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10. The primer dropping method [H. Wong, W. D. Anderson, T. Cheng, K. T. Riabowol, *Anal. Biochem.* **223**, 251 (1994)] was used to amplify genomic DNA by PCR. Forty cycles of 94°C (1 min), 60°C (1 min), and 72°C (1 min) were used to amplify *lacZ* with the following primer pair: 5′-TTG GAG TGA CGG CAG TTA TCT GGA and 3′-TCA ACC ACC GCA CGA TAG AGA TTC. After 20 cycles, primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5′-CGG AGT CAA CGG ATT TGG TCG TAT and 3′-AGC CTT CTC CAT GGT GGT GAA GAC) were added as an internal control.
11. BM cells (22,500 cells/ml in Iscove's modified Dulbecco's medium and 2% heat-inactivated fetal bovine serum were added to MethoCult (Stem Cell Technologies, Vancouver, BC, Canada), supplemented with the appropriate cytokines, plated on 35-mm dishes, and incubated at 37°C in a 5% CO_2 atmosphere. Cytokines used were the following: interleukin-3 (IL-3) (10 ng/ml), IL-7 (10 ng/ml), stem cell factor (50 ng/ml), erythropoietin (3 U/ml; R&D Systems), and IL-6 (10 ng/ml; Novartis).
12. X-Gal working solution [5 mM $K_3F_3(CN)_6$, 5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 2 mM $MgCl_2$ (Sigma)] and X-Gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside; 1 mg/ml; in dimethyl sulfoxide; Molecular Probes) in phosphate-buffered saline (PBS) (pH 7.4) were added to methylcellulose cultures for 8 hours at 37°C.
13. We used splenic cell suspensions prepared by grinding minced organ between frosted Corning slides, BM flushed from femurs using EBSS, and whole blood isolated in 20 mM EDTA. Erythrocytes were lysed with 144 mM NH_4Cl and 17 mM tris-HCl (pH 7.2). Cells were rinsed with fluorescence-activated cell sorting (FACS) buffer (EBSS and 1.0% HIFBS), and 1×10^6 cells were added to 100 μl of FACS buffer supplemented with the appropriate primary antibodies and incubated at 4°C for 30 min. After washing, secondary antibodies were added (where appropriate) and incubated at 4°C for 30 min. For biotinylated antibodies, isotype controls were used to set gates; otherwise, gates were set with cells alone. Cell viability was greater than 95%, by propidium iodide exclusion. Flow cytometric analysis was performed with a FACScan (Becton-Dickinson) with all events gated on the forward and side scatter.
14. Clones AF6-88.5 (H-2K^b) and SF1-1.1 (H-2K^d) (PharMingen) were used.
15. Clones 145-2c11 (CD3e), 1D3 (CD19), and M1/70 (CD11b) (PharMingen) were used.
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19. Single- and triple-label immunocytochemistry was performed as previously described (2) with antibody to nestin, monoclonal antibody to type III β-tubulin (Sigma), glial fibrillary acidic protein antisera (Incstar), and monoclonal antibody to O4 (immunoglobulin M; Boehringer Mannheim).
20. We thank M. Dicay, L. Robertson, D. Schmidt, G. Gobbel, D. Spencer, R. Dawson, and P. Hill for technical assistance; E. Cattaneo and R. McKay for antibody to nestin; and J. Dick for critical assessment of this work. Supported by the Spinal Cord Society, Fergus Falls, MN, and by Comitato Telethon (grant A.116) to A.L.V.

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REPORTS

A Tobacco Syntaxin with a Role in Hormonal Control of Guard Cell Ion Channels

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The plant hormone abscisic acid (ABA) regulates potassium and chloride ion channels at the plasma membrane of guard cells, leading to stomatal closure that reduces transpirational water loss from the leaf. The tobacco *Nt-SYR1* gene encodes a syntaxin that is associated with the plasma membrane. Syntaxins and related SNARE proteins aid intracellular vesicle trafficking, fusion, and secretion. Disrupting Nt-Syr1 function by cleavage with *Clostridium botulinum* type C toxin or competition with a soluble fragment of Nt-Syr1 prevents potassium and chloride ion channel response to ABA in guard cells and implicates Nt-Syr1 in an ABA-signaling cascade.

The size of stomatal guard cells in higher plant leaves is rapidly reversible and is crucial to maintaining the hydrated environment within the leaf. In dry conditions, guard cells respond to the hormone abscisic acid (ABA) to regulate plasma membrane K^+ and Cl^- channels which facilitate solute efflux. The concurrent decrease in turgor and cell volume closes the stomatal pore to reduce transpirational water loss (*1*). Response to ABA depends on guanosine triphosphatases (GTPases), protein (de-)phosphorylation, and changes in cytosolic-free Ca^{2+} concentration and pH (*2, 3*) and is associated with substantial alterations in intracellular membrane structure in the guard cells (*4*).

We isolated elements that contribute to ABA signaling in vivo, adapting a strategy similar to that used to identify mammalian receptor and ion channel proteins (*5, 6*). Polyadenylated [poly(A)$^+$] RNA from leaves of drought-stressed *Nicotiana tabacum* was injected into *Xenopus laevis* oocytes. Expression of the *Nicotiana* mRNA led to a cross-coupling between exogenous ABA-sensitive elements and the endogenous signaling pathways of the oocyte, evidenced by activation of the *Xenopus* Ca^{2+}-dependent Cl^- current in the presence of 20 μM ABA (n = 16; Fig. 1A). Current activation was specific to mRNA-injected oocytes and was observed in response to ABA, but not to acetate or kinetin, a plant hormone that stimulates cell division and stomatal opening (*1*). After sucrose gradient fractionation of *Nicotiana* mRNA, the active fraction (mean size, 1.3 kb) was used to construct a cDNA library for expression and screening. Subdivision of library pools yielded clones that promoted the ABA-evoked current and a copurifying background current with similar characteristics but independent of ABA. The background current was isolated to a single clone (Fig. 1B). After depleting this transcript from *Nicotiana* mRNA, no ABA-sensitive current was observed (*7*), indicating that the gene carried by the clone was necessary to evoke the ABA-sensitive current (*8*).

Sequencing the transcript cDNA (*9*) revealed an open-reading frame encoding a syntaxin-related protein (Nt-Syr1; GenBank number AF112863) of 300 amino acids with a predicted molecular mass of 34.01 kD and an isoelectric point of 7.95. Alignments of Nt-Syr1 protein (Megalign, DNAstar, Madison, Wisconsin) showed similarities to the syntaxin-like Knolle gene product of *Arabidopsis thaliana* (38% identity) (*10*), human syntaxin-1A (23% identity) (*11*), and the yeast syntaxins SSO1p and SSO2p (22% identity each) (*12*). Syntaxins are essential for synaptic transmission, they coordinate cellular growth, and are implicated in vesicle trafficking in yeast, plants, and animals (*10*, *13–15*). Features of Nt-Syr1 common to syntaxin proteins (Fig. 2A) include three domains (H1 through H3) with high probabilities for forming coiled-coil structures in protein-protein interactions, a putative membrane-spanning (hydrophobic) domain, and an adjacent domain (within H3) of 84% identity (92% homology) with the epimorphin consensus sequence (*11*). Nt-Syr1 also showed partial conservation of the three sites necessary for binding and cleavage by *Clostridium botulinum* type C neurotoxin (BotN/C) (*16*). Unlike other syntaxin proteins, Nt-Syr1 harbors a putative EF-hand, Ca^{2+}-binding sequence and nucleotide binding site. Southern blot

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analyses using *Nt-SYR1* cDNA indicated a low number of homologous genes in the *Nicotiana* genome and yielded *At-SYR1* from *Arabidopsis* (GenBank number AF112864) encoding a predicted protein with an overall identity of 72% with Nt-Syr1 (*8, 17*).

Northern (RNA) blot and protein immunoblot analyses showed that the Nt-SYR1 transcript and translation product (34 kD) were present in low abundance in leaves of well-watered plants (Fig. 2, B and C). Virtually all Nt-Syr1 was found in the 50,000*g* microsomal pellet and, after two-phase partitioning, was localized primarily to the plasma membrane, paralleling the distribution of the plasma membrane H^+–adenosine triphosphatase (ATPase) (*18*). Remarkably, *Nt-SYR1* [and *At-SYR1* (*8*)] transcript levels rose transiently approximately ninefold within 30-min exposure to ABA and after 48 hours drought stress (n = 3). Nt-Syr1 protein showed a parallel, albeit delayed, transience in ABA as expected for de novo translation and protein accumulation.

We explored syntaxin-related function of Nt-Syr1 by complementation of the H440 strain of *Saccharomyces cerevisiae*, which harbors the lethal deletion of plasma membrane syntaxin genes *SSO1* and *SSO2*, and carries *SSO1* on a plasmid behind the *GAL1* galactose-inducible promoter (*19*). The H440 strain will grow on galactose, but not on glucose (*12*). After transformation, constitutive expression of Nt-Syr1 failed to rescue yeast growth on glucose (*8*). To examine Nt-Syr1 function in the plant, we used BotN/C which disrupts secretion by cleavage of syntaxins containing specific recognition sites (*16, 20*). Western blot analysis (*21*) of *Nicotiana* leaf microsomal proteins showed that Nt-Syr1, which contains homologs of the BotN/C-recognition sites, was cleaved by BotN/C, but not by BotN/D toxin (Fig. 3A), which targets the vesicle-associated protein synaptobrevin (*16*). Loss of BotN/C cleaved fragments (30 kD) was probably related to product instability and breakdown by endogenous proteases. The specificity of BotN/C action was indicated by the fact that cleavage was observed only when protein extracts were pretreated with ATP, which in synaptic protein complexes is required to expose syntaxin through complex disassembly by NSF ATPase (*22*). These results, and observations of antibody binding to high molecular weight bands (*8*), implicate Nt-Syr1 in similar complexes in planta.

We tested the effects of BotN/C and BotN/D on ABA-mediated control of guard cell K^+ and Cl^- channels in *Nicotiana*. ABA treatment normally results in a 40 to 60% inactivation of inward-rectifying K^+ channel current ($I_{K,in}$), a two- to fourfold stimulation of current through the Cl^- channels (I_{Cl}) and slowing of I_{Cl} gating (*2, 23, 24*). Voltage clamp recordings (Fig. 3B) (*25*) showed that cytosolic loading with BotN/C, but not with BotN/D, prevented ABA action on Cl^- channel gating, and a similar loss of sensitivity to ABA was found for $I_{K,in}$ after BotN/C loading (Fig. 3C). Equivalent results were ob-

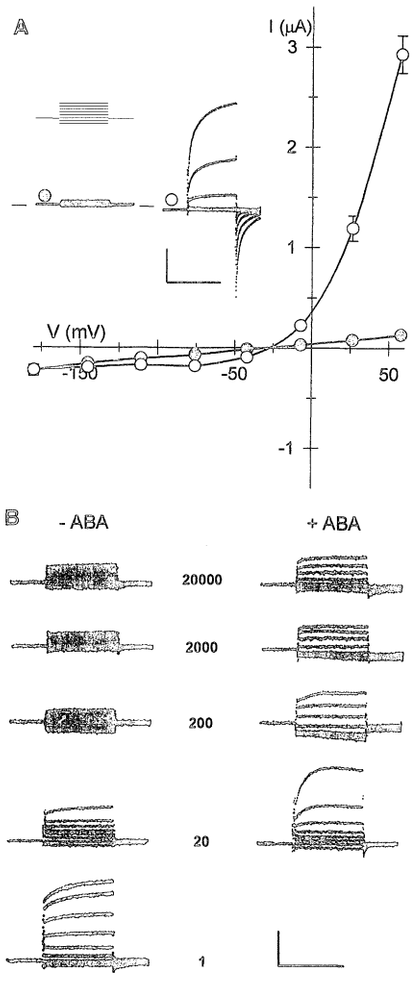


Fig. 1. Expression cloning of *Nt-SYR1*. (**A**) Voltage clamp recordings of *Xenopus* oocytes expressing poly(A)$^+$ RNA from drought-stressed *Nicotiana* leaves. Current-voltage (I-V) curves from one set of injections (means ± SE, n = 3 cells) recorded at the end of 1-s voltage steps before (●) and 30 s after (○) adding 20 μM ABA. (Inset) Currents from one cell cross-referenced by symbol. Voltage protocol (above): conditioning voltage, –120 mV; test voltages (8 cycles), –180 to +60 mV. Scale: horizontal, 1 s; vertical, 1 μA. The ABA-evoked current was identified with Cl^- channels by tail current analysis (E_{Cl} = –26 mV, n = 4) (*8*). (**B**) Sib-selection cloning of *Nt-SYR1*. *Nicotiana* leaf poly(A)$^+$ RNA was size-fractionated on a 10 to 30% sucrose gradient. The fraction yielding the ABA response was used to construct a cDNA library in the pSPORT vector with the Superscript plasmid system (Gibco-BRL). DNA derived from pools of clones was linearized with Not I and transcribed in vitro with the T7 RNA polymerase. *Xenopus* oocytes were injected with this cRNA and assayed for ABA sensitivity. Currents before (left) and during (right) challenge with 20 μM ABA are shown from representative oocytes injected with progressively smaller cRNA pools. Scale: horizontal, 1 s; vertical, 500 nA.

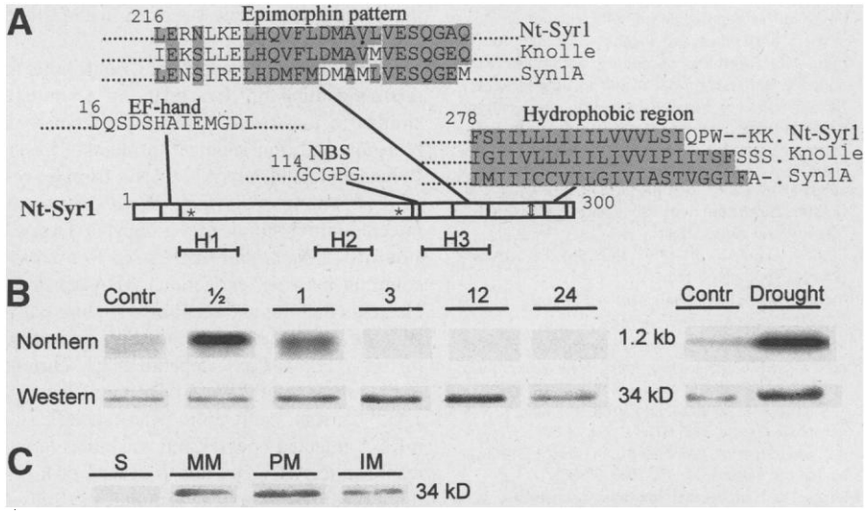


Fig. 2. Structure and expression analysis of Nt-Syr1 (*37*). (**A**) Key features of Nt-Syr1 include putative Ca^{2+}- (EF-hand) and nucleotide-binding (NBS) sites, partially conserved domains for recognition (*) and cleavage (⇳) by BotN/C, and putative coiled-coil domains (H1 through H3). High amino acid conservation with Knolle (*10*) and syntaxin-1A (Syn1A) (*11*) is found in the epimorphin domain and hydrophobic COOH-terminus. (**B**) Northern blot (above) and protein immunoblot (Western; below) analyses showing transient enhancement of *Nt-SYR1* transcript and protein levels by treatment with 20 μM ABA and drought stress. Total RNA (15 μg/lane) and crude protein extracts (10 μg/lane) were isolated from *Nicotiana* leaves. Ribosomal RNA was used as a loading control (*8*). Northern blots probed with full *Nt-SYR1* cDNA and protein immunoblots probed with Anti-Sp2 antiserum (*18*). Contr, control; 1/2 to 24, hours of ABA exposure; Drought, 48 hours. (**C**) Subcellular localization of the Nt-Syr1 by protein immunoblot analysis of fractionated *Nicotiana* leaves (MM, 50,000*g* microsomal membrane; S, soluble fraction; PM, two-phase partitioning plasma membrane; IM, inner membrane).

tained in recordings from *Vicia faba* guard cells (*8*).

In separate experiments we used the C-truncated Nt-Syr1 protein Sp2 (*18*) to "poison" Nt-Syr1 functioning. By analogy with the action of C-truncated Syntaxin-1A in secretion (*20*), we reasoned that if a protein complex with Nt-Syr1 was necessary for ABA signaling, adding the truncated protein—including the protein-protein interaction domains, but lacking the COOH-terminal membrane anchor—might compete with Nt-Syr1 for partners and prevent normal complex functioning. Voltage clamp records (Fig. 4A) (*25*) showed that current through the outward-rectifying K^+ channels ($I_{K,out}$) was enhanced two- to threefold in ABA, while $I_{K,in}$ was reduced, at –200 mV, to roughly 25% of the control. ABA also shifted the voltage-sensitivity of $I_{K,in}$ (Fig. 4C), consistent with its Ca^{2+}-sensitivity and ABA action on its gating (*2*). In guard cells loaded 20 or 100 μM Sp2 protein $I_{K,in}$ and $I_{K,out}$ showed complete loss of sensitivity to ABA, and a similar loss of sensitivity was found for I_{Cl} (Fig. 4B).

We interpret these results to indicate a central role for Nt-Syr1 in early steps of ABA signaling and to implicate its functioning in a heteromultimeric complex with other proteins. This idea accords with the homology of Nt-Syr1 to other syntaxins, its presence in high–molecular weight components, and the action of Sp2 on the ABA response of guard cell ion channels. The target (t-)SNARE syntaxin takes part in a number of protein-protein interactions essential for vesicle trafficking, secretion and endocytosis (*13, 26*). Syntaxin binding partners at the presynaptic membrane include SNAP-25 and the vesicle (v-)SNARE synaptobrevin, which form a stable ternary complex for vesicle fusion (*13*). Less is known of syntaxin function in stimulus perception, although its interaction with other elements is likely to be important for signaling (*27, 28*). Syntaxins do interact with other proteins that may not be related to secretion processes directly. Syntaxin-1A

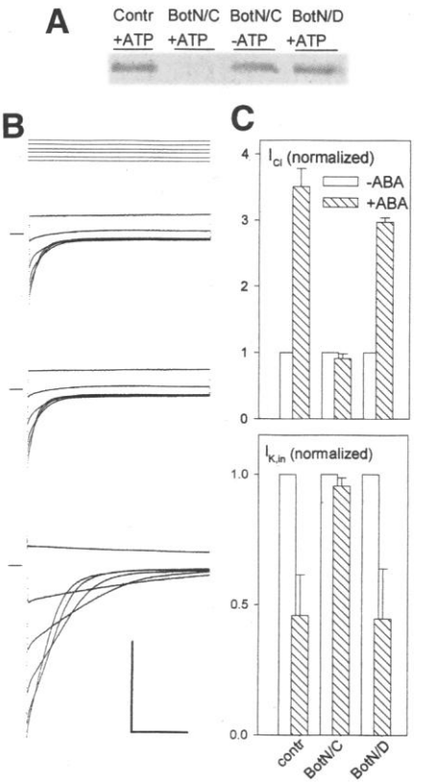
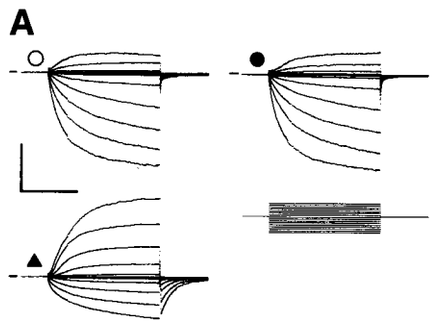
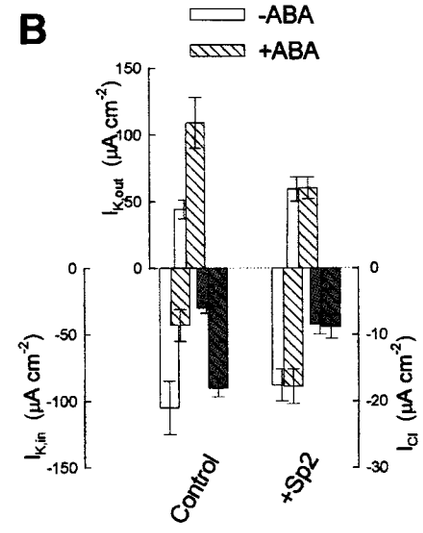


Fig. 3. Neurotoxin BotN/C, but not BotN/D, targets Nt-Syr1 and blocks ion channel response to ABA in *Nicotiana* guard cells. (**A**) Microsomal protein fractions were isolated from *Nicotiana* leaves, pretreated either with or without 1 mM ATP, incubated with BotN/C and BotN/D, separated by SDS-PAGE (6 μg/lane), and assayed by protein immunoblot analysis. (**B**) Voltage clamp analysis of Cl^- channel response to ABA in guard cells with and without BotN/C. Voltage steps (above): conditioning voltage (5 s), +30 mV (*8*); test voltages (6 cycles), –160 mV to +30 mV. Currents are from one guard cell loaded with 0.1 μM BotN/C before (top) and 8 min after (center) adding 20 μM ABA. Data from a second cell in ABA (bottom) is shown for comparison. No significant difference in current characteristics were observed without ABA between nonloaded cells and cells loaded with either toxin. Scale: vertical, 100 μA cm^{-2}; horizontal, 2 s. Zero current level is on the left. (**C**) Means ± SE of the ABA response of the Cl^- current (I_{Cl}; top) and inward-rectifying K^+ current ($I_{K,in}$; bottom) from nonloaded (contr) and BotN/C- and BotN/D-loaded (0.1 μM) guard cells ($n \geq 5$). Currents were taken at –200 mV and normalized to the corresponding measurements taken before ABA treatments.





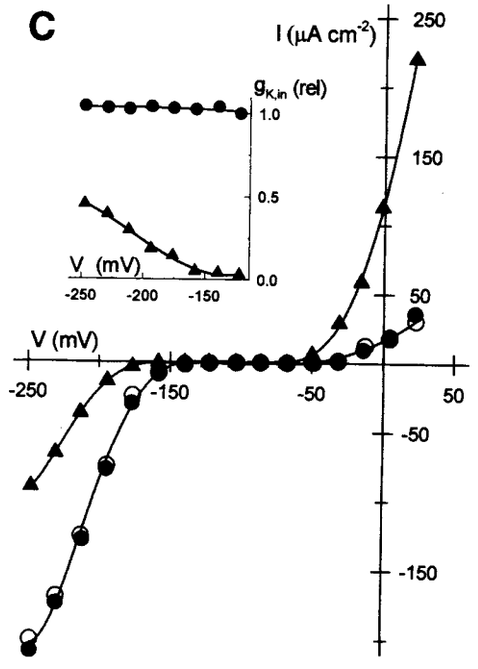


Fig. 4. Truncated Nt-Syr1 protein (Sp2) blocks ion channel response to ABA in *Nicotiana* guard cells. (**A**) Voltage clamp analysis of K^+ channel response to 20 μM ABA. Voltage protocol (bottom right): conditioning voltage, –100 mV; test voltages (16 cycles), –250 mV to +30 mV; tailing voltage, –100 mV. Currents are shown from one guard cell loaded with 20 μM Sp2 before (○) and 10 min after (●) adding ABA. Data from a second cell in ABA (▲) for comparison show characteristic slowing of inward current by ABA (*2*). No significant difference in current characteristics were observed without ABA between nonloaded cells and cells loaded with Sp2. Scale: vertical, 100 μA cm^{-2}; horizontal, 1 s. Zero current levels are on the left. (**B**) Means ± SE of steady-state currents before and 10 min after adding ABA in nonloaded (Control) and Sp2-loaded (+Sp2) cells. Data are for $I_{K,in}$ (open bars, down) and $I_{K,out}$ (open bars, up) and for I_{Cl} (shaded bars, down). Currents were recorded at –200 mV ($I_{K,in}$), +30 mV ($I_{K,out}$), and –100 mV (I_{Cl}). (**C**) Steady-state current-voltage relationships for $I_{K,in}$ and $I_{K,out}$ from (A) and cross-referenced by symbol showing characteristic shift in voltage-sensitivity for $I_{K,in}$ in ABA (▲). (Inset) Conductances ($g_{K,rel}$) of $I_{K,in}$ ± Sp2 in ABA (●, ▲) relative to conductances without ABA.

copurifies with the orphan G-protein–coupled receptor CIRL (*29*) and interacts with tomosyn, a microfilament-associated protein (*30*). Syntaxins also bind Ca^{2+} channels and CFTR Cl^- channels, influencing their regulation (*13, 31, 32*). Significantly, Ca^{2+} channels and SNARE proteins interact with the syntaxin-1A epimorphin domain, and peptides synthesized to this domain, corresponding to that in Sp2, prevent secretion (*13, 20, 32*).

In plants, SNARE proteins have been implicated in cell growth and development (*33*). During stomatal movements, the plasma membrane surface area of guard cells can change by 50%, much more than can be accommodated by lateral expansion and compression of the bilayer (*1, 33*). Guard cell membrane structure undergoes substantial change during stomatal movements (*4*), and protoplast volume is coupled to membrane trafficking (*34*). Thus, we anticipate a crucial role for SNARE proteins in ABA-evoked changes in secretion and endocytosis. It is interesting that Nt-Syr1 harbors putative nucleotide and Ca^{2+}-binding domains, especially in light of Ca^{2+} action as a second messenger during ABA signaling (*2, 3*). For synaptic transmission Ca^{2+} facilitates the later stages of vesicle fusion, but binds to synaptotagmin, not to syntaxin (*13*). These domains may therefore indicate regulatory activities unique to Nt-Syr1.

Most importantly, our data point to a new function for this putative SNARE protein as a key element in a hormonal signal cascade, and not simply as a component of the response mechanics. ABA control of K^+ and Cl^- channel gating cannot be linked readily to membrane trafficking per se. It is equally difficult to explain concurrent regulation of all three ion channels through a direct interaction with each of the channel proteins, especially as syntaxins are not known to associate with K^+ channels. Hence, disruption of ABA signaling by BotN/C and Sp2 in vivo implies an action of Nt-Syr1 upstream, possibly close to the primary event of ABA binding. How might Nt-Syr1 contribute to ABA signaling? At present, there is almost no data that bears on SNARE function in intracellular (nonsynaptic) signal transmission. Nonetheless, several lines of evidence suggest more intimate roles for SNARE proteins beyond the mechanics of vesicle trafficking, including scaffolding and nucleation (*27, 30, 35*) that are essential features of many signaling cascades. SNARE proteins have also been suggested to contribute to the sensing of osmotic stress (*36*). At present our data do not speak directly to the possibility of ABA binding to Nt-Syr1, or to one or more possible roles as a scaffolding protein, a second messenger element, or a modulator to the poise of one or more signaling elements or ion channels. Identifying proteins that interact with Nt-Syr1 will help to resolve this issue and to gain further insights into its function.

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7. Hybrid depletion was carried out (*6*) using poly(A)$^+$ RNA from drought-stressed *Nicotiana* leaves. Antisense *Nt-SYR1* cRNA was hybridized [20 ng per 1 μg/μl poly(A)$^+$ RNA] in 0.1 M NaCl and 50 mM sodium phosphate (pH 6.5) at 65°C for 2 min, at 42°C for 10 min, and then chilled on ice. Oocytes were injected with hybrid-depleted poly(A)$^+$ RNA, and in parallel with nonhybridized poly(A)$^+$ RNA, and assayed for ABA-evoked Cl^- current.
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17. An *Arabidopsis* cDNA library in λYES (Clontech) was screened by colony hybridization with *Nt-SYR1* cDNA. Positive inserts were sequenced, and a full-length open reading frame identified by homology with *Nt-SYR1*.
18. *Nicotiana* plants were grown from seed at 25°C and 80 to 90% relative humidity (RH) on a 12:12 hour day-night cycle. Plants at the three to five leaf-pair stage were drought-stressed by withholding watering and transfer to <60% RH for 48 hours. Plants were treated with ABA by foliar misting and watering with 20 μM ABA. Crude protein extracts and microsomes were prepared (*14*) and plasma membrane was purified as described [M. J. Daniels, T. E. Mirkov, M. J. Chrispeels, *Plant Physiol.* **106**, 1325 (1994)]. Antibodies were generated against the Sp2 protein corresponding to the first 279 amino acids of Nt-Syr1. His-tagged Sp2 was purified using the QIAexpressionist kit (Qiagen) and anti-Sp2 sera collected from white rabbits (UKC, Canterbury). Antisera were titered against Sp2 expressed in *Escherichia coli* and were specific for Nt-Syr1 when expressed in *Saccharomyces* (below). The anti-PMA_2 plasma membrane H^+-ATPase antibody was a gift of M. Boutry [Louvain-la-Neuve, A. D. Dexaerde *et al.*, *J. Biol. Chem.* **270**, 23828 (1995)]. Western blot analysis was carried out using anti-rabbit IgG-alkaline phosphatase conjugate for immunodetection [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Boston, 1988), pp. 1–726].
19. *Nt-SYR1* was cloned in the pMA91 vector behind the PGK1 promoter. High constitutive expression of Nt-Syr1 was evidenced by protein immunoblot analysis. *Saccharomyces* strain H440 (*12*) was transformed in PEG Li^+-acetate [R. Elble, *Biotechniques* **13**, 18 (1992)] and selected on SD medium without uracil and containing 2% galactose. Complementation screening was carried out on YPD (containing 2% glucose) in 1% agar at 28°C.
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37. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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