A Grapevine Gene Encoding a Guard Cell K⁺ Channel Displays Developmental Regulation in the Grapevine Berry

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SIRK is a K⁺ channel identified in grapevine (Vitis vinifera), belonging to the so-called Shaker family. The highest sequence similarities it shares with the members of this family are found with channels of the KAT type, although SIRK displays a small ankyrin domain. This atypical feature provides a key to understand the evolution of the plant Shaker family. Expression in Xenopus laevis oocytes indicated that SIRK is an inwardly rectifying channel displaying functional properties very similar to those of KAT2. The activity of SIRK promoter region fused to the GUS reporter gene was analyzed in both grapevine and Arabidopsis. Like other KAT-like channels, SIRK is expressed in guard cells. In Arabidopsis, the construct is also expressed in xylem parenchyma. Semiquantitative reverse transcriptase-polymerase chain reaction experiments indicated that SIRK transcript was present at low levels in the berry, during the first stages of berry growth. After veraison, the period of berry development that corresponds to the inception of ripening and that is associated with large biochemical and structural modifications, such as evolution of stomata in nonfunctional lenticels and degeneration of xylem vasculature, the transcript was no longer detected. The whole set of data suggests that in the berries SIRK is expressed in guard cells and, possibly, in xylem tissues. The encoded channel polypeptide could therefore play a role in the regulation of transpiration and water fluxes in grapevine fruits.

The grapevine (Vitis vinifera) is a highly productive water stress-adapted plant and the most economically important fruit crop in the world (Coombe, 1989; Kanellis and Roubelakis-Angelakis, 1993). The grape berry provides, at the plant physiology level, a model for investigating non-climacteric fruit development, the present knowledge in this field being very poor. Also, with vacuolar pH values being very low (approximately 2.2–3.5; Hrazdina et al., 1994), the grape berry is of interest as a model for acid fruit physiology (Ros et al., 1995; Terrier et al., 2001). The growth pattern of the grape berry follows a double sigmoidal curve, in which three successive phases can be distinguished: two growth periods, separated by a phase of slow growth (Kanellis and Roubelakis-Angelakis, 1993). Immediately after flowering, the first phase results from rapid cell division followed by marked cell enlargement, because of rapid accumulation of organic acids (tartaric and malic acids) in the vacuolar compartment. The second phase begins approximately 7 to 10 weeks after flowering. The berry displays slow or no growth. Further accumulation of organic acids makes the berry acidity reach a maximum at the end of this stage. The entry into the second growth period begins with the sudden onset of ripening, called veraison. This transition, which may occur within 24 h, results in and is characterized by berry softening (Coombe, 1992). Growth after veraison is rather due to flesh cell expansion than to cell division. A rapid accumulation of sugar (up to 1 M) and amino acids sets up, as well as a decrease in organic acid content, essentially due to malate degradation. Before veraison, the grape berry is photosynthetically active and its stomata are functional. The berry
is connected to the rest of the plant mainly via the xylem vessels for water and solutes importation. After veraison, the berry becomes a strong sink and depends on phloem sap flux not only for sugars and other organic molecules but also for mineral ions and water, because xylem vessels are no longer functional. Discontinuities of xylem occur by the time of veraison in the vascular tissues that connect the berry to the stalk (Düring et al., 1987; Findlay et al., 1987). At the same time, berry stomata evolve into nonfunctional lenticels, the stomatal vestibule being progressively blocked by accumulation of polyphenolics, suberin, silicon, and calcium (Blanke et al., 1999).

The mechanisms of grape berry development, as those of other non-climacteric fruits, are still poorly understood at the molecular level. Most studies have focused on genes, the expression of which is modified at veraison (Robinson et al., 1997; Tattersall et al., 1997; Davies and Robinson, 2000). A particular attention has been given to sugar transporters and enzymes involved in Suc metabolism (Davies and Robinson, 1996; Fillion et al., 1999; Ageorges et al., 2000; Manning et al., 2001). The aim of the present study was to clone and characterize vine K⁺ transporters implicated in ionic homeostasis of the berry. K⁺ is the most abundant cation in the cell, being weakly chaotropic and compatible at high concentration, in the 0.1 M range, with water and protein structure (Clarkson and Hanson, 1980).

It is involved in a number of basic functions linked together at the cellular or the whole plant level, e.g. control of cell turgor, and thereby control of cell enlargement or guard cell movements, and electrical neutralization of organic acids stored in the vacuolar compartment. At the agronomic level, and concerning wine quality, a large set of data has demonstrated that an increase in grape berry K⁺ content, due to e.g. increased K⁺ levels in the soil, or root stock efficiency in taking up and transporting K⁺, leads to decreased vacuolar acidity and changes in relative concentrations of organic acids in the ripe berry, resulting in poor quality wine (Hale, 1977; Delas et al., 1989).

Here, we report the identification and characterization of a vine K⁺ channel, belonging to the so-called Shaker family. In Arabidopsis, channels of this family have been shown to play a role in K⁺ uptake from the soil solution, K⁺ secretion into the xylem sap, and probably K⁺ redistribution via the phloem sap, as well as stomatal movements (Zimmermann and Sentenac, 1999). When expressed in Xenopus laevis oocytes, the newly identified vine channel is gated by voltage and endowed with inwardly rectifying activity. A GUS reporter gene approach revealed that expression of this channel in vegetative organs is restricted to leaf and stem guard cells. Semiquantitative reverse transcriptase (RT)-PCR experiments indicated that its expression in the grape berry is strongly decreased after veraison. Finally, analysis of the vine channel sequence provided interesting clues regarding evolution of the Shaker K⁺ channel family in plants.

RESULTS

Cloning of a Gene Encoding a Shaker-Like K⁺ Channel from Vine

A vine genomic library was screened with a probe consisting in an equimolar mix of the Arabidopsis AKTI (Sentenac et al., 1992) and KAT1 (Anderson et al., 1992) Shaker K⁺ channel cDNAs. Of 500,000 clones (equivalent to about 10 times the grapevine genome, i.e. 10 times 475 Mb, as estimated by Lodhi and Reisch [1995]), 10 positive phages were obtained after the first screening round, two of which were discarded during further purification rounds. Recombinant DNA from the purified phages was amplified and digested by several restriction enzymes (SacI, BamHI, EcoRI, NotI, PstI, and HindIII). The restriction patterns of five of the eight clones were identical, reducing to four the number of clones different from each other. Southern blots of these four clones displayed hybridization with the AKTI/KAT1 probe. For two of the clones, the hybridization signals corresponded to two SacI restriction fragments, exhibiting sizes of 2.8 and 3.4 kb. BamHI digestion of the third clone led to a 4-kb fragment also revealed by hybridization. Finally, a 12-kb fragment resulting from digestion of the fourth clone by NotI was also hybridized, but because of its size, it was not further characterized. PCR analyses and sequencing revealed overlaps of the SacI and BamHI fragments, allowing reconstruction of a single gene, with a coding sequence of about 5 kb and a promoter region of 3 kb. The predicted coding sequence showed similarities with K⁺ channels of the Shaker family. The gene was named SIRK, for Stomatal Inward Rectifying K⁺ channel (see below). SIRK shares the strongest similarities (see below) with members of the so-called KAT subfamily (Ache et al., 2000), which comprises KAT1 and KAT2 in Arabidopsis and KST1 in potato (Solanum tuberosum).

Cloning of SIRK cDNA

Assuming that SIRK was expressed in the same organs or cell types as the Arabidopsis KAT1 and KAT2 genes, i.e. mainly guard cells (Nakamura et al., 1995; Pilot et al., 2001), RT-PCR experiments were performed on leaf RNAs, with several primers designed from the predicted exons of the SIRK gene. Analyses of the sequence of the amplified fragments proved that SIRK was effectively expressed in grapevine leaves. Amplification of the complete cDNA in a single reaction failed, for unclear reasons. It was observed that SIRK mRNA accumulation level was...
Figure 1. The grapevine SIRK gene encodes a Shaker-like K⁺ channel related to the Arabidopsis KAT2 channel. A, Schematic representations of the predicted domains of SIRK and KAT2 channels. S1 through S6, Six membrane-spanning segments (light gray) and linkers (dark gray) forming the channel transmembrane domain. P, Conserved pore-forming domain. K HA, C-terminal domain supposed to be involved in channel subunit tetramerization and/or clustering in the membrane. B, Dot matrix comparison (DNA Strider program [Marck, 1990]) polypeptide homology matrix; stringency = 7, window = 15 amino acids) of the deduced SIRK amino acid sequence (accession no. AF359521) with that of KAT2 (accession no. AJ288900). C, Alignment of SIRK and KAT2 voltage sensor (S4) and pore sequences. Identical amino acids are written in white, similar amino acids are written in black on a gray background. D, Phylogenetic tree of plant potassium channels deduced from full-length cDNA. This tree was generated by the Darwin program (Gonnet et al., 1992) with the complete protein sequences of the channels. Arabidopsis sequences are underlined. Protein accession numbers are AKT1, S23606; SKT1, T07651; ZmK1, T03939; SPIK, AJ309323; AKT6, CAA22577; LKT1, X96390; KAT1, S32816; KAT2, T04931; KST1, S55349; AKT2, AAA97865; NpK1, BAA84085; SKT2, T07052; VIK1, T12177; SPIK1, AAD16278; SPIK2, AAD39492; ZmK2, CAB54856; AtKC1, G7488024; KDC1, AJ249962; SKOR, CAIA1280; TaAKT1, AF207745; GORK, AJ279009; SPORK1, AJ299019; PTK2, AJ271447; PTORK, AJ271446; Mkt1, AF267753; and SIRK, AF359521.
low, and splicing intermediaries were often obtained when performing the above-mentioned RT-PCR experiments (not shown). The complete cDNA therefore was amplified as two fragments that were joined thanks to a unique NsiI restriction site in the coding sequence. Sequence comparison confirmed that the reconstructed cDNA corresponds to the SIRK gene, and not to an artifactual chimera obtained from transcripts of two different genes.

The resulting cDNA is 2.4 kb long, encoding a 791-amino acid protein, with a predicted molecular mass of 98.1 kD. The hydropathy profile of the polypeptide suggests the presence of a hydrophobic core composed of six membrane-spanning segments (not shown). Detailed analysis indicated that the protein exhibits the same structure as Shaker channels of the KAT type (Fig. 1A; see “Discussion”): (a) a short, cytoplasmic N-terminal region; (b) a hydrophobic core composed of six transmembrane segments and a pore-forming domain (P) between the last two transmembrane segments; and (c) a long C-terminal region containing a putative cyclic nucleotide binding domain (cNBD) and the so-called KHA domain, rich in hydrophobic and acidic amino acids (Fig. 1A). SIRK shares 70% identical amino acids with KAT2 and 56% with KAT1 (Table I). These similarities are mainly detected in the regions lying upstream of the putative cNBD (Fig. 1B). In particular, the P domain (pore) and the S4 segment (involved in voltage sensing) of SIRK differ from those of KAT2 by only two residues (Fig. 1C). However, SIRK displays a particular feature when compared with KAT2 and all the KAT-like channels cloned so far: It possesses a small ankyrin domain, located downstream from the putative cNBD, and reminiscent of the ankyrin domain found in plant Shaker channels of the AKT1, AKT2, and SKOR subfamilies. But whereas AKT- and SKOR-like polypeptides possess five to six ankyrin repeats (33 amino acids each; Lux et al., 1990) in tandem, SIRK displays only one complete repeat, surrounded by two one-half repeats. It is worthwhile to note that this ankyrin domain is responsible for a decrease in similarity between SIRK and KAT2: When this domain is not considered, the two proteins share 83% identity, a score higher than that observed between KAT1 and KAT2, the two members of the KAT subfamily in Arabidopsis. In other words, when the ankyrin domain is ignored, the grapevine channel SIRK is found more related to the Arabidopsis KAT2 channel than the twin Arabidopsis channels KAT1 and KAT2 to each other (72% identity; see Table I).

The close relationships between KAT1, KAT2, and SIRK were also assessed by a phylogenetic approach. These three channels are closely associated on the tree presented in Figure 1D, i.e. on the same branch, whereas KST1, which is a KAT-like channel expressed in potato guard cells, is located on a different branch. Thus, the SIRK small ankyrin domain did not influence the positioning of this channel in the phylogenetic tree, i.e. SIRK was not placed in a group of channels possessing ankyrin repeats. Therefore, the usual classification of Shaker channels in proteins with or without an ankyrin domain (Chérel et al., 1996) must be reconsidered.

Comparison of the cDNA and gene sequences allowed determination of intron positions, number and length. Like the KAT2 gene, SIRK contains 10 introns (KAT1 possesses only eight introns). In both KAT2 and SIRK genes, these introns are positioned exactly at the same places within the same codons and follow the AG/GT rule for splice junctions (Hanley and Schuler, 1988). It is therefore remarkable that the presence of an ankyrin domain in SIRK does not result in altered gene structure when compared with the KAT2 gene. The introns in SIRK are, in general, slightly longer than in KAT2, but remain in the range of plant intron mean lengths (Hawkins, 1988). Finally, the SIRK 5′-untranslated region was shown to be very long (data not shown). 5′-RACE experiments suggested that this region is about 1 kb long (the longest untranslated 5′ region we isolated was 850 bp long). When analyzing the corresponding genomic sequence, a putative transcription start site (ATATCA) was identified at 967 bp from the translation start codon. A putative TATA box (TTATT) was localized at about 25 bp upstream from this transcription start site, which is in agreement with the consensus distances generally found between these two regulation sites.

<table>
<thead>
<tr>
<th>Channel</th>
<th>AKT1</th>
<th>AKT2</th>
<th>SKOR</th>
<th>KAT1</th>
<th>KAT2</th>
<th>SIRK</th>
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<tr>
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<td>24</td>
<td>76</td>
<td>–</td>
<td>70</td>
</tr>
<tr>
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<td>58</td>
<td>50</td>
<td>50</td>
<td>67</td>
<td>81</td>
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</table>

Number of SIRK Copies in the Grapevine Genome

Southern blots of leaf genomic DNA (grapevine cv Pinot noir) were performed, using a fragment of SIRK cDNA (Fig. 2B) as a probe (Fig. 2). The banding pattern (Fig. 2A) for any of the three tested restriction enzymes is as expected from the restriction pattern of the SIRK gene (Fig. 2B). Furthermore, low-stringency Southern-blot analysis of grapevine genome with a KAT1/KAT2 probe resulted in the same banding pattern (not shown). These data indicate that SIRK is probably a single-copy gene. If
SIRK was mainly carried by K⁺ ions. Determination of reversal potential in pseudo bi-ionic conditions (Bruggemann et al., 1993) allowed determination of relative permeability ratios (not shown). SIRK displays the following permeability sequence: \( K^+ > Rb^+ \gg Na^+ \) (Table II).

SIRK gating parameters were determined and proved to be close to those of KAT1 and KAT2: the values of the gating charges for the three channels are 2.8 for SIRK, 2.5 for KAT2, and 1.6 for KAT1, and the half-activation potential values are \(-161 \text{ mV} \) for SIRK, \(-152 \text{ mV} \) for KAT2, and \(-130 \text{ mV} \) for KAT1 (Table II; Pilot et al., 2001). Based on these values, the gating properties of SIRK are more related to those of KAT2 than to those of KAT1.

In our experimental conditions (expression level, size of patch), macroscopic SIRK currents mimicking whole-oocyte currents could be recorded in the cell-attached patch-clamp configuration (Fig. 3D, current trace labeled 1). Upon patch excision, however, the SIRK current decreased rapidly (inside-out configuration; Fig. 3D, current trace labeled 2). SIRK rundown could be overcome and the initial current partly restored by cramping the patch into the oocyte (Fig. 3D, current trace labeled 3). This suggested that SIRK opening required intracellular factors, available in the oocyte cytoplasm.

The patch-clamp technique was also used to investigate single-channel properties of SIRK expressed in X. laevis oocyte. Figure 4A shows an example of the most frequent single-channel activity recorded in membrane patches from SIRK-injected oocytes. At steady state, this activity resulted only in inward currents and was observed only at potentials negative to \(-80 \text{ mV} \). The sum of 200 current records obtained at \(-140 \text{ mV} \) revealed that the single-channel current shown in Figure 4A was mediated by SIRK: The resulting curve was clearly reminiscent of the macroscopic current trace recorded by the two-electrode voltage-clamp technique (Fig. 4B). Analysis of the single-channel current-voltage relationship allowed determination of the single-channel slope conductance (Fig. 4C): 13 pS in 100 mM external potassium concentration. The voltage sensitivity of the single channel current (Fig. 4D) was also reminiscent of that of the macroscopic current (Fig. 3A).

Block by external Cs⁺ is a classical feature of plant and animal K⁺ channels that is believed to involve binding of Cs⁺ to some site within the pore (Hille, 1992). Need of pore penetration by Cs⁺ for its blocking action is deduced from the voltage dependence of the K⁺ channel block as reported for the KAT1 channel (Véry et al., 1994; Becker et al., 1996). Addition of 0.1 mM Cs⁺ resulted in a strong block of SIRK inward currents (more than 80%; Table II). This effect was poorly voltage dependent, similar to the Cs⁺ block of the KAT2 channel (Pilot et al., 2001).

**Functional Characterization in X. laevis Oocytes**

In X. laevis oocytes injected with SIRK cRNA, hyperpolarization of the membrane beyond \(-120 \text{ mV} \) elicited an inward current (Fig. 3A) that was not recorded in control oocytes injected with water (data not shown). The exogenous macroscopic current displayed slow activation (Fig. 3A) and deactivation (data not shown). No inactivation could be seen even during hyperpolarizing pulses lasting 50 s (not shown). The steady-state current (Fig. 3B) and the G/G\textsubscript{max}-voltage plots (Fig. 3C) showed a strong inward rectification with a threshold potential of about \(-120 \text{ mV} \). The G/G\textsubscript{max}-voltage plot shows that the gating parameters (\(E_{\text{act}}\), the half-activation potential and \(z_g\), the so-called gating charge) are insensitive to external K⁺ concentrations (Fig. 3C). The reversal potential (\(E_{\text{rev}}\)) of SIRK current was determined at different external K⁺ concentrations and was found to remain close to \(E_K\) (Fig. 3B, inset), indicating that the inward current mediated by SIRK was mainly carried by K⁺ ions. Determination of reversal potential in pseudo bi-ionic conditions (Bruggemann et al., 1993) allowed determination of relative permeability ratios (not shown). SIRK displays the following permeability sequence: \( K^+ > Rb^+ \gg Na^+ \) (Table II).

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Figure 3. SIRK is an inward-rectifying voltage-gated channel, as assessed by macroscopic current analysis in *X. laevis* oocytes. A through C. Analysis by two-electrode voltage-clamp. The bath solution contained 1 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-NaOH (pH 7.4), and 100 mM (K + Na) Cl (K<sup>+</sup> concentration indicated below). A. SIRK currents elicited by 1.5-s voltage pulses from 0 to −165 mV in 12 steps (−15-mV increments) from a holding potential of −40 mV in a 100 mM K<sup>+</sup> solution. B. The steady-state current at the end of the activation step was plotted against membrane potential for three different external K<sup>+</sup> concentrations (in mM): 100 (circles), 10 (squares), and 2 (triangles). Inset. Plotting the reversal potential (E<sub>rev</sub>) for the SIRK current (black circles) versus the external concentration of K<sup>+</sup> revealed that E<sub>rev</sub> shifted by 56.5 mV for a 10-fold increase in external K<sup>+</sup> concentration, as expected for a highly selective K<sup>+</sup> channel. Solid line in the inset, K<sup>+</sup> equilibrium potential (E<sub>K</sub>) calculated with the Nernst equation assuming a 113 mM intracellular K<sup>+</sup> concentration (i.e. E<sub>rev</sub> = E<sub>K</sub> in 100 mM external K<sup>+</sup>). C. SIRK activation level at steady state for three different K<sup>+</sup> concentrations (in mM): 100 (diamonds), 10 (squares), and 2 (triangles); n = 3 oocytes. A two-state Boltzmann relation (dotted line; Lacombe and Thibaud, 1998) modeled the G/G<sub>max</sub> ratio. D. Analysis of macroscopic currents assessed by patch-clamp (patch-excision and patch-cramming). Both bath and pipette solutions contained 100 mM KCl, 2 mM MgCl<sub>2</sub>, and 10 mM HEPES-NaOH (pH 7.4). In the cell-attached patch-clamp configuration, the current-voltage curve (1) is reminiscent of those obtained by two-electrode voltage-clamp (see B). After patch excision (inside-out configuration), the current amplitude decreased very quickly (trace 2, obtained 10 s after the patch excision). Patch cramming into the oocyte produced an increase in current (trace 3). Data shown are representative of five independent patch-cramming experiments.

Localization of SIRK Expression

Localization of SIRK expression was investigated by the reporter gene approach. The promotor sequence of SIRK (3 kb) was fused to GUS cDNA (encoding the *Escherichia coli* β-glucuronidase), and the resulting construct was used for *Agrobacterium tumefaciens*-mediated transformations of both Arabidopsis and grapevine. About 1 year is necessary after the transformation steps (performed on embryogenic calli) to regenerate transgenic vines.

In Arabidopsis, reporter gene activity was analyzed on F<sub>1</sub> and F<sub>2</sub> progeny of 10 independent transgenic plants. β-glucuronidase (GUS) activity was systematically detected in guard cells of leaves, stems, and petioles (Fig. 5, A–C). It was also detected in leaf xylem tissues, but with different patterns depending on the lines: The plants could present either a coloration in all the xylem tissues, or in the primary vessels, or only in the minor veins. Some leaves displayed no staining at all. GUS activity was never detected in roots.

GUS activity was also detected in each of the 13 independent grapevine lines obtained. The staining was restricted to guard cells in cotyledons, leaves, and petals assessed by patch-clamp (patch-excision and patch-cramming). Both bath and pipette solutions contained 100 mM KCl, 2 mM MgCl<sub>2</sub>, and 10 mM HEPES-NaOH (pH 7.4). In the cell-attached patch-clamp configuration, the current-voltage curve (1) is reminiscent of those obtained by two-electrode voltage-clamp (see B). After patch excision (inside-out configuration), the current amplitude decreased very quickly (trace 2, obtained 10 s after the patch excision). Patch cramming into the oocyte produced an increase in current (trace 3). Data shown are representative of five independent patch-cramming experiments.

All the Arabidopsis Shaker-like K<sup>+</sup> channels characterized so far at the functional level are sensitive to changes in external pH. The outwardly rectifying channels SKOR and GORK and the weakly inwardly rectifying channel AKT2 are inhibited by external acidification (Marten et al., 1999; Ache et al., 2000; Lacombe et al., 2000a, 2000b) while the inwardly rectifying channels KAT1 and KAT2 are activated (Pilot et al., 2001, and references therein). Decrease in external pH from 7.4 to 6.0 induced a positive shift of SIRK activation potential (Table II), leading to an increase in current amplitude at a given potential. Analyses of the corresponding G/G<sub>max</sub> ratio versus potential curves (not shown) showed that, whereas the apparent gating charge (z<sub>a</sub>) was not changed, the half-activation potential (E<sub>rev</sub>) was shifted by approximately +30 mV when the pH was decreased from 7.5 to 6.0 (Table II). At external pH 7.0, a decrease in internal pH from 7.4 to 7.0 was obtained using acetate solution as described previously (Lacombe et al., 2000b). This cytosolic acidification also induced a positive shift of the half-activation potential (close to +10 mV) without affecting the gating charge (Table II).

**Channel in Grapevine Guard Cells**
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Table II. Functional features of SIRK channel

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<th>Selectivity</th>
<th>$P_{Rb}/P_K$</th>
<th>$P_{Na}/P_K$</th>
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<tr>
<td>pH effect on gating ($n = 4$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$pHe/pHi$</td>
<td>7.5/7.4</td>
<td>6.0/7.4</td>
</tr>
<tr>
<td>$Z_e$</td>
<td>$2.79 \pm 0.09$</td>
<td>$2.8 \pm 0.07$</td>
</tr>
<tr>
<td>$E_{c50}$</td>
<td>$-158 \pm 2$</td>
<td>$-129 \pm 2$</td>
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<tr>
<td>Caesium block (0.1 mM, inhibition in % of control, $n = 3$)</td>
<td></td>
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<tr>
<td>$-160$ mV</td>
<td>$-140$ mV</td>
<td>$-120$ mV</td>
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<td>$83 \pm 2$</td>
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SIRK belongs to the Shaker-like family of plant K$^+$ channels. The members of this family share sequence similarities and structural homologies with animal Shaker channels (Jan and Jan, 1997). Since the initial cloning of KAT1 (Anderson et al., 1992) and AKT1 (Sentenac et al., 1992) in Arabidopsis, the plant Shaker family has increased (Ache et al., 2000), with nine members identified in Arabidopsis (Zimmermann and Sentenac, 1999) and at least 16 in other plants (see Fig. 1D). As their animal counterparts, plant Shaker channels are thought to be tetrameric structures, formed by four α-subunits arranged around a central pore (Mackinnon, 1991; Daram et al., 1997; Urbach et al., 2000). The transmembrane hydrophobic core of the Shaker subunit harbors six membrane-spanning segments, named S1 to S6 and a pore-forming domain, P, between S5 and S6. S4 is characterized by the presence of regularly spaced basic amino acids rendering the channel sensitive to changes in transmembrane electrical potential (Zei and Aldrich, 1998; Bezanilla, 2000). Movements of S4 within the membrane in response to changes in membrane polarization result in conformational changes of the protein, leading to pore opening or closing. The channel is said to be voltage gated, and S4 is defined as the channel voltage sensor. The P domain is usually the most conserved domain in Shaker proteins, and petioles (Fig. 5, D–H). To our knowledge, this work is the first in which a reporter gene approach using a specific promoter has been developed in grapevine. Thus, in parallel experiments, we also transformed calli with a control construct, namely the fusion of a minimal 35S promoter to GUS cDNA. In the seven independent lines analyzed, GUS activity was systematically detected in the vascular tissues (particularly in the minor veins) of leaves (Fig. 5, I) and in root tips (not shown). This pattern is not as extended as previously observed with more strengthen-d versions of the 35S promoter (Baribault et al., 1997) and in root tips (not shown). This pattern is not as extended as previously observed with more strengthen-d versions of the 35S promoter (Baribault et al., 1997; Urbach et al., 2000). A few sequences corresponding to aquaporins and to a (H$^+$) pyrophosphatase are accessible in databases. To our knowledge, SIRK is the first K$^+$ transport system cloned in vine.
channels. It presents a hallmark motif, GYGD, typical of highly selective K⁺ channels. The C-terminal part of the polypeptide is cytoplasmic (Uozumi et al., 1998). In plant Shaker channels, three regions can be distinguished within it. The first one is a putative cyclic nucleotide binding site, followed by a variable domain, which may be composed of ankyrin motif repetitions (AKT1, AKT2, and SKOR subfamilies) or sequences sharing no similarity with other proteins (KAT and AtKC subfamilies). The third region corresponds to the so-called KHA domain, rich in hydrophobic and acidic residues. Two-hybrid (Daram et al., 1997) and green fluorescent protein fusion (Ehrhardt et al., 1997) experiments suggest that this domain is involved in Shaker polypeptide tetramerization and/or channel clustering in the membrane.

Some Arabidopsis Shaker channels have been shown to be involved in long-term, wholesale K⁺ transport. Integrated approaches combining electrophysiological analyses in planta and/or in heterologous systems, expression studies, and reverse genetics have led to the demonstration that AKT1 is involved in K⁺ nutrition (Lagarde et al., 1996; Hirsch et al., 1998; Spalding et al., 1999), SKOR in K⁺ secretion into the xylem sap (Gaymard et al., 1998) and SPIK in pollen tube growth (Mouline et al., 2002). Expression studies support the hypothesis that AKT2 plays a role in K⁺ transport in phloem tissues in both source and sink organs (Lacombe et al., 2000b). KAT1 (Nakamura et al., 1995), KAT2 (Pilot et al., 2001), and GORK (Ache et al., 2000) are expressed in guard cells. KAT1 and KAT2 are inwardly rectifying channels (Schachtman et al., 1992; Pilot et al., 2001) supposed to mediate K⁺ influx during stomata opening. On the other hand, the outwardly rectifying K⁺ channel GORK could mediate the K⁺ efflux leading to stomata closure (Ache et al., 2000). RT-PCR experiments suggest that other Shaker channel genes, AKT1, AKT2, and AtKC1, could be expressed in guard cells, and characterization of an Arabidopsis mutant knock-out for the KAT1 gene has recently revealed that the encoded polypeptide is not essential for stomatal opening (Szyroki et al., 2001).

SIRK shares the highest similarities with the members of the KAT subfamily within the plant Shaker family (Table I). It is most related to the Arabidopsis KAT2 channel, based on sequence analysis of the deduced polypeptides. Similarities are also found at the gene level, because SIRK and KAT2 have strictly the same number of introns, placed at the same positions, referring to the coding sequence. These data suggest that SIRK and KAT2 have been derived from a common ancestral gene that was subsequently duplicated and independently adapted for different functions in guard cells and other tissues.

Figure 4. Single channel analysis of SIRK in X. laevis oocytes. Both bath and pipette solutions contained 100 mM KCl, 2 mM MgCl₂, and 10 mM HEPES-NaOH (pH 7.4). A, SIRK single-channel currents in a X. laevis oocyte recorded at −100, −125, and −140 mV. C, Closed state; O1, O2, and O3, one, two, and three open channels, respectively. B, Upper trace, Sum of 200 pulses at −140 mV from a holding potential of 0 mV. Bottom trace, Current at −140 mV measured by two-electrode voltage-clamp (see Fig. 3A). Both traces were normalized to the steady-state value for easier comparison. C, Single-channel current-voltage relationship measured by patch-clamp experiments (n = 5). Single channel conductance: 13 pS in K⁺ 100 mM. D, Voltage-sensitivity of open probability of SIRK.
suggest that SIRK is the orthologous gene of KAT2 in vine.

As the Arabidopsis Shaker channels KAT1 and KAT2, SIRK expressed in X. laevis oocytes behaves as a voltage-gated, inwardly rectifying channel, highly selective for K/H^+ in accordance with the presence of the tetrapeptide GYGD in its pore. Detailed comparison of the functional features of SIRK with those of KAT1 and KAT2 indicates that SIRK is more related to KAT2, with closer activation potentials, gating charges, and pH sensitivities. Furthermore, SIRK and KAT2 are both blocked by Cs/H^+ in an essentially voltage-independent way, whereas the block of KAT1 by this cation is clearly voltage dependent (Véry et al., 1994). Therefore, sequence comparison between KAT1, KAT2, and SIRK could help in investigating the structure-function relationship of these channels.

In summary, both sequence analysis and functional characterization lead to the conclusion that SIRK is most related to KAT2 among the members of the Shaker family in Arabidopsis. KAT1 and KAT2 are both expressed in Arabidopsis guard cells (Nakamura et al., 1995; Pilot et al., 2001). SIRK promoter also directs expression of the GUS reporter gene in guard cells. In addition, KST1, a KAT-type Shaker channel cloned in potato, is expressed in guard cells (Müller-Röber et al., 1995). These data suggest that the expression pattern of KAT-like genes could be conserved among different plant species. Such a situation would be different from that described for the plasma membrane H^+-ATPase gene family, because no relationship can be proposed between H^+-ATPase gene expression patterns in Arabidopsis and in tobacco (Nicotiana tabacum; Morosmon and Boutry, 2000).

KAT1 and KAT2, and all other channels belonging to the KAT subfamily identified so far possess no ankyrin domain (KST1 in potato; sequence BAA96150 in rice [Oryza sativa]; ZmKT1 in maize [Zea...
Despite its high similarities with KAT-type channels and its positioning on the same branch as KAT1 and KAT2 in the phylogenetic tree, SIRK harbors an ankyrin domain. When fewer plant Shaker channels had been identified, they were classified by taking into account the presence or absence of the ankyrin domain (Chérél et al., 1996), the channels being said of the SIRK or KAT type, respectively. The cloning of SIRK demonstrates that KAT-like channels can have ankyrin domains. We propose that the presence of this ankyrin domain is an indication of the evolution of plant Shaker channels: The common ancestor of all plant Shaker channels may have possessed the ankyrin domain, and the ancestor of KAT-type genes would have lost this domain. Thus, SIRK could represent an intermediate step in the loss of this domain, with only a small remaining ankyrin domain (one complete motif, surrounded by two half-motifs, instead of six repeats as in AKT-type channels). Ankyrin domains are involved in protein-protein interactions (Hoffman, 1991). The ankyrin domain present in plant Shaker channels does not seem to be involved in interactions between the four polypeptides forming the functional channel (Daram et al., 1997). It is therefore likely that this domain interacts with other proteins, cytosolic or associated to plasma membrane. In the absence of any information regarding these targets and the role of the ankyrin domain in AKT type channels, the meaning of conservation or loss of this domain in plant Shaker channels cannot be assessed. It is worth noting that no ankyrin domain has been identified in any Shaker channel identified in animal cells up to now. The presence of such a domain seems to be typical of plant channels.

The expression pattern of the SIRK:GUS reporter gene construct was investigated here in both grapevine and Arabidopsis. In both the homologous and heterologous context, the SIRK promoter region was consistently active in guard cells. Thus, Arabidopsis could provide an interesting alternative for studying grapevine promoters and identifying regulatory boxes, considering the time necessary for regeneration of transgenic vines. In Arabidopsis, additional activity of the reporter gene was often detected in leaf xylem parenchyma. This expression could be artifactual, because of the heterologous genetic background. However, it cannot be excluded that SIRK is expressed in xylem tissues in grapevine, but in organs or at developmental stages that could not be tested, or in specific environmental conditions. Checking the latter hypothesis will be possible when the transgenic lines are older and grown in field conditions.

RT-PCR experiments showed that SIRK was expressed in the grape berries. This result will be verified by analysis of the vine transgenic lines expressing the SIRK:GUS construct, when the plants are old enough to produce berries (this requires about 2 years). Semiquantitative RT-PCR analysis indicates that SIRK transcript accumulation varies during berry development, the amount of SIRK transcript decreasing drastically by the time of veraison. As stated in the introduction, veraison is a period of deep physiological and histological modifications in the berry. Nutritional status of the berries switches from (photosynthetic) source to (no longer photosynthetic) sink, whereas functional stomata and xylem tissues are lost by these organs. Based on the available expression data (Fig. 5), we propose that the decrease in SIRK transcript accumulation in the berry after veraison is due to the fact that SIRK is expressed in guard cells and/or in xylem tissues of this organ (only before veraison). Therefore, SIRK could be involved in the regulation of berry water loss and/or K⁺ loading before veraison. The identification of the SIRK gene is therefore likely to provide the first molecular tool for investigating these aspects of berry development.

MATERIALS AND METHODS

Plant Material

Aerial tissue samples were collected from grapevine (Vitis vinifera cv Pinot noir), from Agro-M/Institut National de la Recherche Agronomique collections (Montpellier, France), in the 1999 season. Roots were collected from 1-year-old rooted canes planted in “Perlite” 10-L containers and maintained with Hoagland solution once a week. Embryogenic calli of grapevine cv Portan were maintained as described by Torregrosa (1998).

Library Screening

A genomic library prepared in λ-GEM-12 (Sarni-Manchado et al., 1997) was screened using standard plaque-lift method (Sambrook et al., 1989). The probe consisted of a mix of AKT1 (Sentenac et al., 1992) and KAT1 (Anderson et al., 1992) channel cDNAs radiolabeled with [32P]dATP and dCTP (random priming kit, Promega, Madison, WI). Filters were prehybridized at 65°C in Church buffer (Church and Gilbert, 1984) for 2 h and then hybridized in the same solution with added radiolabeled probe at 65°C for 48 h. Filters were washed twice in 2× SSC and 0.1% (w/v) SDS for 10 min each at room temperature and once in 0.1× SSC and 0.1% (w/v) SDS for 15 min at 65°C. The positive fragments were subcloned in pBS SK+, sequenced, and compared with the GenBank data library.

RNA Extraction

Samples were collected and immediately frozen in liquid nitrogen and stored at −80°C until use. As described by Fillon et al. (1999), two different procedures were performed to achieve RNA isolation, one for vegetative tissues and the other one for grapevine berry pericarp.
RT-PCR

Ten micromgrams of RNA was denatured at 65°C for 5 min, and RT was performed for 30 min at 42°C in 30 μL in the presence of 200 units of Superscript II (Gibco-BRL, Cleveland) and 5 pmol of specific primer (SIRK reverse, 5′-CATTATAGGTTTCTGAACTTACCAGG-3′). Then, 1 μL from this reaction was amplified in a final volume of 50 μL with 5 pmol of each primer (SIRK forward, 5′-GTTGCTGTCTGCAACCGG-3′; SIRK reverse, see above) and 0.5 units of Thermus brokianus polymerase (Extrapol I, Eurobio, Les Ulis, France), following the manufacturer’s protocol.

Southern-Blot Analysis

of the two cDNAs by calculating the ratio of the amount of the two cDNAs by calculating the ratio of the amount of

Semiquantitative RT-PCR

For each developmental stage of berries, three RT reactions were performed as described above, with three different batches of RNA. The products of these three reactions were pooled to alleviate the differences in reaction efficiency. One microliter of these pools was then used for PCR amplification. Three PCR reactions were repeated independently for each developmental stage and for both the SIRK gene and a control gene, EF1γ. The PCR reactions were performed as described above, with 24 cycles consisting of 45 s at 95°C, 45 s at 50°C, and 90 s at 72°C. The primers used for these amplifications were SIRK forward and SIRK reverse (see above), EF1 forward (5′-ACGTITTTACGCGGCAAAGATACC-3′) and EF1 reverse (5′-TTTGGATAGGTAACGTATCACTTAAATAAC-3′). The amplified fragments were about 300 bp long. Every two cycles between cycle 10 and cycle 24, 2.5 μL was taken from the PCR reaction and blotted on a nylon membrane as dots. These dots were hybridized with 300-bp probes (SIRK or EF1) corresponding to the amplified fragments. Hybridization was performed at 45°C in 50% (v/v) formamide, 5× SSC, 10% (w/v) dextran sulfate, and 1% (w/v) N-laurylsarcosine. Filters were washed twice in 2× SSC, 0.1% (w/v) SDS for 10 min each at room temperature and once in 0.1× SSC and 0.1% (w/v) SDS for 15 min at 50°C. Signals on the hybridization membranes were quantified by a PhosphorImager (STORM, Molecular Dynamics, Sunnyvale, CA) and analyzed with the software Image-Quant (Molecular Dynamics).

Statistical analyses indicated that the amplification was linear (as a semilogarithmic function) between cycles 14 and 22 for both cDNAs. We estimated the relative levels of both cDNAs by calculating the ratio of the amount of the two cDNAs by calculating the ratio of the amount of

Grapevine Transformation

The SIRK promoter:GUS construct was introduced into A. tumefaciens MP90 (Höfgen and Willmitzer, 1988). Arabidopsis (ecotype Columbia) was transformed using the floral dip method (Clough and Bent, 1998). Selection of T1 seedlings was performed in vitro on Murashige and Skoog/2 medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) Suc, 0.7% (w/v) agar, and 30 μL L⁻¹ hygromycin under the following conditions: 21°C/18°C day/night temperature, 16-h photoperiod, and 150 μE m⁻² s⁻¹. For GUS assay, plants were cultivated either in vitro on the same medium and in the same conditions as described above or grown in greenhouse on attapulgite-peat compost (Gaymard et al., 1998).

Arabidopsis Transformation

The SIRK promoter:GUS construct was introduced into A. tumefaciens EHA105 (Li et al., 1995). Twenty milliliters of A. tumefaciens culture (A550 = 0.4 (~1.10⁶ colony forming units mL⁻¹)) was added to 1 g of embryogenic callus, vigorously shaken and incubated for 10 min at room temperature. Calli were then separated from the liquid phase with a 100-μm nylon net (Millipore, Bedford, MA), and transferred onto GS₁CA medium (Franks et al., 1998) for a 2-d coculture. Calli were then washed in half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) with 1 mg mL⁻¹ Augmentin (Duchefa, Haarlem, The Netherlands) and transferred to 55-mm petri dishes containing GS₁CA plus 1 mg mL⁻¹ Augmentin. After 3 weeks, calli were transferred onto 55-mm plates containing selective GS₁CA plus 10 μg mL⁻¹ hygromycin, cefotaxim, and Augmentin. Subsequent subcultures occurred every month under the same conditions except that hygromycin was progressively increased to 25 μg mL⁻¹.

To stimulate germination of putative transformed embryos, the embryogenic calli were transferred on Murashige and Skoog/2 medium plus 25 μg mL⁻¹ hygromycin and 10 μg mL⁻¹ cefotaxim and Augmentin. Only develop-
ing embryo-like structures were further subcultured. To stimulate caulogenesis of germinating embryos, whole root meristem regions plus cotyledon upper parts were discarded and cotyledon basal parts containing the shoot meristem were scarified. This tissue was transferred on BFe2 medium (Torregrosa and Bouquet, 1995) and incubated under attenuated light (approximately 15 μE s⁻¹ m⁻²). Axillary shoots emerged in a few weeks and elongated after two to three subcultures enabling the transfer to rooting medium (Murashigie and Skoog/2 + 5 μM IAA) and plant regeneration.

GUS Assay

GUS histochemical staining was performed according to Lagarde et al. (1996). Cross sections of GUS-stained material were prepared on 4% (w/v) agarose embedded tissues with a Vibracut (Bio-Rad, Hercules, CA).

Expression in Xenopus laevis Oocytes and Electrophysiology

In vitro-transcribed SIRK cRNA were injected into X. laevis oocytes (purchased from Centre de Recherche de Biochimie Macromoléculaire, Centre National de la Recherche Scientifique, Montpellier, France) using a 10- to 15-μm tip-diameter micropipette and a pneumatic injector (20 nL of 1 μg μL⁻¹ RNA solution per oocyte). Control oocytes were injected with 20 nL of deionized water.

Whole-cell currents were recorded as previously described (Lacombe and Thibaud, 1998) using the two-electrode voltage-clamp technique, 3 to 7 d after injection on oocytes continuously perfused with bath solution (see figure legends). Quantitative analyses of macroscopic current that yielded the gating parameters were performed as previously described (Lacombe and Thibaud, 1998). Intracellular pH was monitored using pH-sensitive microelectrodes prepared and used as previously described (Lacombe et al., 2000a).

Patch-clamp experiments were performed on devitellinized oocytes as previously described (Lacombe et al., 2000a). Voltage-pulse protocol application, data acquisition, and data analyses were performed using pClamp (Axon Instruments, Foster City, CA), Winascd (Dr. G. Droogmans, University of Leuven, Belgium) and Sigmaplot (Jandel Scientific, Erkrath, Germany) software.

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