evolution, there was a relaxation of the strict En-mediated repression of *ci* that occurs in the posterior compartment of *Drosophila*. Furthermore, the coincidence of the expression domains of *en/inv* and *ci*, rather than of *en/inv* and *hh*, indicates that during focal establishment *en* and *inv* are targets, rather than inducers, of Hh signaling.

In *P. coenia*, as in most species of butterflies, eyespots are found only in the posterior compartment of the wing, the normal domain of *hh* and *en/inv* expression. However, a few butterfly families contain species with eyespots in the anterior compartment. If *hh* signaling plays a role in focal establishment and all butterfly eyespot foci develop by the same mechanism, as is suggested by correlations in their variance (*22*), then expression of Hh signaling components and *en/inv* should be associated with foci located in the anterior as well as the posterior compartment. To test whether Hh signaling accompanies focal establishment regardless of its location in the wing, we examined hindwing imaginal discs of *Bicyclus anynana*, a species that forms eyespots on both sides of the A/P compartment border (Fig. 3A). Both En/Inv and Ci are coexpressed in all *B. anynana* eyespot foci, including the one in the anterior compartment (Fig. 3, B and C). Thus, the expression of the Hh signaling pathway and *en/inv* is associated with the development of all eyespot foci and has become independent of A/P compartmental restrictions.

The novel expression patterns of *hh*, *ptc*, *ci*, and *en/inv* could result from independent or dependent changes in their regulation during eyespot evolution. Experimental evidence from *Drosophila* and comparative analysis of butterflies leads us to infer that some changes in the expression of *hh* pathway components were primary whereas others were secondary consequences. For instance, beginning in the late third instar of the developing *Drosophila* wing disc, the ability to express *en/inv* in response to Hh signaling is a general property of Ci-expressing cells. This competence is present throughout the anterior compartment (*23*) but is only used just anterior to the A/P boundary to pattern intervein tissue (*24*, *25*). Expression of *en/inv* just to the anterior of the A/P boundary in *P. coenia* and *B. anynana* indicates that this regulatory circuit is conserved in butterflies (note overlap of En/Inv and Ci in Fig. 3C). The similarity between the induction of *en/inv* by Hh at the A/P boundary and in eyespot foci in late wing development suggests that during eyespot evolution, the Hh-dependent regulatory circuit that establishes foci was recruited from the circuit that acts along the A/P boundary of the wing (Fig. 4).

For this Hh regulatory circuit to operate in focal development, two primary spatial regulatory changes must have evolved. First, mechanisms must have evolved that modulate levels of *hh* expression along the proximodistal axis of the wing field. Second, because reception of the Hh signal depends on expression of the Ptc receptor, which in turn depends on Ci function, the restriction of *ci* from the posterior compartment must have been relaxed. *ptc* and *en/inv* expression would then evolve as secondary consequences of these regulatory changes. This recruitment of an entire regulatory circuit through changes in the regulation of a subset of components increases the facility with which new developmental functions can evolve and may be a general theme in the evolution of novelties within extant structures.

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29. We thank P. M. Brakefield for *B. anynana* larvae; N. Patel for antibodies; C. Brunetti, R. Galant, G. Halder, and R. ffrench-Constant for comments on the manuscript; and J. Wilson for help with manuscript preparation. D.N.K. was supported by an NIH Training Grant to the Department of Genetics, D.L.L. was supported by an NIH postdoctoral fellowship (F32 GM18162), R.L.J. was supported by a Damon Runyon–Walter Winchell Foundation Fellowship (DRG 1218) and a Walter and Idun Berry Postdoctoral Fellowship, and this work was supported by NSF grant IBN-948449. M.P.S. and S.B.C. are investigators of the Howard Hughes Medical Institute.

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Turning Brain into Blood: A Hematopoietic Fate Adopted by Adult Neural Stem Cells in Vivo

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Stem cells are found in various organs where they participate in tissue homeostasis by replacing differentiated cells lost to physiological turnover or injury. An investigation was performed to determine whether stem cells are restricted to produce specific cell types, namely, those from the tissue in which they reside. After transplantation into irradiated hosts, genetically labeled neural stem cells were found to produce a variety of blood cell types including myeloid and lymphoid cells as well as early hematopoietic cells. Thus, neural stem cells appear to have a wider differentiation potential than previously thought.

Stem cells have been identified in adult tissues that undergo extensive cell replacement due to physiological turnover or injury such as the hematopoietic, intestinal, and epidermal systems (*1*). These cells have been found in the central nervous system (CNS) (*2*), a tissue thought to be capable of extremely limited self-repair. CNS stem cells can generate the three major cell types found in the adult brain: namely, astrocytes, oligodendrocytes, and neurons (*3*). This is consistent with the view that the developmental potential of stem cells is restricted to the differentiated elements of

the tissue in which they reside. However, some developmental peculiarities suggest certain cells may be able to differentiate into cell types that are not of the same dermal origin (*4*). Hence, we sought to determine whether neural stem cells (NSCs) could produce hematopoietic progeny.

A bone marrow (BM) repopulation assay (*5*) was used to test this hypothesis. Hematopoietic stem cells from unfractionated adult BM (10^7cells per animal) or NSCs cultured from either the embryonic or adult forebrain (10^6 cells per animal) (*6*) of ROSA26 mice were systemically injected into sublethally irradiated Balb/c recipient animals (*7*). ROSA26 animals were selected as the source of donor tissue because they are of a different immunological background than Balb/c and are transgenic for *lacZ* (*8*), which encodes for the *Escherichia coli* enzyme β-galactosidase. Importantly, to eliminate possible contamination of NSCs with cells of mesodermal origin (*9*), we also injected clonally derived adult ROSA26 NSCs (Fig. 1).

Polymerase chain reaction (PCR) (*10*) was used to assay for the presence of *lacZ* in splenic DNA isolated from animals transplanted 5 to 12 months earlier. The *lacZ* gene was not detected in samples from either unirradiated or irradiated Balb/c mice that received vehicle (EBSS) (Fig. 2), whereas a strong signal was observed from both untreated ROSA26 animals and irradiated Balb/c mice that received ROSA26 BM (Fig. 2). A strong *lacZ* signal was also detected in animals injected with embryonic, adult, or clonally derived adult NSCs (Fig. 2). Because the behavior of NSCs derived from three clones (2H1, 4E8, and 3C6) was indistinguishable from that of bulk cultures, only results from a representative clone (2H1) and embryonic NSCs will be considered for the remainder of this report.

To test whether engrafted NSCs adopted a hematopoietic identity, we used in vitro clonogenic assays, immunocytochemistry, and flow cytometric analysis. For the clonogenic assays, cells from the BM of transplanted animals were plated in methylcellulose in the presence of defined cytokines (*11*). Ten to 14 days after plating, colonies founded by single hematopoietic progenitor cells were subjected to X-Gal histochemistry to detect β-galactosidase activity (*12*), thereby identifying hematopoietic precursors of a NSC origin. None of the colonies derived from the BM of irradiated Balb/c animals injected with EBSS stained positively for β-galactosidase (Fig. 3A). Conversely, BM isolated from recipients of either embryonic or adult NSCs formed colonies that reacted strongly to X-Gal (Fig. 3, B and C). A few colonies (<5%) did not stain positively for β-galactosidase (Fig. 3C), showing that some endogenous hematopoietic progenitors had survived the sublethal irradiation. Different types of colonies generated from BM isolated from adult NSC recipients included pure granulocyte (13%), granulocyte-macrophage (Fig. 3, D, E, and F) (30%), and pure macrophage (Fig. 3, G, H, and I) (22%), as well as mixed colonies (19%). Megakaryocytic and B cell colonies were also present, although at lower frequencies (<1% and <10%, respectively). Erythroid cells were not taken into account in this analysis because their evaluation cannot be reliably carried out by X-Gal staining. None of the NSC cultures proliferated or formed colonies when used in the same clonogenic assay before injection. Thus, ROSA26-derived NSCs can give rise to hematopoietic precursors after engraftment into irradiated Balb/c hosts.

To further demonstrate the engraftment of NSCs into the hematopoietic system, we exploited the fact that distinct cell surface antigens are expressed by ROSA26 (H-$2K^b$) and Balb/c (H-$2K^d$) mice. We assayed cells isolated from the spleen, BM, and peripheral blood of transplanted and control animals by flow cytometry (*13*) using antibodies specific to H-$2K^b$ and H-$2K^d$ (*14*). Whereas no H-$2K^b$–positive (H-$2K^{b+}$) cells were found in animals injected with EBSS alone, numerous H-$2K^{b+}$ cells were detected in animals that received either ROSA26 BM or, more importantly, NSCs (Fig. 4A). By this approach, early experiments showed effective engraftment with between 10^5 and 10^7 NSCs per animal. With 10^6 NSCs per animal and 10^7 BM cells per animal, 100% of BM, 100% of embryonic NSCs, 70% of adult NSCs, and 63% of clonal-adult NSC recipients showed positive engraftment by flow cytometric analysis (n = 20, 20, 40, and 30 animals per group, respectively). In addition, donor-derived (H-$2K^{b+}$) cells were first detected in the peripheral blood of NSC recipients 20 to 22 weeks after injection compared with 16 to 18 weeks for ROSA26 BM recipients. After initial detection in peripheral blood, spleen cells were harvested and processed for dual-label flow cytometry (*13*) with

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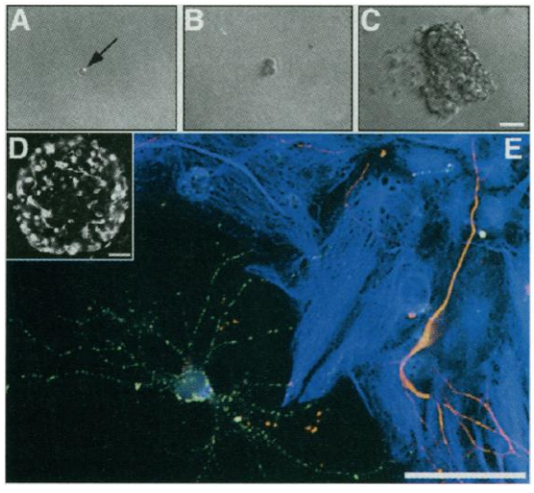

Fig. 1. Cloning of adult ROSA26 CNS stem cells. (**A**) Single adult ROSA26 NSC plated in isolation in a single well (arrow). After (**B**) 1 and (**C**) 8 days, a cluster of cells formed, which was serially subcultured every 4 days to establish a continuous culture. (**D**) All cells expressed the NSC antigen, nestin. Differentiation was induced by plating a fraction of these cells in 1% fetal bovine serum, in the absence of growth factors. (**E**) The simultaneous detection of neurons [red; 25.3 ± 0.9% of total cell number (TCN); n = 6, ± SEM], astroglia (blue; 71.6 ± 6.3% TCN; n = 6), and oligodendroglia (green; 0.9 ± 0.01% TCN; n = 6) among the progeny of this cell indicate its tripotentiality (*19*). Secondary clones of the cell displayed in (A) produced an average of 48 ± 3.2 (n = 6) cells capable of producing tertiary, tripotential clones, thereby demonstrating self-maintenance (*2*). Data are from clone 2H1. Bars: (A) to (C), 90 μm; (D), 60 μm; (E), 40 μm.

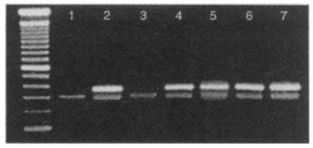

Fig. 2. Detection of *lacZ* in splenic DNA isolated from animals injected with ROSA26 NSCs by PCR. The *lacZ* gene was not detected in control (lane 1) or irradiated Balb/c animals injected with vehicle (lane 3). ROSA26 animals (lane 2) and Balb/c mice that received either ROSA26-derived BM (lane 4), embryonic NSCs (lane 5), adult NSCs (lane 6), or clonal adult NSC 2H1 (lane 7) produced strong amplification signals for *lacZ* (upper band; 374 base pairs). GAPDH was also amplified in the same reaction tube as an internal control (*10*) (lower band; 309 base pairs).

antibodies to H-2K^b in combination with either antibodies to CD3e (anti-CD3e) (T lymphocytes), anti-CD19 (B lymphocytes), or anti-CD11b (myeloid cells) (*15*). A significant number of ROSA26-derived NSCs gave rise to B and T lymphocytes or myeloid cells after transplantation into Balb/c hosts (Fig. 4, A and B). None of the hematopoietic antigens tested was expressed by any NSCs before transplantation.

Thus, NSCs isolated from the embryonic and adult murine forebrain, which generate neurons and glia, engraft into the hematopoietic system of irradiated hosts to produce a range of blood cell types. This demonstrates that the actual differentiation potential of adult NSCs, which are currently viewed as tripotent neural precursors, is much broader than expected.

We chose sublethal irradiation because it eliminates a significant fraction of the endogenous hematopoietic precursors without killing the mouse. We felt this would be necessary because NSCs would likely need more time to acquire a hematopoietic fate than a lethal dose would allow. That the repopulation of the immune system took on average 3 weeks longer, combined with a slightly weaker engraftment for NSC compared with BM recipient animals, seems to supports this idea. This extra time required suggests that NSCs undergo additional steps of fate determination, differentiation, and maturation with respect to BM cells to produce hematopoietic progeny.

It has been suggested by studies in various model systems that most somatic cell specialization may not involve irreversible genetic changes (*16*). The seminal demonstration of conserved genomic totipotentiality in adult somatic cells was provided in a study that describes the cloning of an adult ewe (*17*). Our work is complementary to these findings and suggests that the reactivation of dormant genetic programs may not require nuclear transfer or experimental modification of the genome. NSCs appear naturally endowed with the appropriate machinery required to express an otherwise silent genomic potentiality in response to an appropriate pattern of stimulation.

Given the fact that human NSCs can be continuously expanded for extended periods of time (*18*), the finding presented here may have implications for the treatment of a number of human disorders. If they behave similarly to their murine counterparts, human NSCs may provide a renewable, characterized source of cells that could be used in approaches aimed at hematopoietic reconstitution in various blood diseases and disorders.

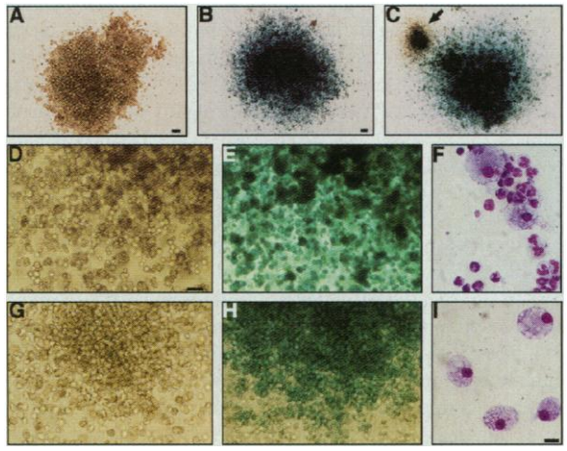

Fig. 3. NSCs produce early hematopoietic cells after transplantation into irradiated Balb/c recipients. An in vitro clonogenic assay (*11*) was used to analyze hematopoietic precursors in Balb/c mice injected with either embryonic or clonal adult NSCs, both of which yielded identical results. X-Gal histochemistry was used to identify hematopoietic clones derived from NSCs after 10 to 14 days in culture (*12*). Whereas none of the colonies from Balb/c BM stained positively for β-galactosidase (**A**), colonies from the BM of animals that received adult NSCs displayed β-galactosidase activity (blue) (**B**). In the same cultures a few unlabeled clones were found in Balb/c mice injected with clonal adult NSCs (**C**) (arrow), showing the persistence of a small number of endogenous (Balb/c) hematopoietic precursors in recipient animals. NSC-derived (β-galactosidase–reactive) colonies were further characterized by morphology. Colonies with distinctive granulocyte-macrophage (**D** and **E**) and pure macrophage (**G** and **H**) characteristics before (D and G) and after (E and H) reaction with X-Gal are shown. The X-Gal reaction was stopped prematurely to allow for morphological identification of individual cells within these colonies after staining. High-power microphotographs show the identifying morphology of the cells derived from the two types of colonies as stained by May-Grünwald-Giemsa [(**F**), granulocyte-macrophage; (**I**), pure macrophage]. Bars: (A), 40 μm; (B) and (C), 90 μm; (D), (E), (G), and (H), 40 μm; (F) and (I), 15 μm.

A

% H-2K^b	Balb/c	ROSA 26	EBSS	Bone Marrow	Adult NSCs	Embryonic NSCs
Peripheral Blood	2.35±0.40	94.2±1.13	1.68±0.43	56.7±12.6	43.1±6.87	43.9±6.98
Spleen	0.92±0.15	97.3±1.03	1.68±0.44	95.3±2.45	65.4±23.5	60.7±11.0
Bone Marrow	2.26±0.55	42.3±4.09	1.59±0.51	38.8±2.48	35.8±10.0	40.4±6.61
CD3e/H-2K^b	0.52±0.08	33.0±5.16	0.77±0.05	31.9±2.62	28.1±14.2	9.86±4.69
CD11b/H-2K^b	0.47±0.09	30.0±2.24	0.43±0.02	14.5±3.85	14.9±10.9	19.5±6.88
CD19/H-2K^b	0.57±0.08	51.0±1.90	0.59±0.07	56.2±2.50	26.8±11.7	31.8±1.38

B

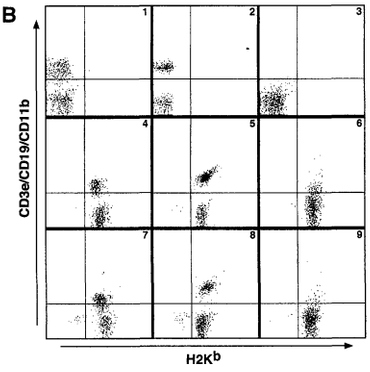

Fig. 4. Identification of differentiated hematopoietic cell types derived from ROSA26 NSCs. Hematopoietic cells from transplanted mice were processed for flow cytometry (*13*). (**A**) A significant number of H-2K^{b+} cells were found in the BM, spleen, and peripheral blood of Balb/c animals injected with ROSA26 BM, and embryonic or adult clonal NSCs, but not in unirradiated Balb/c or EBSS recipients. Similarly, none of the CD3e, CD19, or CD11 immunoreactive cells identified in the spleens of control animals (Balb/c, EBSS) expressed H-2K^b. Conversely, a significant proportion of CD3e, CD19, or CD11b immunoreactive cells isolated from the spleens of ROSA26 animals as well as ROSA26 BM, embryonic, or adult clonal NSC recipients were H-2K^{b+}. All percentages were calculated relative to the total events gated (n = 6, ±SEM; * indicates $P <$ 0.05 compared with Balb/c by analysis of variance). (**B**) Representative dual-label FACS plots identifies CD3e (panels 1, 4, and 7), CD19 (panels 2, 5, and 8), and CD11b (panels 3, 6, 9) immunoreactive cells (y axes) that express H-2K^b (x axes) in Balb/c animals that received EBSS (panels 1 to 3), and in ROSA26 BM (panels 4 to 6) or adult clonal NSC (panels 7 to 9) recipient animals.

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7. Male and female Balb/c mice 6 to 8 weeks old (Charles River) received whole-body irradiation (cobalt source) with a fractionated dose of 450/400 rads (4.5/4 Gy) separated by 4 hours. Mice were injected with 1 × 10^6 NSCs or 1 × 10^7 ROSA26 BM in 200 μl of Earle's buffered saline solution (EBSS; Gibco-BRL) through the tail vein 16 hours after irradiation (n = 4 independent experiments). All animal experimen-

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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.
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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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