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Mining secreted proteins that function in pepper fruit development and ripening using a yeast secretion trap (YST)

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ABSTRACT

Plant cells secrete diverse sets of constitutively- and conditionally-expressed proteins under various environmental and developmental states. Secreted protein populations, or secretomes have multiple functions, including defense responses, signaling, metabolic processes, and developmental regulation. To identify genes encoding secreted proteins that function in fruit development and ripening, a yeast secretion trap (YST) screen was employed using pepper (*Capsicum annuum*) fruit cDNAs. The YST screen revealed 80 pepper fruit-related genes (*CaPFRs*) encoding secreted proteins including cell wall proteins, several of which have not been previously described. Transient GFP-fusion assay and an *in planta* secretion trap were used to validate the secretion of proteins encoded by selected YST clones. In addition, RNA gel blot analyses provided further insights into their expression and regulation during fruit development and ripening. Integrating our data, we conclude that the YST provides a valuable functional genomics tool for the identification of substantial numbers of novel secreted plant proteins that are associated with biological processes, including fruit development and ripening.

1. Introduction

The growing number of sequenced plant genomes resulting from innovative sequencing technologies, is generating an enormous repository of primary DNA sequence information, defined gene structures, and predicted proteomes. A key subsequent goal is the complete functional annotation of plant genes/proteins, or the determination of the functional proteome. Plant cells are highly compartmentalized and the subcellular localization of a protein is intrinsic to its function. Therefore, information about the subcellular localization of their products can provide insight into the functions of genes. Among the various plant subcellular proteomes, secreted proteins or secretomes have central roles in growth and development, plant–pathogen interactions, abiotic stress responses, self- and interorganismal recognition, signaling, and metabolism. [1,2].

Fruit development and ripening are complex developmental processes, and numerous biochemical and regulatory pathways that contribute to physiochemical changes and the determination of their final composition [3]. Many of these phenomena, including changes in fruit size and texture, are influenced by the secretome or cell wall proteome [4]. A comprehensive understanding of the secretome is a prerequisite for understanding critical aspects of fruit development and ripening, but much remains to be learned of its composition and dynamics. Direct proteomic approaches can potentially allow the identification of proteins expressed in cell wall or apoplast and have the advantage of direct analysis of actual gene products [5]. However, developing a comprehensive catalog of the cell wall proteome is generally more challenging than for intracellular organelles that can be isolated in highly purified fractions, relatively free from nonspecific protein contamination [2].

As an alternative to functionally screening the secretome using time-consuming and labor-intensive techniques, the yeast secretion trap (YST) assay has been employed to screen cDNA libraries to identify secreted proteins in plants and mammals [6]. YST involves fusing cDNA libraries from organs, tissues or cell types of interest to a yeast (*Saccharomyces cerevisiae*) invertase gene lacking a signal peptide and transforming the resulting fusion library into an invertase-deficient yeast strain [7,8]. The transformants

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containing cDNAs with signal peptide are consequently able to grow on a medium containing sucrose as the sole carbon source as the secreted invertase fusion protein rescues the mutant.

In this study, the YST assay was performed to characterize the pepper fruit secretome and to gain insight into pepper fruit development and ripening. To confirm the secretion of the identified proteins in plant cells, transient GFP-fusion assays and an *in planta* secretion trap assay [8] were then applied. In addition, the expression pattern of fruit secretomes was evaluated at various fruit developmental stages. The information from the secretome analysis will provide a better understanding of the diverse extracellular and cell surface biological processes in pepper fruit. Furthermore, this study underscores the effectiveness of using the YST and *in planta* secretion trap assays for high-throughput screening of plant secretomes.

2. Materials and methods

2.1. Plant materials

Pepper (*Capsicum annuum* cv. SF11) plants were grown in a greenhouse at Suwon, Korea. Fruits were staged based on size or color: immature green (IG), mature green (MG), breaker (BR; appearance of red pigment), turning (TU; approximately 30% red), immature red (IR; approximately 70% red), and red ripe (RR; 100% red). For cDNA library construction and Northern blot analysis, fruit pericarp tissues were immediately frozen in liquid nitrogen following seed and placenta removal and stored at −80 °C until use.

2.2. Pepper fruit yeast secretion trap (YST) screen and protein structure predictions

A YST cDNA library was constructed using polyadenylated mRNA from a mixture of equal fresh weights of the sequential developmental and ripening stages of pepper pericarps. The library was screened as previously described [7,8]. Two vector systems, pSMASH [9,10] (CaPFR1–CaPFR44) and pYST [7,8] (CaPFR45–CaPFR80), were used for YST screening. The cDNA library constructed in *Escherichia coli* was approximately 1.0×10^6 cfu/mL. Each clone was confirmed at least twice with the YST assay. Sequences of the positive YST clones were used to search the GenBank database (http://www.ncbi.nlm.nih.gov). To supplement the limited sequence information for some YST clones, ESTs from the pepper EST database (http://210.218.199.240/pepper) and SGN database (http://solgenomics.net) that exactly matched the YST clone sequences were also used for gene annotations. YST clone sequences were translated *in silico* and signal peptide and putative cleavage sites were predicted using SignalP 3.0 software (http://www.cbs.dtu.dk/services/SignalP) [11].

2.3. Northern blot analysis

Total RNA (10 μg) from pepper pericarps materials was separated on 1.2% agarose gels containing formaldehyde and blotted onto Hybond-N^+ membranes (Amersham-Pharmacia Biotech, Piscataway, NJ). Membranes were hybridized at 65 °C overnight with [α^{32}P]-labeled cDNA from selected YST clones. Hybridization was performed as described previously [12]. After hybridization, membranes were washed with 2× SSC and 0.1% SDS at 65 °C for 10 min, with 1× SSC and 0.1% SDS at 65 °C for 10 min, and with 1× SSC and 0.1% SDS at room temperature for 10 min. Membranes were exposed to X-ray film with an intensifying screen at −80 °C for 24 or 48 h.

2.4. Subcellular localization of CaPFR24-smGFP fusion protein

To obtain full-length cDNA sequences, rapid amplification of cDNA ends (RACE)-PCR was performed using a SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA). The termination codon of the *CaPFR24* cDNA was removed after PCR and the PCR-amplified product was fused in-frame to the coding region of soluble-modified green fluorescent protein (smGFP) [13]. For transient expression analyses, plasmid DNA of the fusion construct (1 μg) was introduced into the onion epidermal cell using 1.1-μm tungsten microcarriers and a helium-driven PDS2000 particle delivery system (Bio-Rad, Hercules, CA). After bombardment, tissues were incubated at 22 °C for 16 h. For smGFP expression analysis, onion epidermal cells were examined by confocal laser-microscopy (TCS SP2 system, Leica Microsystems, Wetzlar, Germany) with an argon laser excitation wavelength of 488 nm.

2.5. In planta secretion trap assay for secretion of CaPFR33 in tobacco and pepper

A full-length *CaPFR33* ORF without the termination codon was prepared using PCR amplification with *CaPFR33* cDNA as the template. Two *CaPFR33* gene-specific oligonucleotide primers, 5́-GCGCGCGCTCGAGCGATGGATCGGAAAAATGCT-3́ and 5́-GCGCG CGCGATCGCCCGATCAGCAAGTAGAGGAG-3́, containing *Xho*I and *Sgf*I sites, were synthesized and used to amplify the *CaPFR33* ORF. After *Xho*I and *Sgf*I digestion, PCR-amplified products were purified from agarose gels and inserted into the *Xho*I and *Sgf*I site of the pART-NIPΔ^{SP} plasmid [8].

Nicotiana benthamiana and *C. annuum* cv. ECW plants grown in a greenhouse for 4–6 weeks were used for agro-infiltration experiments. Leaves were infiltrated with *Agrobacterium tumefaciens* strain C58C1 as previously described [14] with the following modification. Bacterial suspensions for infiltration were derived from fresh (1- to 2-d-old) cultures grown on petri plates containing LB media amended with appropriate antibiotics.

For agro-infiltration, suspensions of transformed C58C1 cells were adjusted to an OD_{600} of 0.1–0.2 in 10 mM $MgCl_2$ and 150 mM acetosyringone and maintained at room temperature for 2–3 h. Infiltration was conducted using needleless 1-mL disposable syringes on the abaxial surface of fully-expanded leaves. Sufficient amounts of bacterial suspensions were used to completely infiltrate leaves and give a water-soaked appearance. Plants were then maintained in a growth chamber at 24 °C with a 12/12 h, light/dark photoperiod.

3. Results and discussion

3.1. Yeast secretion trap (YST) screen using pepper fruit cDNAs

To identify secreted proteins that affect pepper fruit development and ripening and to gain molecular insights into these processes, the fruit secretome was characterized with the yeast secretion trap (YST) assay using pepper pericarp cDNAs. A total of 80 non-redundant YST clones (Table 1) was identified and their secretion under the YST system was confirmed by retransforming each plasmid into DBYα2445. The clones obtained from the YST library were designated as *CaPFR*s indicating pepper fruit-related clones.

Since the invertase protein lacked its secretion signal, and the 80 *CaPFR*s fused with invertase were able to complement the yeast mutant strain lacking invertase, the deduced polypeptides of these genes were inferred to include a signal peptide. This was evaluated computationally using SignalP 3.0 [11]. The putative cellular locations of the CaPFR proteins were classified based on the signal

Table 1
Pepper fruit secretome.

Clone name	Protein name	Functional annotation				SignalP HMM	Score (NN)
		SGN No.	GenBank No.	*E*-value	Species		
CaPFR1	Lipid transfer protein	U202926	NP_849837	8.8e-15	*Arabidopsis thaliana*	1	(0.945)
CaPFR2	Prohibitin	U196766	T03843	4e-121	*Nicotiana tabacum*	0.344	(0.42)
CaPFR3	Non-specific lipid transfer protein	U196359	AAF23460	1.4e-53	*Capsicum annuum*	0.492	(0.898)
CaPFR4	Germin homolog	U199898	T07004	2e-45	*Solanum tuberosum*	0.957	(0.885)
CaPFR5	Osmotin precursor	U196407	P12670	3e-137	*Solanum lycopersicon*	0.981	(0.926)
CaPFR6	Unknown	U198877	NP_564256	2.1e-15	*Arabidopsis thaliana*	0.87	(0.955)
CaPFR7	Putative protein disulfide isomerase	U197846	NP_849696	4.7e-39	*Arabidopsis thaliana*	0.965	(0.833)
CaPFR8	Thaumatin-like protein	U196408	AAK97184	1e-136	*Capsicum annuum*	0.99	(0.941)
CaPFR9	Defensin J1-1 precursor	No	Q43413	2e-13	*Capsicum annuum*	0.962	(0.911)
CaPFR10	Putative expansin	U198800	AAM12782	6e-117	*Capsicum annuum*	0.999	(0.917)
CaPFR11	Unknown	No	NP_566460	5e-06	*Arabidopsis thaliana*	0.903	(0.961)
CaPFR12	Hypothetical protein	KS12074E11	No	No	No	0.991	(0.856)
CaPFR13	Hypothetical protein	No	No	No	No	0.911	(0.728)
CaPFR14	Unknown	U196529	NP_911279	8.2e-34	*Oryza sativa*	1	(0.95)
CaPFR15	Pistil-specific protein sts15	KS18039D08	T07677	9e-33	*Solanum tuberosum*	0.999	(0.874)
CaPFR16	Wound-induced protein WIN1 precursor	U196300	P09762	4.3e-94	*Solanum tuberosum*	0.998	(0.922)
CaPFR17	Antifungal protein	U196085	AAL73184	5.5e-43	*Capsicum annuum*	1	(0.969)
CaPFR18	Allergen-like protein BRSn20	U196250	AAF16869	9.2e-40	*Sambucus nigra*	1	(0.964)
CaPFR19	Defensin J1-2 precursor	U198096	O65740	1e-24	*Capsicum annuum*	0.97	(0.934)
CaPFR20	Unknown	U196911	NP_910312	6e-27	*Arabidopsis thaliana*	0.975	(0.713)
CaPFR21	Expressed protein	KS13013H01	No	No	No	0.998	(0.931)
CaPFR22	Putative miraculin	U196573	CAC40756	1.7e-11	*Atropa belladonna*	1	(0.978)
CaPFR23	emp24/gp25L/p24 family	KS26034B01	NP_172429	2e-28	*Arabidopsis thaliana*	0.979	(0.921)
CaPFR24	Arabinogalactan-protein	U196664	AAA66362	4.7e-06	*Nicotiana alata*	0.99	(0.935)
CaPFR25	Proteinase inhibitor I-B	U196517	Q03199	2.7e-40	*Nicotiana tabacum*	1	(0.912)
CaPFR26	HR7	U196169	BAA76516	1.1e-12	Hyoscyamus niger	0.773	(0.753)
CaPFR27	Subtilisin-like proteinase	U200656	NP_569048	6.4e-80	*Arabidopsis thaliana*	0.989	(0.818)
CaPFR28	Expressed protein	KS18006B09	No	No	No	0.999	(0.898)
CaPFR29	Endo-beta-1,4-glucanase	U204800	CAA65827	7.1e-87	*Capsicum annuum*	1	(0.888)
CaPFR30	Putative endomembrane protein 70	No	NP_172919	2e-16	*Arabidopsis thaliana*	0.718	(0.788)
CaPFR31	Lipid transfer protein	U196357	AAB07486	2.8e-44	*Solanum pennellii*	0.998	(0.899)
CaPFR32	Unknown	KS20064H09	G86378	1e-09	*Arabidopsis thaliana*	0.823	(0.741)
CaPFR33	Expressed protein	U198336	No	No	No	0.986	(0.867)
CaPFR34	Invertase inhibitor precursor	No	T07380	4e-08	*Solanum lycopersicon*	0.993	(0.942)
CaPFR35	Fruit-ripening protein	No	J04099	3e-04	*Solanum lycopersicon*	0.252	(0.915)
CaPFR36	Chitinase	U196233	AAF02299	1e-21	*Brassica juncea*	0.979	(0.966)
CaPFR37	Putative nonspecific lipid transfer protein	U196205	JQ2342	8.9e-25	*Zinnia elegans*	1	(0.951)
CaPFR38	Endochitinase precursor	U196327	Q40114	0.082	*Lycopersicon chilense*	0.431	(0.92)
CaPFR39	Putative preprocysteine proteinase	U196350	AAD29084	2e-99	*Solanum melongena*	0.999	(0.884)
CaPFR40	Thionin-like protein	U196050	AAF16413	5.8e-44	*Capsicum annuum*	0.988	(0.93)
CaPFR41	Acidic endochitinase Q precursor	U196327	Q05540	2e-137	*Solanum lycopersicon*	1	(0.957)
CaPFR42	SOUL-related protein	U197351	NP_913279	2.9e-52	*Oryza sativa*	0.997	(0.943)
CaPFR43	Expressed protein	U197385	No	No	No	0.846	(0.901)
CaPFR44	Expansin-like protein	U196809	CAE12163	7e-83	*Quercus robur*	1	(0.956)
CaPFR45	Hypothetical protein	No	No	No	No	0.999	(0.971)
CaPFR46	Hypothetical protein	KS15056G11	No	No	No	1	(0.962)
CaPFR47	Aleurone ribonuclease	U198622	NP_917957	1.9e-16	*Oryza sativa*	1	(0.967)
CaPFR48	Hypothetical protein	No	No	No	No	0.979	(0.926)
CaPFR49	Glycosyl hydrolase family	U199561	NP_177697	8e-05	*Arabidopsis thaliana*	0.571	(0.727)
CaPFR50	Hypothetical protein	No	No	No	No	0.994	(0.943)
CaPFR51	Unknown	No	NP_564669	1e-08	*Arabidopsis thaliana*	1	(0.873)
CaPFR52	Glycine-rich protein Tfm5	U196044	T07381	1.8e-34	*Solanum lycopersicon*	0.998	(0.943)
CaPFR53	U-Lim protein	U196077	AAR83883	2e-23	*Capsicum annuum*	0.998	(0.964)
CaPFR54	Hypothetical protein	No	No	No	No	0	(0.017)
CaPFR55	Hypothetical protein	KS17012B06	No	No	No	0.997	(0.947)
CaPFR56	Unknown	KS01074H03	T04501	3e-05	*Arabidopsis thaliana*	0.884	(0.886)
CaPFR57	Glycine-rich protein TomR2	No	AAP83840	2e-09	*Solanum lycopersicon*	0.996	(0.969)
CaPFR58	Expressed protein	U196025	No	No	No	0.999	(0.936)
CaPFR59	Hypothetical protein	No	No	No	No	0.999	(0.971)
CaPFR60	Hypothetical protein	No	No	No	No	0.999	(0.908)
CaPFR61	Hypothetical protein	No	No	No	No	0.055	(0.727)
CaPFR62	GAST1 protein precursor	U196364	P27057	1.1e-54	*Solanum lycopersicon*	0.998	(0.879)
CaPFR63	Putative peroxidase	U196574	CAC42086	9e-151	*Solanum tuberosum*	1	(0.954)
CaPFR64	Glycine-rich protein	*KS26031H11*	S14977	3e-05	*Solanum lycopersicon*	0.997	(0.961)
CaPFR65	Unknown	U197679	NP_174162	9.6e-05	*Arabidopsis thaliana*	0.999	(0.909)
CaPFR66	Pectin methylesterase-like protein	U198423	BAB09534	1.8e-94	*Arabidopsis thaliana*	1	(0.955)
CaPFR67	Cell division cycle protein 48 homolog	U202536	Q96372	9e-67	*Capsicum annuum*	0	(0.018)
CaPFR68	Hypothetical protein	KS15055F04	No	No	No	0.999	(0.909)
CaPFR69	Hypothetical protein	No	No	No	No	0.988	(0.937)
CaPFR70	Defensin J1-1 precursor	KS18062B04	Q43413	3e-10	*Capsicum annuum*	0.958	(0.904)
CaPFR71	Antifungal protein	KS18042A09	AAL73184	1e-20	*Capsicum annuum*	1	(0.971)
CaPFR72	ABA and stress inducible protein	KS18038H03	Q09134	0.002	*Medicago sativa*	0.997	(0.945)
CaPFR73	Cell wall protein	U196110	AAF63514	3.5e-41	*Capsicum annuum*	0.995	(0.967)

Table 1 (*continued*)

Clone name	Protein name	Functional annotation				SignalP HMM	Score (NN)
		SGN No.	GenBank No.	*E*-value	Species		
CaPFR74	Hypothetical protein	No	No	No	No	0.948	(0.66)
CaPFR75	Vicilin precursor	No	P09799	3e-07	*Macadamia integrifolia*	0.998	(0.93)
CaPFR76	Vacuolar ATP synthase	U197192	Q40585	3.4e-38	*Nicotiana tabacum*	0.936	(0.456)
CaPFR77	Polygalacturonase	KS17025B08	CAA32235	7e-34	*Solanum lycopersicon*	0.998	(0.937)
CaPFR78	Defensin protein precursor	U196271	AAL35366	1.9e-20	*Capsicum annuum*	0.991	(0.982)
CaPFR79	Putative pectate-lyase	U203014	AAM12784	1.1e-29	*Capsicum annuum*	1	(0.975)
CaPFR80	Unknown	U197590	NP_564318	3e-101	*Arabidopsis thaliana*	0.998	(0.931)

sequence information obtained from the YST assays. These classifications included: cell wall/apoplast proteins (9%), ER/Golgi localized proteins (5%), vacuolar proteins (1%), plasma membrane proteins (1%), mitochondrial proteins (1%), nuclear proteins (1%), and unknown for specific target organelle (32%). Of the 80 CaPFR proteins, 77 were predicted to contain N-terminal secretion signal peptides with mean *S* scores >0.48. Our results indicate that the majority of CaPFRs contained signal peptides at their N-terminus enabling their secretion in the yeast system. Two CaPFRs were predicted to localize to mitochondria (CaPFR2) and the nucleus (CaPFR67), and one had no predicted location (CaPFR54).

Several non-classical eukaryotic protein secretion routes have been uncovered through the characterization of proteins that do not have typical cleavable N-terminal signal peptides for targeting and co-translation into the ER and subsequent transport through the endomembrane system [2]. The three CaPFRs lacking N-terminal signal peptides might be secreted through non-classical pathways or might represent false positives since some truncated proteins can exhibit abnormally exposed N-terminal hydrophobic or highly-basic regions that can function to cause secretion as an artifact. Moreover, there are clear instances of dual-targeted proteins in plants [15,16] suggesting potential additional subcellular localizations of CaPFR2 and CaPFR67.

3.2. Functional cataloging of the CaPFRs

Based on the functional annotation by the BLASTX program, the 80 CaPFR proteins were categorized into nine functional groups (Fig. 1). These included cell wall structural proteins (9%), cell wall modifying proteins (13%), proteases/protease inhibitors (4%), defense/stress-related proteins (32%), plasma membrane proteins (1%), ER/Golgi localized proteins (5%), other (4%), unknown (10%), and hypothetical proteins (22%). Glycine-rich proteins (GRPs), arabinogalactan protein (AGP), and U-rim protein were identified in the cell wall structural proteins group. In the cell wall modifying proteins group, there were several ripening-related proteins such as polygalacturonase (PG), xyloglucan endotransglucosylase/hydrolase (XTH), β-1,4-glucanase, pectate-lyase, pectic methylesterase (PME), and expansin [17]. Three lipid transfer proteins were classified in this group that may play important roles in antimicrobial defenses, signaling, and cell wall loosening [18].

Among the nine classes, the largest (32%) represents defense-related proteins. Most are annotated as established pathogenesis-related proteins, such as chitinase, antifungal protein, osmotin, and peroxidase. Of these, defensin, thaumatin-like protein, and thionin-like protein are developmentally regulated during fruit ripening to protect reproductive organs against biotic and abiotic stresses [19,20]. However, their specific roles in fruit biology are still unknown, but many have allergenic properties that must be considered for breeding purposes. The large proportion of defense-related proteins was unexpected as the number of defense-related proteins was much lower in an YST screen of tomato fruit (Lee et al., unpublished data). Pepper is generally considered to be a non-climacteric fruit that does not exhibit a burst of respiration and ethylene production at the onset of ripening, whereas tomato fruit are climacteric and show ethylene-mediated induction of ripening-related genes [21]. We propose that some of these defense-related CaPFRs may have additional roles in non-climacteric ripening.

Hypothetical proteins (22%) sharing no homology with other proteins in current databases and unknown proteins (10%) that are homologous with proteins of no characterized function were also identified. Thus, the YST screen is effective tool to identify novel secreted proteins. Such a large proportion of unknown secreted

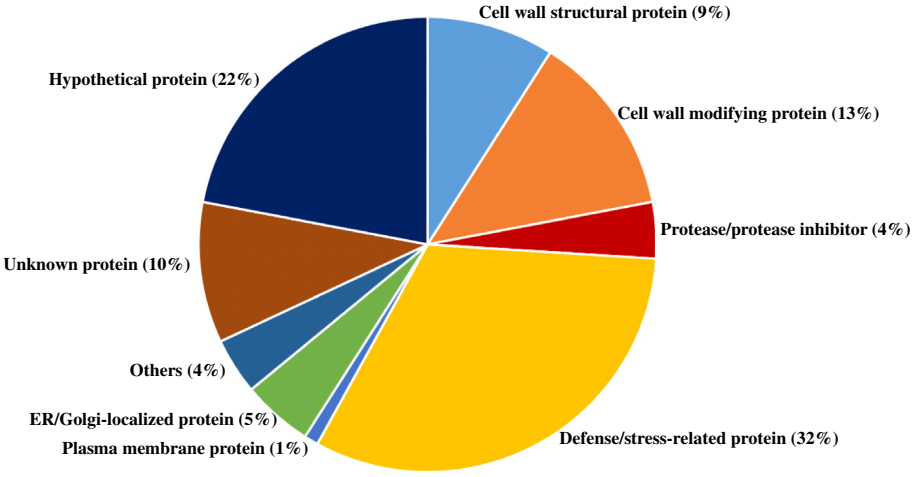

Fig. 1. Functional classification of pepper secretome. YST genes were grouped to nine classes depending on their functions. The functions of all proteins were assumed according to the BLASTP program on NCBI (www.ncbi.nim.nih.gov/blast). The percentage described in each group is in parenthesis.

proteins suggests that mechanisms affecting fruit development and ripening remain to be elucidated.

Several organellar proteins were also identified in the YST screen. ER/Golgi-localized proteins (5%) included *CaPFR7* (protein disulfide isomerase), *CaPFR23* (emp24/gp25/p24 family), and *CaPFR30* (endomembrane protein). Some *CaPFRs* were annotated to encode organellar proteins such as a vacuolar protein (CaPFR76; vacuolar ATPase), mitochondrial protein (CaPFR2; prohibitin), and nuclear protein (CaPFR67; cell division cycle protein 48 homolog).

Approximately 4% of the YST clones were classified as proteases/protease inhibitors. This group contains a cysteine protease (CaPFR39), a subtilisin-like protease (CaPFR27), and protease inhibitor I-B (CaPFR25). Proteases and protease inhibitors in fruit contribute to a wide range of biological processes, including antimicrobial activities and abiotic stress resistance to protect seeds [22].

Among the 80 identified *CaPFR* genes, 23 were able to be positioned in a pepper genetic map [23] relative to frame markers as previously described (Supplemental Fig. S1). This map information will be useful for prioritizing candidate genes with fruit-related traits.

3.3. Gene expression analysis of CaPFRs

The expression patterns of 16 *CaPFRs* during fruit development and ripening were monitored by Northern blot analysis, resulting in the identification of distinct expression patterns (Fig. 2). Based on these patterns, the genes were grouped into four classes: consistent expression, ripening-stage specific expression; abundant expression in the breaker-stage, and early-developmental stage-specific expression.

In the case of *CaPFR46,* a hypothetical protein, the transcripts were detected in all developmental stages of fruit. There was a slight decrease in its transcript level at the immature green stage. However, *CaPFR46* showed consistent expression patterns compared to others.

The expression pattern of *CaPFR28* (XTH) and *CaPFR77* (PG) was ripe fruit-specific suggesting a role in ripening. XTH and PG are cell wall modifying enzymes that are believed to contribute to fruit softening in tomato [17,24]. Thus, *CaPFR28* and *CaPFR77* may similarly play roles in pepper fruit softening. Indeed, a pepper polygalaturonase, the sequence of which corresponds to CaPFR77, has been reported to be a genetic determinant of soft flesh and deciduous fruit in pepper [25].

Several genes, including *CaPFR61* (hypothetical protein), *CaPFR39* (putative preprocystein protease), *CaPFR56* (unknown protein), and *CaPFR23* (protein trafficking-related protein) showing maximum expression at the breaker stage were identified. This stage is the transition point of ripening that includes synthesis and trafficking of new proteins resulting in metabolic shifts [26,27]. These CaPFRs may be involved in this transition to modulate protein homeostasis and changes in fruit metabolism.

Half of the *CaPFRs* examined showed decreased or no expression during ripening. *CaPFR10* (expansin) was only expressed in immature green fruit, at which point cell expansion is very rapid [17]. We presume that *CaPFR10* contributes to changes in cell wall properties that occur during green fruit cell division, expansion, and maturation. The CaPFRs with decreased or undetectable expression following the mature green stage may contribute to establishing fruit shape and size, mechanical support, and/or defense responses.

Studying the differential expression patterns of *CaPFR* clones during different fruit developmental stages was informative for assessing putative functions of the gene products of several clones during the fruit developmental process. Information from this study may contribute to a better understanding of complex extracellular fruit metabolism. Several of the unigenes retrieved by our YST screen may play important roles during pepper fruit ontogeny, particularly those for which no function or homolog was found. However, further investigation of the role of each gene during fruit development is necessary. A comparison of secretome profiles and expression patterns from related genotypes, such as mutants or isogenic lines, may provide critical insight for understanding fruit development and ripening. Detailed functional analyses of these proteins will provide a better understanding of the dynamic biological processes in fruit.Taken together, these results indicated that the YST approach is effective in isolating developmentally-regulated secreted proteins.

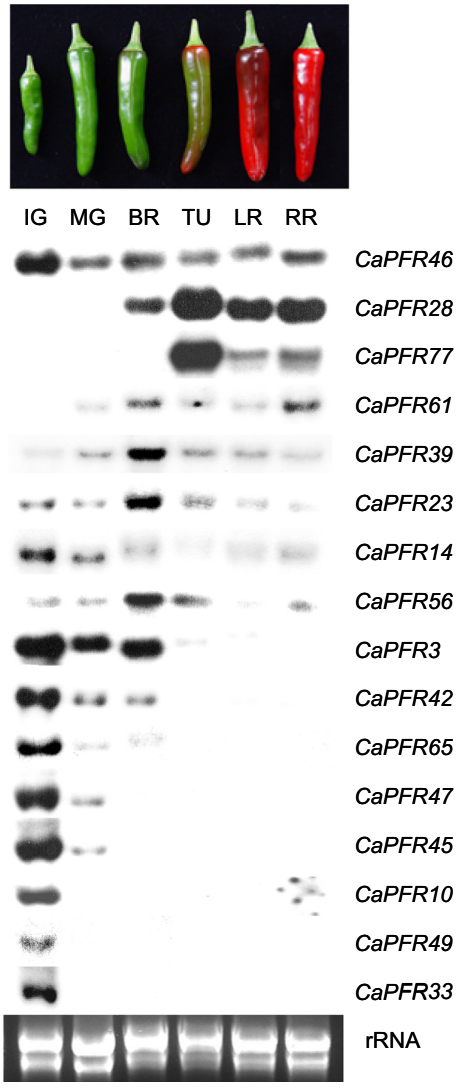

Fig. 2. Northern blot analysis of total RNA isolated from developing and ripening pepper pericarps. Total RNA of 10 μg was electrophoresed on 1.2% formaldehyde agarose gel. Each blot was hybridized with probes from YST clones. IG, immature green; MG, mature green; BR, breaker; TU, turning; LR, light red; RR, red ripe. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Validation of secreted proteins

The YST approach is based on the conservation of signal peptide function among eukaryotic cells. Although the YST identified the presence of signal sequences in the CaPFRs, we wanted to confirm

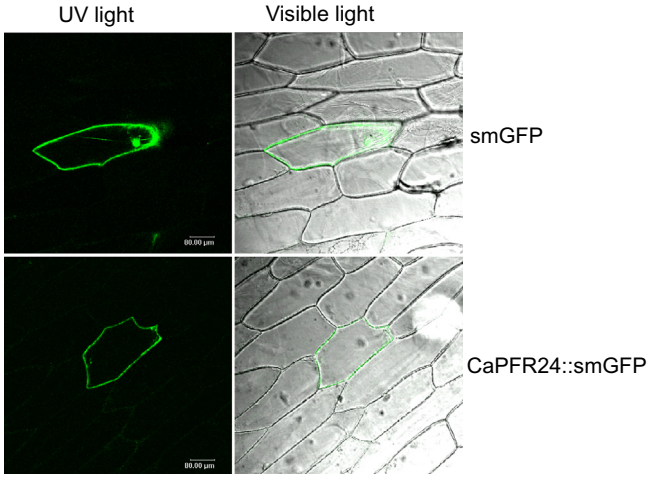

Fig. 3. Subcellular localization of transiently expressed CaPFR24::smGFP fusion protein in onion epidermal cells. The CaPFR24::smGFP fusion and smGFP were introduced into onion epidermal cells by biolistic gene bombardment. The fluorescent signal from GFP fusion protein was monitored 20 h later using confocal laser scanning microscopy. The bar on the bottom of the microscopic image indicates 80 μm.

their secretion *in planta*. To do this, we performed transient GFP fusion protein expression assays in onion epidermal cells and *in planta* secretion trap assays in tobacco and pepper [8].

CaPFR24, a putative arabinogalactan protein (AGP), was selected for this study. *In silico*, this protein contains a 22-amino acid signal peptide at its N-terminal region. AGPs are proteoglycans localized to the plant extracellular matrix that have diverse functions in many biological processes, including cell proliferation and survival, pattern formation and growth, and defenses [28].

After isolating full-length cDNA by RACE, the complete *CaPFR24* cDNA without the termination codon was fused to the soluble-modified green fluorescent protein (smGFP) gene [13]. The resulting construct was introduced into onion epidermal cells. Localization of smGFP and the CaPFR24::smGFP fusion protein was analyzed by fluorescence microscopy (Fig. 3). Following transient expression, the CaPFR24:smGFP fusion protein was mainly detected in the plasma membrane with no nuclear localization. In contrast, unmodified smGFP accumulated both in the cytoplasm and the nucleus. These results suggest that CaPFR24 localized to the plasma membrane and traffics via the secretory pathway.

To confirm the secretion of CaPFR proteins in plant cells, an *in planta* secretion trap assay was employed. pART-NIP$^{\Delta SP}$ [8], containing the necrosis-inducing protein (NIP) [29] without its own signal peptide, was used. The unknown protein CaPFR33 was selected to verify its secretion *in planta*. The complete *CaPFR33* cDNA without its termination codon was fused to the NIP2 gene. The fused gene was transiently expressed in *N. benthamiana* and *C. annuum* using *A. tumefaciens* C58C1 (Fig. 4). *Agrobacterium*-mediated transient expression of NIP lacking its own signal peptides did not induce any visible necrosis on the leaves, whereas transient

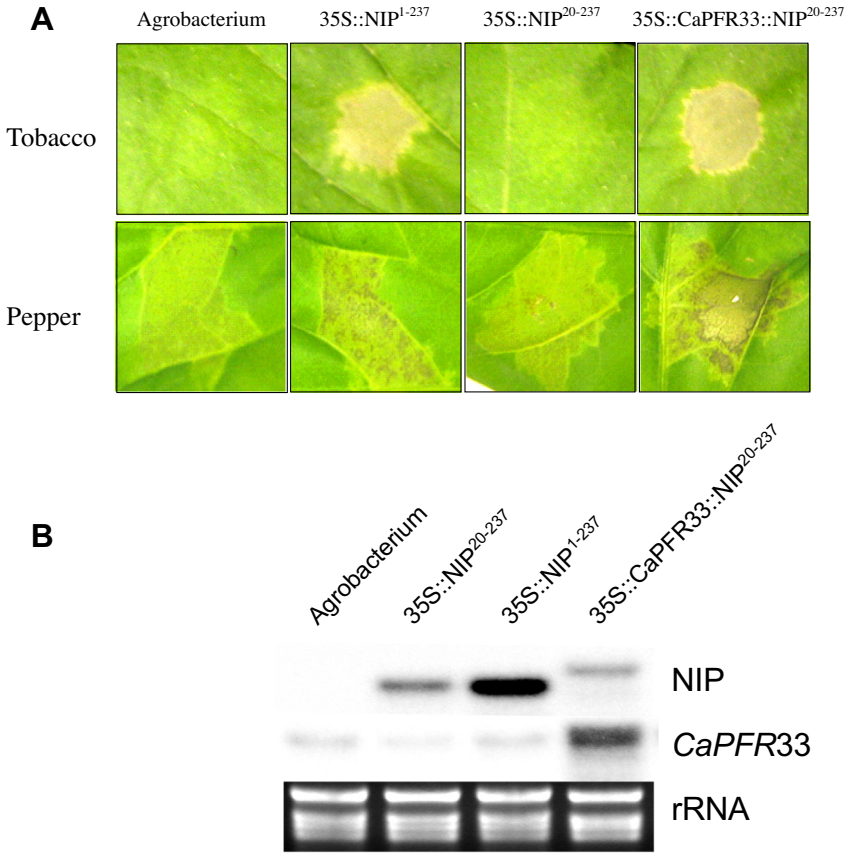

Fig. 4. *In planta* secretion trap. (A) *in planta* secretion trap assay in *N. benthamiana.* and *C. annuum*. pART::NIP [8] was used for *in planta* secretion trap. NIP (Necrosis-inducing protein) [29] without its own signal peptides (NIP$^{20-237}$) was used as a reporter gene. The results were confirmed by at least three independent experiments. (B) Northern blot hybridization of RNA isolated from tobacco leaves that were infiltrated with *A. tumefaciens* C58C1 carrying 35S::NIP$^{1-237}$, 35S::NIP$^{20-237}$, and 35S::CaPFR33::NIP$^{20-237}$. The blot was hybridized with probes from cDNAs of *NIP* and *CaPFR33*. Total RNA was harvested from leaf discs surrounding the inoculation sites immediately after the onset of necrosis.

expression of unmodified NIP showed clear necrosis in both plants. *Agrobacterium*-mediated transient expression of the CaPFR33::NIP$^{\Delta SP}$ fusion protein induced necrosis in both plants, suggesting that CaPFR33 is secreted into the apoplast *in planta*.

The *in planta* secretion trap is a straightforward tool for confirming protein secretion in plants. Subcellular localization using a GFP fusion protein has also been widely used to validate a functional signal peptide, but it is time and labor intensive steps. The limitations were the introduction of the GFP-fused protein into plant cells by biolistic bombardment and the detection of fluorescent signals using a confocal laser scanning microscope. However, the *in planta* secretion trap as a follow-up to mining for secreted proteins using the YST screen provides a powerful tool for facilitating high-throughput screening of the secretome *in planta*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.022.

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