Natl Acad Sci USA **86:** 9084–9088

Vatamaniuk OK, Mari S, Lu YP, Rea PA (1999) AtPCS1, a phytochelatin synthase from *Arabidopsis:* isolation and in vitro reconstitution. Proc Natl Acad Sci USA **96:** 7110–7115

Zhu YL, Pilon-Smits EAH, Tarun AS, Weber SU, Jouanin L, Terry N (1999) Cadmium tolerance and accumulation in Indian Mustard is enhanced by overexpressin gamma-glutamylcysteine synthetase. Plant Physiol **121:** 1169–1177