Vesicle Trafficking Patterns in Developing Peanut Fruits Related to *Aspergillus flavus* Resistancy and Development

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Abstract Innate immunity mediated by vesicle trafficking plays important roles in plant defense. In this study, using QPCR and bioinformatics methods, we demonstrated that two different distinctive vesicle trafficking patterns played major function in peanut developing seeds of *A. flavus* resistant strain C20R and sensitive strain TFR. We also found that VAMP726 and RMR were the preferred vesicle trafficking components in *A. flavus* resistant strain C20R, and VSRs VTI1a, b in *A. flavus* sensitive strain TFR. During fruit developmental process, the dynamic transcription tendency of these vesicle trafficking components was consistant to that of sets of genes in the developing seeds of C20R and TFR respectively at whole genomic transcription levels. Thus, we proposed that two vesicle transport patterns existed in C20R and TFR developing seeds respectively, which was related to the expression differences at whole genomic transcription levels in the developing seeds between C20R and TFR. Such differences could begin as early as at the end of protein synthesis and at the entry of ER as the begining of transport pathway.

Key words RMR; VSR; TIP; vesicle trafficking; VAMP726; SNARE; peanut

花生发育果实小泡运输类型与黄曲霉抗性和发育有关

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摘要 小泡运输介导的先天免疫在植物防卫中起重要作用。采用定量PCR和生物信息学的方法, 该研究揭示两种不同的小泡运输类型分别在花生黄曲霉抗性品种C20R和敏感品种TFR发育的种子中起主要作用。VAMP726和RMR是黄曲霉抗性品种C20R中主要的小泡运输组分, VSRs VTI1a, b是黄曲霉敏感品种TFR中主要的小泡运输组分。在果实发育过程中, 这些小泡运输组分的转录动态在整体转录组水平分别与相应的花生黄曲霉抗性品种C20R和敏感品种TFR差异表达的一系列基因表达趋势一致。因此, 我们认为两类不同组合的小泡运输分别在黄曲霉抗性品种C20R和敏感品种TFR果实发育中起着主要运输作用, 与发育中转录组水平基因表达的差异一致。这种差异早在蛋白质合成结束和运输起始阶段就已经显示, 导致果实代谢和发育方向的差异, 造就黄曲霉抗性的不同。

关键词 RMR; VSR; TIP; 小泡运输; VAMP726; SNARE; 花生

In the evolution process, plant developed diverse correspondent fine tuned execution mechanism on the entry route of microbe, to terminate microbial colonization both extrally and intracellularly[1-2]. Vesicles as

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cargo loading with set of original materials for the battle via specific pathway reflect their specific function.

SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) are tetrameric complexes that bringing two lipid bilayer membranes together and matching vesicles with their destinations to deliver specific membrane proteins and soluble cargo[3]. The SNARE complexes contain both target SNARE (t-SNARE) that associated with destination membrane and vesicle SNARE (v-SNARE) associated with vesicles. The components of tetrameric complex are desiginated as Qa, Qb, Qc, and R. *Arabidopsis* has 18 of each Qa, Qb, and Qc, and they were divided into 8 classes (SYP1-8, syntaxins in plants)[3].

The early secretory pathway has changed little in land plant, but the additional SNARE genes associate with increased complexity of multicellularity in green plants. ER/Golgi pathway SNAREs are clustered with Qa (SYP8, 3), Qb (SEC20, MEMB1, GOS1), Qc (USE1, BET1, pSFT1) and R (SEC22). TGN/Endosomal pathway SNAREs are Qa (SYP2, 4), Qb (VTI1), Qc (SYP6, 5) and Q (YKT6, VAMP71, VAMP714). Secretory SNAREs are Qa (SYP13, 112, 124, KNOLLE, PEN1/ROR2), Qb+Qc (SYP33), Qb (NPSN1), Qc (SYP7) and R (VAMP72, VAMP 724, VAMP 727)[4].

SYP1 is a class of plasma membrane-binding proteins. The ternary SNARE complexes formed by AtSYP121, AtSYP122, SNAP33, and vesicle-associated membrane protein VAMP 721 or VAMP 722, play a specialized fusion function in a polarized secretion pathway at position of defense upon attack by fungus[2,5]. SNAP33 and VAMP721/722 can cooperate with plasma membrane syntaxins other than PEN1 and SYP122 in plant growth and development reproduction[5-6]. SYP132 (NbSYP132) from *N. benthamiana* and SYP132 (AtSYP132) from *Arabidopsis* function in accumulation of extracellular PR-1, which is a member of pathogenesis related protein (PRP) family, and SYP132 acts through different ternary SNARE complexes other than VAMP 721 or VAMP722[7]. This indicates existing distinct cellular compartment secretion pathway for specific extracellular components. These components in vesicle pathway all have dual roles in both defense and development[5-7], suggesting that plant reorganizes the cell components economically in defense response[2].

SYP2 from the t-SNARE family probably targets to the vacuole, a major destination of vesicle trafficking[3]. From trans-Golgi network (TGN) to vacuole, two major transport pathways exist. They differ at specific sorting signals on TGN and types of cargo transported (lytic and storage). A pre-vacuolar compartment (PVC)/multivesicular mechanism is involved in this process. From PVC, trafficking components can be recycled back to TGN[3]. SYP21 is found in PVC and probably function in targeting to the lytic vacuoles; SYP22/VAM3 locates in both vacuole and PVC, possibly functioning in fusion to tonoplast, affecting both growth and shoot gravitropism[3]. SYP22/VAM3 was reported synergistically interact with VAMP727 to form a complex, together with Qb-VTI11 and Qc-SYP51[8]. SYP22 and VAMP727 co-localized at an endosomal sub domain associated with the vacuolar membrane and on the PVC. Duplication of the VAMP727 can suppress the mutation of *syp22-1*, possibly replace the syp22 or increase the incorporating efficiency of other redundant Qa SNAREs[8]. VTI11 forms SNAREs with SYP2- and SYP5-, while VTI12 forms SNAREs with SYP4- and SYP6-. VTI11 and VTI12 function differently in transport pathways, but they can substitute each other while one of them mutated[9].

Tonoplast intrinsic proteins (TIPs) belong to one of four types of Aquaporins (AQPs)[10]. Different TIPs function on different types of vacuoles. Seed-type protein storage vacuoles (PSV) are marked by α and δ-TIP, which are co-localized on PSV membrane[11], or by α, δ and γ-TIP; vacuoles storing vegetative storage proteins and pigments are marked by δ-TIP alone or δ and γ-TIP; lytic vacuoles marked by γ-TIP alone, while autophatic vacuoles marked by α-TIP alone[12]. δ-TIP marks the vesicles storing vegetative storage proteins in response to wounding and developmental switches affecting carbon and nitrogen sinks[13]. High γ-TIP gene expression was reported associated with cell elongation and/or differentiation[14]. In young cotyledons of *Arabidopsis*,

γ-TIP is associated with intricately folded bulbs in the continuous vacuolar membrane[15]. And protein storage vacuoles in seeds contain matrix, crystalloid and globoid three morphologically distinct regions. The matrix and crystalloid contain storage proteins, whereas the content in globoid are phytic acid crystals, processed proteins and proteases. The globoid surrounded by a unit membrane is marked by vacuolar H^+ -pyrophosphatase (V-PPase), γ-TIP and soluble protein marker for LV. The globoid is the internal LV inside of PSV, a prevacuole compartment (PVC). Thus PSV is a large multivesticular body, containing different compartments with different functions[16-17].

In developing seeds, one vesicle trafficking pathway is mediated by AP1 or EpsinR1-AtVTI11-tSYP21[18-19] through clathrin-coated vesicles (CCV). The cargo exits from TGN, via PVC by CCV, then transported to vacuoles. It can also be transported to PSV to form a large multivesticular body[16-17]. This pathway is a two directional trafficking pathway between TGN and PSV through PVC. AtVSR1 (VTI11) plays a salvage role for recycle of VSR and membrane proteins back to TGN[19-20]. Mutated *atvsr1* seeds accumulate storage protein precursors, and mis- sort the storage proteins to extracellular space[21]. AtVSR1 (VTI11) also play key roles in vascular patterning and auxin transport[9]. The other way is mediated by AtRMR (receptor-like membrane Ring-H2)[11,22]. RMR is co-localized with DIP (dark intrinsic protein, the EST sequence is similar to δ-TIP) in the crystalloid region within the organelle labeled with δ-TIP, and δ-TIP is co-localized with α-TIP on organelle within PSV[10]. In pea, storage proteins are transported to vacuole via a pathway without CCV[23]. In *Arabidopsis*, the storage proteins and their processing proteases are packaged separately in different vesicles in CGN[24]. The storage proteins vesicles contain AtVSR1, while the proteases containing vesicles do not. The storage protein containing vesicles exit from all parts of Golgi bodies but not CGN. Both types of vesicles fuse into prevacuolar multivesicular body (MVB) and the storage proteins are processed in MVB[25]. The formation and functions of PSVs in developing seeds are possibly through multiple different ways by complicated sorting mechanism. And sorting directions of proteins synthesized should be adapted to their function. It is still remains in vague in this area.

In *Aspergillus flavus*-resistant developing seeds of peanut, special structures and chemicals have been formed. Seed coat has thicker cell wall, is less infiltrative to water, and has less and smaller apertures between seed coat cells[26-28]. Resistant peanut fruit shell is rich in lignin and phenoloids, and in middle region has condensed lignified cells, strong and resistant to broken[29]. Substances inhibiting growth and aflaxtoxin production of *A. flavus* in peanut are phenoloids[30-31], tannin[32], and flavonoids. Peanut can also produce phytotoxins, pathogenesis related protein (PRP), and other resistant proteins, including chitinase, proteinase inhibitor etc[33-37].

Previous macroarray study on differential gene expression of developing peanut seed coat from *A. flavus* resistant strain KB153 versus that from sensitive strain JH1012 revealed that a vesicle associated membrane protein similar to *G.Max BF423736* was up regulated in KB153[38]. In this paper, *BF423736* was used to search a cDNA library from developing seed of KB153 strain, and this led to a EST JM18, which was mostly similar to a *Arabidopsis* vSNARE At*VAMP726*. We designed a pair of primers to *JM18*, and examined the expression of *VAMP726* in different parts of the developing fruits from resistant peanut strain KB153 and sensitive strain JH1012 by QPCR. We also demonstrated the expression difference of genes in the pathways of vesicle-transport between resistant and sensitive strain, and provided some hint on function of immune response to *Aspergillus flavus*-resistance and cytology basis for understanding the mechanism of *Aspergillus flavus*-resistance by fruit structure and chemicals.

1 Materials and methods

1.1 Materials

EST sequences were blasted from Genbank database according to similar genes from *Arabidopsis* and known sequence from peanut[39]. Sequence of *JM18* was

from cDNA library of KB153 developing seeds. EST sequences were translated with AUGUSTUS[40]. Peanut materials used for QPCR were from developing small fruits (seed could not be seperated), fruit shells (seeds could be moved), seed coats 1 (white), 2 (red), young leave of cultivar KB153 (*Aspergillus flavus*-resistant) and JH1012 (*Aspergillus flavus*-sensitive)[38]. Sets of ESTs data collected from Genbank[41] are from peanut cultivar 'Tifrunner' (TFR) (susceptible to *Aspergillus* infection with higher aflatoxin contamination and resistant to TSWV) and 'GT-C20' (C20R) (resistant to *Aspergillus* with reduced aflatoxin contamination and susceptible to TSWV). The developmental stages of C20R and TFR seeds were R5 (beginning of seeds), R6 (full seeds) and R7 (beginning maturity).

1.2 Methods

RNA extraction used modified CTAB method[42]. The first cDNA chain was synthesized with high efficiency reverse transcriptase PrimeScript. The reaction mix contained RNA 8.75 μL, P3 primer (10 pmol/L) 5μL (mixed and heated at 60 °C for 10 min), 5×RT buffer 4 μL, dNTPs Mix (each 10 mmol/L) 1 μL, PrimeScript 0.75 μL, Rnase inhibitor 0.5 μL. 42 °C reacted for 40 min, then 70 °C 15 min to stop the reaction.

Quantitative PCR was conducted using SYBR Green Real-time PCR Master Mix QPK-201 (ToYoBo) and Rotor-Gene 2000 (Corbett Research). The reaction mix contained ddH_2O 4.4 μL, *JM 18* forward and reverse primers each 0.2 μL, DNA template 0.2 μL, and SYBR® Green PCR Master Mix 5 μL. The reaction process for *JM* 18 was: 95 °C 90 s, into cycle, 95 °C 25 s, 49 °C 30 s, 72 °C 20 s for 45 repeat, and 57~99 °C hold for 5 s. Threshold was 0.02. The reference gene was peanut actin cDNA (GenBank: GO340080.1). The reaction process for *Actin* is: 95 °C 90 s, into cycle, 95 °C 25 s, 54 °C 30 s, 72 °C 35 s for 40 repeat, and 57~99 °C hold for 5 s.

Primer sequence of *P3* was: 5′-CGC GGA TCC TTA GTA CAA GAA AGT GGG TTT TTT TTT TTT TT TTT VN-3′, *JM18* forward primer was: 5′-TTC TCT TCA TAC TTA-3′. *JM18* reverse primer was: 5′-AGC GAC CTC TGG TTC TGC-3′. *Actin* forward primer was: 5′-CAC ACA CAT TCC CCG TTT-3′, *Actin* reverse primer was: 5′-CAC CGT CTC CAG AGT CCA-3′.

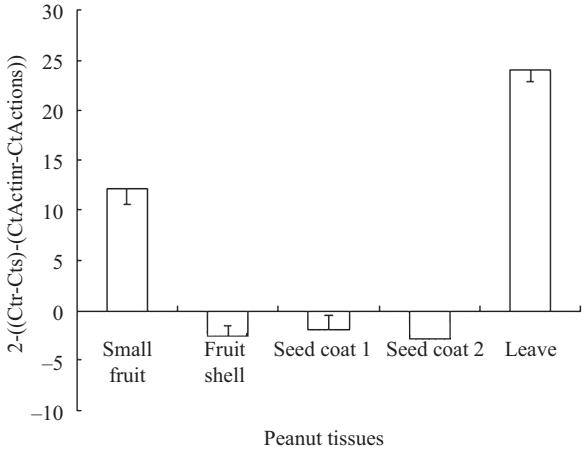
The software GENEDOC version 2 (1989) was used for sequences comparison. MICROSOFT OFFICE EXCEL (2003) was used for expression patterns assay on transcriptoms from C20Rs and TFRs, and the data were from Guo *et al* [41] and genbank.

Calculation[43] for QPCR: $2^{-((Ctr- Cts)-(CtActinr -CtActins))}$, (in which Ct represents quantitative fluorescent PCR relative value, r and s represent resistant and sensitive) showed in Fig.1 as: IF (N>1, N, -1/N); standard deviation in Fig.1 as: $M=2^{-((STDr- STDs)-(STDActinr -STDActins))}$, IF(M>1, M, -1/M). All data were from averages of three repeats of Ct value. MICROSOFT OFFICE EXCEL (2003) was used for this. For each stage of each material, there were three repeats of RNA samples.

2 Results

2.1 Main SNAREs and vesicle sorting proteins in the developing seeds show differences in the C20R and TFR

The results of QPCR on *AhVAMP726* revealed that the *VAMP726-like* gene was more up regulated in the



VAMP726 has higher expression in small fruit, leave of *A. flavus* resistant cultivar KB153. The data was calculated from Ct values according the formulas described in methods. Ctr: Ct of QPCR value for sample from resistant cultivar. Cts: Ct of QPCR value for sample from sensitive cultivar.

Fig.1 Differential expression of *VAMP726* in different parts and stages of *A. flavus* resistant and sensitive peanut cultivar KB153 and JH1012

Table 1 Expression patterns of SNAREs in different peanut cultivars related to *A. flavus* resistance

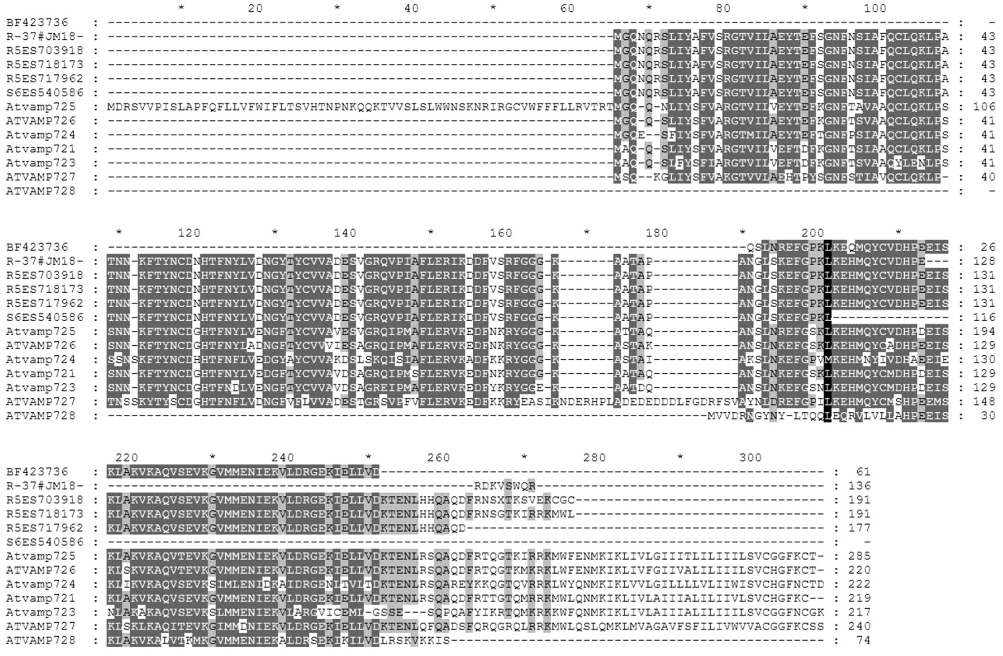
	R(v)-SNAREs				tSNAREs		TIPs		
Cultivars & Stages	VAMP726	VSR1	VSR3	RMR	SYP22	SYP132	α-TIP	δ-TIP	γ-TIP
TFR5	*ES540587*	***ES711701**** *ES721198* *ES721302*	*ES721764*		*ES709034* *ES710276*		8	4	0
TFR6	*ES540586*	***ES714818***	*ES723921* *ES714797*		*ES712573*		8	2	2
TFR7		***ES724326***				*ES724229*	6	0	0
C20R5	*ES718173* *ES717962* *ES703918*	***ES718366***		*ES717644*	*ES717852*		6	1	0
C20R6							1	0	0
C20R7					*ES707916*		4	0	0
KB153	*JM18*								

*Bold marks the same sequences.

small fruit stage of the resistant strain KB153 than that of the sensitive strain JH1012 (Fig.1).

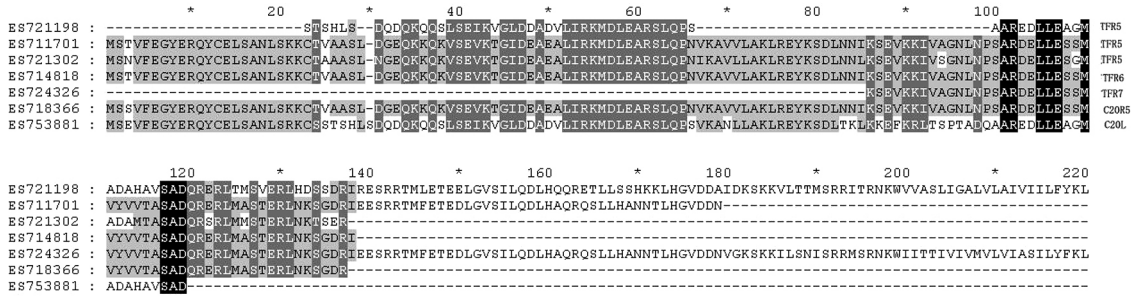
Similar ESTs expressed in developing seeds of *A. flavus* sensitive strain TFR and *A. flavus* resistant strain C20R obtained by blasting with sequences of *JM18* (*VAMP726*), *VTI1s* from *G.Max*, *AtVSR* and *RMR* (*AF218807* and *AF218808*) are listed in Table 1. Expression patterns of *ahVAMP726* and *VTI1s* in C20R5 and TFR5 are opposite. Among three stages of C20Rs, only in stage 5, three ESTs of *ahVAMP726* were detected, while among three stages of TFRs, only none coding *ES540587* in TFR5 and one coding *ES540586* in TFR6 similar to *VAMP726* were detected (Table 1, Fig.2).

For *VTI1*(*VSR1*), only one EST of *VTI1* was detected in seed stage 5 of C20R, none in stage 6 and 7 of C20R seeds, while three EST of *VTI1s* were found in TFR5, and one was detected in stage 6 and 7 respectively (Fig.3).



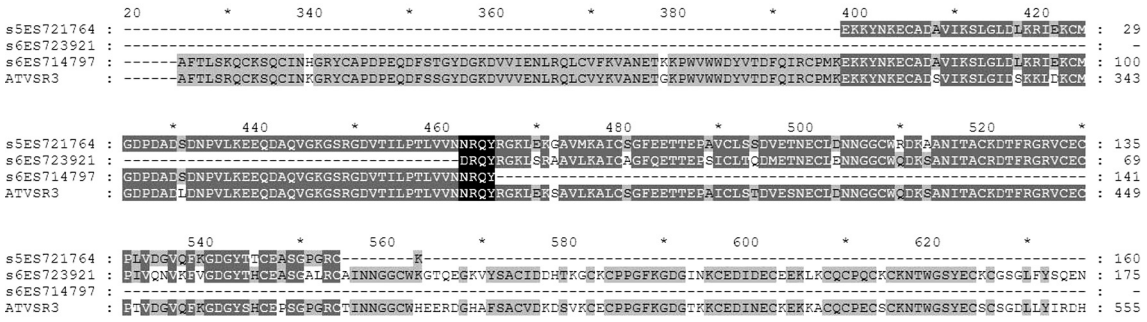
Only *ES540587* (not shown) from TFR5 can not be translated into functional VAMP. Other VAMP726s are highly conserved. R- from resistant cultivar, S- from sensitive cultivar.

Fig.2 Comparison of protein sequences between VAMP726s from different peanut cultivars



ES711701 from TFR5, ES714818 from TFR6, ES724326 from TFR7 and ES718366 from C20R5 are conserved, but ES723102 and ES721108 have differences from others at multiple positions, and they are more similar to VSR1 (ES753881) from leave.

Fig.3 Comparison of protein sequences of VSR1 from peanut developing seeds of *A. flavus* resistant and sensitive cultivars



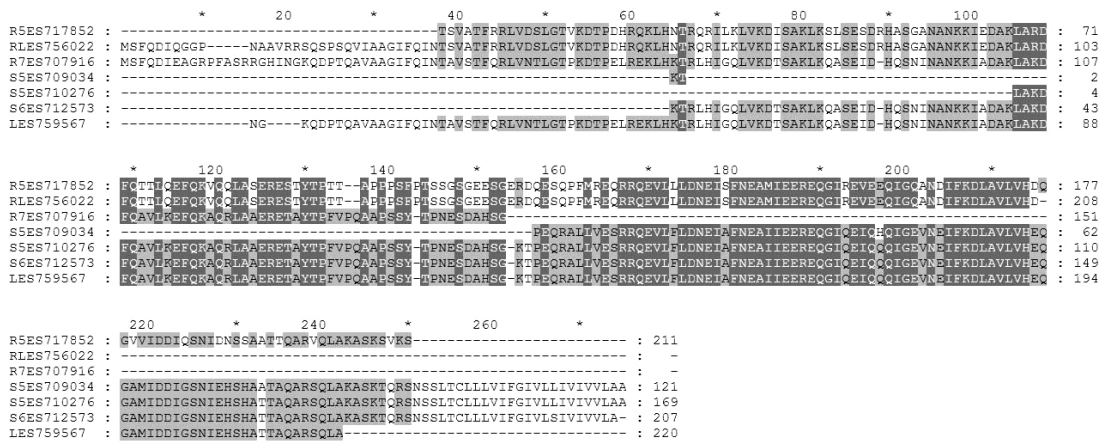
The sequence ES723921 from TFR6 is different from ES721764 expressed in TFR5. S5, 6, 7: from stages 5, 6, 7 of sensitive cultivar; R5, 6, 7: from stages 5, 6, 7 of resistant cultivars.

Fig.4 Comparison of protein sequences of VSR3 from peanut developing seeds of *A. flavus* resistant and sensitive cultivars

One EST of the same type of *VSR1* are found in TFR5, 6, 7 and C20R5 (Table 1, bold marked ESTs Fig.3). The sequence of EST *ES721198* of *VSR1* from TMSV resistant but *A. flavus* sensitive cultivar TFR5 is the same as that of *ES753881* from the leaf cDNA of C20R, which is *A. flavus* resistant. One additional EST of *VSR1* and two ESTs of *VSR3* were detected in stage 5 and 6 of TFR seeds, none in C20R seeds (Fig.4, Table 1). The sequences of two ESTs of *VSR3* in TFR5 and TFR6 are different (Fig.4). In C20R5, besides the only one *VSR1*, there is an *AtRMR* similar EST *ES717644*, which is only detected in C20R5. Total five v(R)-type SNAREs were detected in stage 5 of developing seeds of leaf spot resistant but *A. flavus* sensitive cultivar TFR and the same number of v(R)-type SNAREs were detected in all stages of *A. flavus* resistant but TMSV sensitive cultivar C20R, but the major type of v-SNARE are different. In C20R5, three ESTs of *VAMP726* are detected, while in TFR5, three ESTs of *VSR1s* are detected. Since different types of v-SNAREs may reflect different transporting mechanism, thus the expression patterns of v-SNAREs indicated possible different vesicle trafficking mechanisms function between developing seeds of cultivars C20R and TFR. In C20R, the major pathway is mediated by VAMP726, and in TFR, the major pathway is mediated by VSR1 and VSR3.

2.2 Syntaxin mRNAs are differentially expressed in late seed developing stage 7 of C20R and TFR

Two and one ESTs of t-SNARE *SYP22* were detected respectively in TFR5 and 6 developing seeds. There is one more *SYP22* EST expressed than that in both C20R5 and 6. But in stage 7, only one *ES707916* expressed in C20R7, while none in TFR7, and the protein sequence is similar to that of SYP22 from TFR5 and 6 and one types of SYP22 from leaf (Table 1). The protein encoded by *ES717852* from C20R5 is different from others, and it is the only SYP22 in C20R5, which is similar to another type of SYP22 from leaf (Fig.5). *SYP22/VAM3* was also up regulated in KB153[43]. Conversely, an EST of *SYP132* was found only at stage 7 of



ES717852 in C20R5 is different from other SYP22s in both TFR and C20R. S5, 6, 7: from stages 5,6,7 of sensitive cultivar; R5, 6, 7: from stages 5, 6, 7 of resistant cultivars.

Fig.5 Amino sequences comparison of SYP22 from peanut developing seeds of *A. flavus* resistant and sensitive cultivars

A. flavus sensitive cultivar TFR, not in any stage of *A. flavus* resistant strain C20R or other stages of *A. flavus* sensitive strain.

The ESTs of *SYP132* and *SYP124* were also found in developing seed of VBL6. In Luhua14 developing seed, another type of syntaxin *SYP121* was found. SYP1 family was reported targeting to plasma membranes, and related to defense in leaf. TFR is a strain with resistance to TSWV. Possibly the expression of *SYP132* in TFR is a storage protein storing for later seedling defense function.

2.3 mRNAs for transport intrinsic membrane proteins are differentially expressed in developing seeds of C20R versus TFR

The numbers of ESTs of α, δ and γ-*TIP* from developing seeds of TFR and C20R were listed in Table 1. α-*TIP* ESTs are expressed at higher level in TFR developing seed than that of C20R in stages 6 and 7. It is obvious that α-*TIP* is expressed at lower level in C20R6 than that in C20R5 and C20R7, with highest expression in C20R5, while developing seeds TFR5 and TFR6 have same high α-*TIP* expression level, and at stage 7 still keep high. δ-*TIP* and γ-*TIP* have not been detected in stage 7 of both TFR and C20R, which is a fully matured stage, and not in C20R6, which possibly indicates the earlier maturation of the *A. flavus* resistant seeds. More TIP ESTs were found in stage 5 and 6 in TFR developing seeds than those in C20R developing seeds at the same stages (Table 1). It has been known that α-TIP mainly marks vesicles with seed storage proteins, δ-TIP marks vesicles with vegetative storage proteins and γ-TIP marks LVs. TIPs mark the same way inside the multiple vesicle body. The lack or less of δ and γ-TIP expressed in C20R developing seeds indicates that the less growth activities happened in developing seed of *Aspergillus* resistant cultivar C20R than that of *Aspergillus* sensitive cultivar TFR.

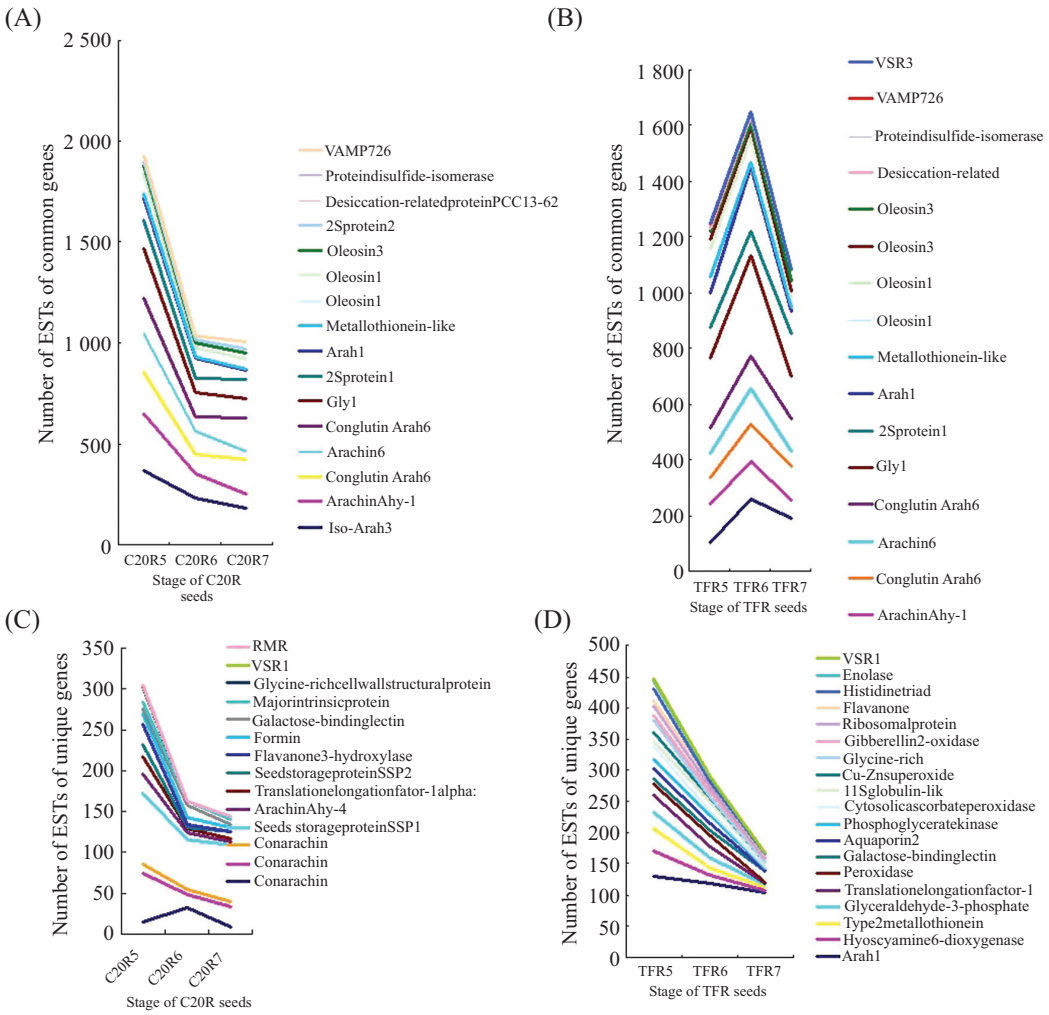
2.4 Expression patterns of highly expressed genes in C20R and TFR developing seeds are consistent with those of vesicle trafficking related genes in the same cultivars

Expression patterns of genes with high expression level during seed developing process in C20R and TFR show the different tendency with time (Fig.6, data are from Guo *et al*.[41]). For genes of most storage proteins expressed in both C20R and TFR developing seeds (here we call them common genes), the highest expression level in C20R seed is at stage 5, and the highest expression level in TFR seeds is at stage 6 (Fig.6A, 6B). The peaks of storage proteins in C20R5 correspond to the higher *VAMP726* expression in C20R5 seed (Fig.6A). In TFR developing seeds, the only EST *ES540587* in TFR5 is not a coding sequence, and possibly is a negative regulation sequence as a miRNA suppressor to repress the level of *VAMP726* mRNA. Thus the expression pattern of functional VAMP726 mRNAs is the same as

that of storage proteins in TFR developing seeds. This indicates that VAMP726 is the major R-SNARE response for sorting of storage proteins expressed in both C20R and TFR seeds (Fig.6B). Besides, VSRs are major R-SNAREs expressed in TFR seeds. The expression pattern of VSR3 in TFR seeds is also similar to that of storage proteins (Fig.6B), implicating that VSR3 is also possibly involved in storage protein sorting in TFR.

Most unique genes in TFR and C20R encode enzymes or other functional proteins. The tendency of genes expressed with the time reflects the synthesis, usage, or dilution of the genes during the development, as well indicates the patterns of vesicle trafficking pathway. The expression patterns of most unique genes in C20R show similar patterns as those of common genes in C20R seeds (Fig.6C, 6A), only the expression pattern of one conarachin gene is the same as the common genes in TFR (Fig.6C, 6B), and those of two conarachin genes are similar as those of unique genes in TFR (Fig. 6C, 6B). Uniform pattern of common genes and unique genes in C20R suggests that in C20R, the trafficking of both common proteins and unique proteins with specific function in C20R are transported via the same major vesicle trafficking pathway mediated by VAMP726 (Fig.6A, 6C).

Through the developmental stages, the time course of expression patterns of most unique genes in TFR seeds have distinct linear down pattern except two stor-



A,B are common genes expressed in C20R and TFR respectively; they are storage proteins. In C20R, R-SNARE VAMP726 has the same expression pattern as that of common proteins. In TFR, VSR3 has also the same patterns as that of common proteins. C,D are unique genes expressed in C20R and TFR respectively. In C20R, R-SNARE VAMP726 has the same expression pattern as that of unique proteins. More storage proteins in C20R unique proteins. In TFR, R-SNARE VSR1 has the same expression pattern as that of unique proteins. Most of TFR uniqie proteins are belong to enzymes involved in metabolism.

Fig.6 Patterns of EST numbers during seed development in TFR and C20R

age proteins Arah1 and arachin Ahy-4 (Fig.6D). The linear down pattern has the similar tendency as expression pattern along the time course of major R-SNARE VSR1 genes, which is 3, 1, 1 at stage 5, 6, 7 respectively, suggesting that VRS1 is the major R-SNARE mediating traffic of unique type of proteins in TFR seeds (Fig.6D). The proteins with unique types in TFR are mostly enzymes or proteins involved in metabolism, possibly involved in morphogenesis of seeds. In C20R, the unique genes have the similar expression pattern as those of VSR1 and RMR (Fig.6C).

The two different expression patterns for unique genes and common genes respectively in TFR developing seed reveals that there are two distinct types of vesicle trafficking pathway. VSR1 mainly function in the unique genes as linear down pattern, which could involve in seed growth. It could also function partially in storage protein transporting. VAMP726 and VSR3 could play major roles in transporting of storage proteins in TFR seeds.

For tSNARE *SYP22*, there are 2, 1, 0 ESTs detected in seeds of TFR5, 6, 7 stages respectively (Fig.6), and they can be translated into functional proteins. The pattern is the same as those of unique proteins in TFR seeds, indicating that SYP22 was tSNARE component interacting with VSR1 in TFR developing seeds.

In C20R, the expression pattern of functional ESTs of *SYP22* in developing C20R is 1, 0, 1 in stage 5, 6, 7 respectively, and it is similar to the patterns of common storage protein genes and unique ESTs in C20R developing seeds (Fig.6), suggesting that SYP22 possibly interact with VAMP726 for major vesicle traffic in developing process of C20R seed. *ES717852* EST encoded protein is different at multiple place with all other SYP22 proteins in TFR and C20R7, and is specific in C20R5. This specificity could possible related to peak of ESTs in C20R5 (Fig.5). Two types of SYP22 are similar to two types of SYP22 from C20R leaf respectively (Fig.5).

δ and γ-TIP were reported as specific TIPs located on vegetative and lytic types of vacuoles respectively, which probably function in usage and redistribution of the proteins. In TFR developing seeds, both expression patterns of α and γ-*TIP* genes are similar to that of common genes, while δ-TIP expression is possibly related to expression patterns of unique genes (Table 1). α and δ-TIP genes have similar expression patterns as both common and unique genes in C20R seed development.

The colligations of all the same types of transcrips along the seed development time courses of common and unique genes, vesicle transport related SNARE and TIPs genes in C20R and TFR exhibit two types of transcription patterns with development process. These two patterns is possibly corresponding to two different combination of vesicle traffic complex components VAMP726-SYP22 in C20R and VSR1-SYP22 in TFR. The higher ratios of transcripts in C20R to TFR (storage proteins (1.41/0.48), system regulation on interaction with environment (0.56/0.33), energy (2.41/1.63), protein fate, protein synthesis, cellular transportand transcription) are possibly related to more vesicle traffic supported by VAMP726. The higher ratios of transcripts in TFR to C20R (development, cell cycle and DNA processing, metabolism,cell rescue, some types of defense and virulence) are possibly related to higher level of VSRs, SPY22 and SYN132, which supporting different vesicle traffic pathway in TFR. While in TFR, VSR1-SYP22 could be the major types of vesicle traffic combination, mainly functioning in growth activities.

3 Discussion

Vesicle traffickings are important and complicated intracellular events that mediate development as well as defense processes. Different combination of complexes of SNAREs, VSRs and other components transport different cargos to different destinations. These vesicles usually contain multiple types of molecular cargos, and they function in multiple aspects of cell activities. For example, PEN1 function in both development and pathogenic defense[44]. *A. flavus* resistant and sensitive peanut cultivars have remarkable structural differences in their fruit and seed coat structures[27-28]. The expression patterns of genes between

TFR and C20R through seed developing stages are different[41], including the total numbers of ESTs, the types of ESTs in each stage, the ratios of genes expressed in certain function categories, and specific metabolic or cell activities. All these differences can be related to differences in vesicle trafficking.

3.1 Differences in vesicle trafficking proteins exist in the TFR versus the C20R developing seeds

In the *A. flavus* sensitive TFR and *A. flavus* resistant C20R strains, distinct sets of vesicle sorting proteins exist in TFR (VSR3) and C20R (RMR) (Table 1). In addition, VAMP726 is the major V(R)-SNARE in C20R developing seeds, but not in the TFR developing seeds. VSRs are the major R-SNAREs in the TFR developing seeds. The expression pattern of VAMP726 and VSR3 in both C20R and TFR seed developing process has the same tendency as storage proteins (Fig.6A, 6B) respectively, indicating that VAMP726 and VSR3 are major R-SNAREs function in storage proteins sorting and transporting. VSR1 has the same pattern as ESTs related to metabolism in TFR seed development (Fig.6D), while in C20R, expression pattern of both VSR1 and RMR are the same as ESTs of unique type of storage proteins and ESTs related to metabolism. This indicates that VSR1 is the major R-SNARE involved in metabolism and growth (Fig.6). In TFR and C20R, major vesicle sorting pattern are different. This also indicates that in early seed developmental stage, *A. flavus* resistant cultivar C20R develop its defense system earlier than *A. flavus* sensitive cultivars TFR with some types of storage proteins, such as trypsin like (Arah3/4). This result can explain that some *A.Flavus* resistant cultivars have smaller seeds, but some *A.Flavus* sensitive cultivars have larger seeds.

3.2 Higher number of genes expressed in stage 5 and 6 of TFR than in C20R is corresponding to higher *VSR1s* and three types of *TIPs* expressed in TFR

At developing early and middle stages 5 and 6, much more genes expressed in TFR seeds than that in C20R seeds. Total number of EST ratio of TFR/C20R for stage 5 is 7 104: 5 184 (1.37), for stage 6 is 4 800:2 304 (2.08). At stage 7, similar expression levels were detected in TFR and C20R as: 2 304: 2 496 (0.92), and C20R is slightly higher[41]. This pattern is similar to that of various *TIP* expression patterns among two strains with slightly difference at stage 7: for α-*TIP* the ratio of TFR/C20R is 8:6 (1.33) at stage 5, 8:1 at stage 6 (8), and 6:4 (1.5) at stage 7 (Table 1). This reveals that α-*TIP* involved vesicle trafficking is major cellular activity in seed developing process. For δ-*TIP*, more ESTs in TFR5 and 6 than C20R5 and 6, show the pattern as *VSR1* does (Fig.6D). For γ-*TIP*, only two ESTs are found in TFR6, none is found in all other stages and strains. The pattern is also consistent with the pattern of *VSR3* and *VAMP726* EST in TFR seed development (Fig.6B). This reveals that vesicle transporting is a very important process in both morphogenesis and defense during seed development, because more TIPs indicates more water transport and thus may relates to more growth activities.

3.3 The different vesicle trafficking pathways in TFR and C20R developing seeds is related to corresponding different set of gene products

Not only the total numbers of total ESTs expressed in each stage between TFR and C20R developing seeds are different, but also the types and ratio of genes in certain types of functional categories are different. The sets of genes expressed in TFR and C20R are different at genomic level[41]. In each stage, there are more unique genes expressed in TFR and C20R than common genes[41]. The storage proteins (1.41/0.48), system regulation of interaction with environment (0.56/0.33), and energy (2.41/1.63) related genes in C20R developing seed are expressed at significantly higher ratio than in TFR, the expression levels of genes in the categories of protein fate, protein synthesis, regulation of interaction with cellular environment, cellular transport and transport mechanism, transcription are also slightly higher in C20R than in TFR. The higher level of genes products are possible related to the higher traffic pattern supported by VAMP726. The level of genes expressed higher in TFR in categories of development, cell cycle

and DNA processing, metabolism, cell rescue, defense and virulence[41] are possibly related to higher VSRs and SYN132 pattern in TFR. This demonstrated that the cellular activities in C20R and TFR have different preference.

In addition to specific targeting by different combination of SNARE proteins, at the end of protein synthesis and beginning of packing of proteins, the specific vesicle transport path could have been marked by specific SR and RPL41 in related to specific microtubules[45-46], thus initiating specific vesicle transport pathway.

Early stage of peanut developing fruit is important for determination of both structure and defense abilities. C20R tends to synthesis of more storage proteins with defense ability, but TFR tends more towards metabolism for growth, thus the set of functional proteins and other components are different. It has been reported that the wax and curtin are major resistant components in seed coat, *A. flavus* resistant strain has higher content of wax and curtin than sensitive strain in seed coat[27], and seed coat structure in resistant strains has smaller pores, more denser package between cells and more regular shape of cells with stronger connection between them[28]. Enzymes and cellular transport activities are possibly different because of these differences.

The specific vesicle transport pathway could package specific combination of proteins and other contents for specific developmental purpose, such as fruit development with stronger disease resistant property but in the value of less growth activity. As for the contents were packaged inside this specific vesicles, they could be clustered by partial common RNA sequences (Yan, unpublished results), function in similar protein synthesis machines or protein signals, and cause the cluster package into the same vesicle. There may be more varied mechanisms in clustered package into same vesicles. All these possibilities need to be further studied. Elucidating the mechanism of vesicle packaging and transporting will help understanding variation in different cultivars with different property, and provide the references for breeding in crops.

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