Title:
Prohibitins, Stomatins, and Plant Disease Response Genes Comprise a Protein Superfamily that Controls Cell Proliferation, Ion Channel Regulation, and Death

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Summary:
Prohibitins, stomatins, and a group of plant defense response genes, are demonstrated to belong to a novel protein superfamily. This superfamily is bound by similar primary and secondary predicted protein structures and hydropathy profiles. A PROSITE formatted regular expression is generated which is highly predictive for identifying members of this superfamily using PHI-BLAST. The superfamily is named PID (Proliferation, Ion, and Death), because prohibitins are involved in proliferation and cell cycle control, stomatins are involved in ion channel regulation, and the plant defense-related genes are involved in cell death. The plant defense gene family is named HIR (Hypersensitive Induced Reaction) because its members are associated with hypersensitive reactions involving cell death and pathogen resistance. For this study eight novel maize genes are introduced, four closely related to prohibitins (Zm-PHB1, Zm-PHB2, Zm-PHB3, and Zm-PHB4), one to stomatins (Zm-STM1), and three to a gene implicated in plant disease responses (Zm-HIR1, Zm-HIR2, and Zm-HIR3). The maize Zm-HIR3 gene transcript is up-regulated in a disease lesion mimic mutation Les9, supporting a role in maize defense responses. Members of this super gene family are involved in diverse functions, but their structural similarity suggests a conserved molecular mechanism, which we postulate to be ion channel regulation.

Keywords:
Apoptosis, cancer, hypersensitive reaction, mitochondria, potassium channels, programmed cell death, PROSITE format regular expression, stomatocytosis

Abbreviations:
PCD, programmed cell death; HIR; hypersensitive induced reaction; HR, hypersensitive reaction; PID, proliferation, ion, and death; PHB, prohibitin; STM, stomatin
Introduction:

Plants frequently respond to pathogen attack with the “hypersensitive reaction” (HR), a rapid localized necrosis at the site of infection that cordons off the pathogen and limits its spread (1-3). The HR cell death phenomenon bears similarities to programmed cell death or apoptosis observed in animals (3). A group of tobacco genes were identified that caused the formation of HR-like lesions on tobacco leaves when ectopically expressed from a tobacco mosaic virus (TMV) expression vector (4). One of these genes, represented by a cDNA called NG1, caused both HR-like lesion formation and induced expression of beta-glucanase, a pathogenesis-related protein marker for defense activation. NG1 was thus interpreted to be a novel activator of the plant HR defense system (4). The predicted peptide for the cDNA NG1 was presented as 64 amino acids with no significant homology to any known proteins (4). Independently, an Arabidopsis NG1-like cDNA (gene 106) was reported to represent a mRNA induced by the plant defense activator isonicotinic acid (5). This induction was associated with systemic acquired resistance or SAR (SAR, reviewed in ref. 6) and occurred independent of de novo protein synthesis (5).

Prohibitins are a group of highly conserved proteins that are thought to control cell cycle, senescence, and tumor suppression (reviewed in ref. 7). Prohibitins negatively control cell cycle in the early G1 phase and specifically inhibit initiation of DNA synthesis (8, 9). Prohibitin genes appear to be expressed in many tissues and organisms, but with some modulation of expression consistent with a role in cell cycle (7,8,10). Mutations or deletions of prohibitin are linked to some human breast and ovarian cancers, supporting the idea that prohibitin suppresses tumors as part of its antiproliferative function involving cell cycle control (11-13). Prohibitins are also implicated in controlling senescence and aging, with which there may be a functional link to their antiproliferative function and cell cycle control (7).

Prohibitins are localized largely in the mitochondria, especially in the inner mitochondrial membrane (7) near the periphery (14). Rat and human prohibitins possess a short transmembrane helix near their N-termini, which may be integrated into mitochondrial membranes (7). As mitochondrial inner membrane proteins often control ion transport and ATP production, it has been speculated that prohibitins
may be involved in these processes, in particular in mitochondrial calcium efflux, which regulates ATP formation (7). Prohibitin and a prohibitin-like protein BAP37 (also called prohibitone) have also been localized to the plasma membrane of mouse lymphocytes, where they together interact with the IgM antigen receptor and may function in signaling apoptotic programmed cell death (15). Prohibitin and BAP37 interact directly with each other in animal mitochondria, and in yeast they control replicative lifespan, possibly through control of mitochondrial membrane ionic potential (16).

Stomatin is an integral membrane protein found in red blood cells. In genetic disorders in which this protein is missing a hemolytic anemia called stomatocytosis results. In stomatocytosis the red blood cells experience high passive diffusion of univalent cations, and are often over-hydrated due to an abnormally high amount of intracellular sodium and low amounts of potassium. These red blood cells assume a mouth-like shape, whence stomatocytosis, from 'stoma', Greek for mouth (17). Stomatin is thought to function as a negative regulator of univalent cation permeability. Stomatin has a single membrane-spanning region near its N-terminus, with the rest of the protein thought to be cytoplasmic (18). The molecular mechanism for stomatin function is unknown, but its cytoplasmic portion has been suggested to act as a ball and chain tether that can directly plug ion channels, and may also interact with the cytoskeleton (17). Northern blots detect stomatin mRNA expression in many human tissues besides red blood cells (18).

In this paper we present eight novel full-length cDNA sequences from maize, four of which are closely related to prohibitins, three to the hypersensitive response inducing protein NG1, and one to stomatins. We demonstrate that these eight novel plant genes, along with many animal, bacterial, plant and fungal sequences representing prohibitins, NG1-like proteins, stomatins, and other membrane proteins, comprise a novel protein superfamily. Although these genes are involved in diverse physiological processes, their structural similarity suggests they possess a related biochemical function.
Experimental Procedures:

Gene isolation - The eight full-length maize cDNAs presented in this study were identified in the EST collection at Pioneer Hi-Bred International, Inc. Messenger RNA sources were from various tissues and treatments. The cDNA libraries were created at Pioneer, and the EST sequences were generated at Human Genome Sciences (HGS). The NG1 (HIR), prohibitin and stomatin homologues were identified with the aid of the IRIS software package from HGS, which includes the Blast algorithm, through which homology was indicated to tobacco NG1 (GenBank = GenBank Accession U66271), to prohibitins from various species, and to human stomatin (GenBank = GenBank Accession U33925). Full-insert sequences were produced at Pioneer by primer walking using an A.B.I. 377 sequencing machine. Sequences were assembled using Sequencher™ version 3.0 (Gene Codes Corporation, Ann Arbor, MI) and/or AssemblyLIGN™ (Eastman Kodak Company, New Haven, CT) software.

Protein Sequence Analysis - Initial public database searches were carried out using the BLASTP program (19), with a chickpea HIR-like gene (accession gi|3928150) as a probe, followed by PSI-BLAST (20) with default parameters (Blosum 62, gap existence cost 11, per residue gap cost 1, lambda ratio 0.85, expect threshold 10). About 24 sequences that appeared as significant hits, both in terms of statistical threshold and the type, were, along with the 8 maize sequences presented herein, multiply aligned by ClustalW program with default parameters (21). The residues were reduced to a consensus sequence according to an 80% consensus generated using the CONSENSUS program of Nigel Brown (http://www. bork. embl-heidelberg. de/Alignment/consensus. html). In order to look for conserved motifs in the 32 members included in the multiple alignment, we applied MEME algorithm which resulted in the detection of three highly conserved motifs (26). Highly conserved residues based in part on the MEME motifs were identified to generate a PROSITE format regular expression profile to perform further database searches by PHI-BLAST program (22). Phylogenetic analysis was carried out by using an option within ClustalW (23) to generate multiple alignments followed by distance calculations and tree constructions with the PROTDIST and Neighbor-joining program of the PHYLIP package (24). Secondary structure predictions were carried out by the DSC algorithm using multiple sequence inputs (25). Further structural
analyses were carried out by hydropathy profiles using Kyte-Doolittle method with a 19 residue sliding window (http://bioinformatics.weizmann.ac.il/hydroph/cmp_hydph.html).

**Gene Expression Analysis** - Plant material for mRNA expression analysis was produced from the following three maize families, each with the *Les9* (a disease lesion mutation of maize) mutation segregating 1:1 among the progeny: family 1 (Mo95 18-15 x sibling wild-type +/-Les9; background M14/Mo20W); family 2 (Mo94S 16-35 x sibling wild-type +/-Les9; background M14W23/W23r); and family 3 (Mo95 24-3 x sibling wild-type +/-Les9; background M14/W23). Of the three families, only family 1 with the Mo20W background suppresses the Les9 lesion mimic phenotype.

For the Affymetrix GeneChip® analysis, wild-type and *Les9* mutant plants from all three families were used. Plants were grown in soil in the greenhouse to the V8 stage, which is when the characteristic *Les9* lesions normally begin to appear. The young, upper leaf of *Les9* phenotype plants that did not yet express a lesion phenotype on that leaf and corresponding tissue from wild-type sibling plants was harvested. Using duplicate equal 2 g samples representing each of these six tissues, total RNA was isolated by the TriReagent® method according to the manufacturer’s recommendations (Molecular Research Center, Inc., Cincinnati, OH). Pooled tissue from three different plants formed one sample, and the plants used for each sample were distinct. For GeneChip® expression analysis, one milligram of total RNA from each sample was used for PolyA+ mRNA isolation by the OligoTex resin binding method according to the manufacturer’s recommendations (Qiagen, Chatsworth, CA).

Protocols for preparing in vitro-transcribed biotinylated cRNA probes from poly-A+ mRNA for Affymetrix GeneChip® gene expression analysis were according to the manufacturer’s recommendations (Affymetrix, Santa Clara, CA; Technical Support tel. 1-888-DNA-CHIP), which are described in reference 27. In brief, per sample 2 µg of poly-A+ mRNA, described above in mRNA isolations, was used for the first strand cDNA synthesis. This involved a T7-(dT)$_{24}$ oligonucleotide primer and reverse transcriptase SuperScript II (Gibco-BRL, Gaithersburg, MD). The second strand synthesis involved *E. coli* DNA Polymerase I (Gibco-BRL, Gaithersburg, MD). The double-stranded cDNA was then cleaned up using phenol/chloroform extraction and phase lock gels (5 Prime-3 Prime, Inc., Boulder, CO) followed
by ethanol precipitation. For the in vitro transcription to produce cRNA, biotin-11-CTP and biotin-16-UTP, in addition to all four NTPs, were used with T7 transcriptase (Ambion, Austin, TX). The IVT product was cleaned up using Rneasy affinity resin columns (Qiagen, Chatsworth, CA). Labeled in vitro transcript (IVT) yields ranged from 60-80 µg per sample. They were stored at -80°C until use. The IVT products were fragmented in acetate buffer (pH 8.1) at 94°C for 35 minutes prior to chip hybridization. Per sample equal amounts (12 µg) of in vitro labeled transcript were used to probe each chip overnight. The biotinylated RNA hybridizing to the chips were labeled with a streptavidin-phycoerythrin conjugate, and scanned using a confocal fluorescence microscope. Expression intensity was determined as described (27). Comparisons of mRNA abundance (cRNA abundance) were made per rep between the Les9 and wild-type samples. The average fold change and standard error for all the repetitions per family were calculated and presented.

The GeneChip® used in these experiments was constructed by Affymetrix using a set of 1501 maize cDNA EST sequences, representing nearly as many genes. The genes used to produce this GeneChip® encompass many physiological processes; perhaps a third could be defense related based on their homology to known or suspected defense-related genes. Two of the 1501 ESTs represented Zm-HIR3 (ESTs CMSAR19R and CBPCC63R). Each cRNA GeneChip® probing was replicated twice or thrice (reps A, B and C). In brief, the 1.28 cm x 1.28 cm GeneChip® contain a high-density array of 20-mer oligonucleotides affixed to a silicon wafer. These oligonucleotides were synthesized in situ on the silicon wafer by a light-dependent combinatorial chemical synthesis (27). The oligonucleotide sequences are complementary to the sense strand of Pioneer Hi-Bred’s cDNA EST sequences. For each gene there are up to forty 20-mer oligonucleotides synthesized. Twenty of these oligonucleotides are exact matches to different, though sometimes overlapping, regions of the EST sequence. The other 20 oligonucleotides contain one base mismatch in the center, which changes hybridization efficiency. (For a minority of genes there were less than 20 oligo probe pairs, but never less than 15 pairs per gene). The perfect match (PM) and mismatch (MM) oligo probe pairs for each gene are tiled in adjacent regions of the GeneChip.
Comparison of the hybridization intensities between different PM oligonucleotides for a given gene, and between PM to MM hybridization intensities for an oligonucleotide pair, are used to determine the overall hybridization to the gene, and hence its level of mRNA abundance in the samples (27).

For northern blot analysis, tissue from wild-type and Les9 mutant plants from family 3 was used. Plants were grown in soil in the greenhouse to the V8 stage. Leaf blades (minus midribs) that had developed Les9 lesions on the mature half of the leaf tissue were harvested and divided into basal, lesion-free zone (BL), transition zone where lesions were starting to form (TR), and leaf tip where lesions had reached a mature stage (TP). Corresponding tissue from wild-type sibs was also harvested. Ten micrograms of total RNA was mixed with running dye containing ethidium bromide and electrophoresed at 60 volts for 15 hours on 0.8% agarose gels in MOPS buffer containing formaldehyde, essentially as described (28). The gels were blotted to nylon-backed nitrocellulose membrane and probed with a 1.35 Kb insert from the CMSAR19R cDNA representing Zm-HIR3.

Results:

Three distinct maize cDNAs with high homology to the tobacco NG1 peptide (GenBank = GenBank Accession Number U66271) were identified in the EST collection at Pioneer Hi-Bred International, Inc., and their complete full-length sequences were produced. These three genes were named Zm-HIR1 (GenBank = GenBank Accession Number AF236373), Zm-HIR2 (GenBank = GenBank Accession Number AF236374), and Zm-HIR3 (GenBank = GenBank Accession Number AF236375), for Zea mays Hypersensitive Induced Reaction genes one, two and three, respectively. Initial searches of maize HIR genes using the BLAST program (19) against the public databases indicated some similarity to prohibitins and stomatins, which prompted a search for maize cDNA clones related to prohibitins and stomatins from the same EST collection. Four distinct full-length prohibitin-like maize clones were identified and sequenced, namely Zm-PHB1 (GenBank = GenBank Accession Number AF236368), Zm-PHB2 (GenBank = GenBank Accession Number AF236369), Zm-PHB3 (GenBank = GenBank Accession Number AF236370), and Zm-PHB4 (GenBank = GenBank Accession Number AF236371). In addition,
one full-length stomatin-like clone, named Zm-STM1 (GenBank = GenBank Accession Number AF236372), was also identified and sequenced. Public database searches did not reveal a previously reported plant stomatin-like gene. Pair-wise alignments of the three maize HIR proteins showed high levels of similarity among themselves (>80% identity) and to HIR-like genes from tobacco, chickpea and Arabidopsis (>80% identity). Pair-wise amino acid similarities of plant HIR and HIR-like genes with maize prohibitins were between 28-36%, and with maize stomatin Zm-STM1 between 34-37%. This suggested that the maize HIR genes were somewhat closer in amino acid sequence to stomatins than to prohibitins.

The non-redundant protein database at NCBI was searched using the PSI-BLAST program (20) with a hypothetical protein from chickpea (accession gi|3928150) as a probe, which has over 90% amino acid similarity with the maize HIR proteins. This search identified many genes, including stomatins and integral membrane proteins (E= <10^{-16}) and prohibitins (E= <10^{-8}), and HFLK/HFLC proteins (E=<10^{-6}). Twenty-four of these public sequences, along with the 8 maize sequences introduced above, were used to generate an unrooted dendogram (Fig. 1). This dendogram revealed a large superfamily with at least four constituent families. The stomatins and integral membrane proteins, including a mechanosensor protein from C. elegans (gi|2493263), formed a large family containing sequences from diverse phyla. A second family was composed of HIR and HIR-like sequences from plants. The family consisting of stomatins and integral membrane proteins was most closely related to the HIR family. The third family was composed of prohibitins and related sequences from diverse phyla. The bacterial membrane proteins HFLK/HFLC formed a small fourth family.

Amino acid sequences for 32 members of this superfamily were also multiply aligned to reveal shared and diverged features (Fig. 2). The coding region lengths for the HIR proteins (242-286 aa) are comparable to those of prohibitins (272-289 aa), and many of the stomatins and other membrane-associated proteins (249-481 aa). Relative to prohibitins and stomatins, the HIR proteins are typically shorter at the N-terminus. Several regions of the protein superfamily are highly conserved and aligned well with fewer gaps. Two residues, an Asp and an Ala (corresponding to amino acid positions 64 and
167, respectively, in Zm-HIR1 - being used here as a reference superfamily member), are completely conserved among all the proteins, suggesting a critical role for these residues in the biological function of these proteins. Other amino acids and structural groups of amino acids are also conserved in the PID superfamily, as shown in the consensus sequence (Fig. 2). Also depicted in figure 2 are the consensus DSC predicted secondary structures of each of the families within the PID superfamily. Each of the four families within the PID superfamily share secondary structural features in the same general relative positions, further indicating a relationship between these proteins.

A systematic search for conserved motifs among the aligned sequences was performed using the MEME algorithm. The MEME motifs have been indicated as reliable indicators of family membership (26). The search resulted in the identification of three conserved motifs (Fig. 2). Using Zm-HIR1 as reference again, the amino acid positions of these motifs are as follows: motif 1, 108-167; motif 2, 56-80; motif 3, 23-88. The relative spatial positions of these three motifs in all these genes appears to be spatially well conserved, indicating the possibility for a similar structural orientation in three-dimensional space. Motif 2 is a subset of Motif 3 and is conserved in all members in the alignment. All three motifs are present in all members of the superfamily, except the HFLK/HFLC proteins, which contain only Motif 2. The HFLK/HFLC are bacterial membrane proteins with protease activity, and are involved in lysogenization. They appear to be more distantly related to the other members of this superfamily.

Based upon the amino acid alignment and the motifs derived from MEME algorithm, in particular MEME motif 1, we created a regular expression and used it to search public protein databases as a pattern seed using the PHI-BLAST program (22). This PROSITE formatted regular expression for the PID superfamily is [ILM]-[RK]-x(2)-[VLI]-[PGA]-x(10,11)-[RK]-x(2)-[VLI]-x(7)-[VLIM]-x(6)-[WFY], and corresponds to amino acid position 105 to 139 on Zm-HIR1 (Fig. 2). Using PHI-BLAST and this regular expression, we retrieved 98 sequences which were above the threshold of 0.001 and displayed very significant E-values. Of these, the HIR proteins, stomatins and other membrane-associated proteins had E values <10^{-4}, and prohibitins had relatively higher E values (E= 0.003 - 10.0). This seed pattern was thus effective at retrieving members for each of these three families within the superfamily. In the PROSITE
dictionary, the stomatin (Band 7) signature has been listed as (R-x(2)-[LIV]-[SAN]-x(6)-[LIV]-D-x(2)-T-x(2)-W-G-[LIV]-[KRH]-[LIV]-x-[KR]-[LIV]-E-[LIV]-[KR]). This PROSITE signature corresponds to amino acid position 121-149 on Zm-HIR1, and so it partially overlaps with the PID signature. However, the PID signature accounts for all superfamily members, not just the stomatins. The C-terminal half of the stomatin PROSITE signature extends beyond the C-terminus of the PID signature, and this portion is very stomatin-specific and diverged from the other PID superfamily members (Fig. 2). The PID signature partially overlaps with the MEME Motif 1, and although this Motif 1 is not well conserved in HFLK/HFLC proteins, the PID signature nonetheless recognizes the HFLK/HFLC proteins, affirming that the HFLK/HFLC proteins are indeed distant members of this superfamily. This regular expression pattern presented herein thus represents a signature for all four families contained within this PID superfamily.

A comparison of protein hydropathy plots of maize HIR sequences with prohibitins from maize and Trypanosoma brucei revealed similar structural profiles. A similar comparison of a stomatin-like gene from Synchocystis sp. with Trypanosoma prohibitin also indicated structural similarity between several regions of these genes (Fig. 3). The shared hydropathy plots further indicate that there are conserved structural features between these diverse proteins from widely diverged phyla. Hydropathy analysis of the HFLK/HFLC proteins indicated that these genes have fewer structural similarities in common with other members in this superfamily.

Plant disease lesion mimics are plant variants or mutants that bear symptoms of disease even though they are not infected. Such lesion mimics are common and diverse and they are under extensive study to understand the link between plant programmed cell death and disease responses (29,30). One such mutant of maize is Les9 (partially dominant), which is characterized by numerous spontaneous chlorotic to necrotic lesions that occur by the 9-14 leaf stage (31). The Les9 mutant also shows enhanced resistance to Bipolaris maydis, and enhanced expression of defense-related proteins (Nasser Yalpani, unpublished results). For this study Les9 leaves of V8 plants, a stage just prior to the formation of spontaneous lesions, were investigated for altered levels of Zm-HIR gene expression using the Affymetrix
GeneChip® microarray mRNA profiling technology. The Zm-HIR3 cDNA was represented twice on a GeneChip® representing 1501 genes. A small set of nearly 70 genes were observed to have a two-fold or more change in mRNA abundance. Many of these genes are defense-related, others are unknowns or genes not generally understood to be defense-related. Among this set of 70 were the two examples of Zm-HIR3. Zm-HIR3 showed 2.5 to 8.1-fold enhanced expression in seedling in immature, lesion-free leaves of Les9 mutants compared to those of wild-type siblings in families 2 and 3 (Table I). Northern hybridizations confirmed these microarray results by showing higher Zm-HIR3 transcript levels in Les9 than wild-type plants in family 3 (Fig. 4). This elevated Zm-HIR3 expression was detected prior to the development of a visible lesion phenotype, and was at a stage when Les9 tissue shows enhanced resistance to B. maydis. When Les9 was crossed into the Mo20W background, which considerably suppresses the Les9 lesion mimic phenotype (family 1), the Zm-HIR3 expression was not elevated; in fact it was reduced 2.2-3.3 fold relative to wild-type siblings in the Mo20W background (Table I). Taken together, these results indicate that the Zm-HIR3 gene exhibits modulation in mRNA expression in correspondence to the Les9 disease-related phenotypes.

Discussion:

The eight novel maize gene sequences introduced in this study are structurally related to previously reported prohibitins, stomatins, and a group of plant defense related proteins that we name HIR. By various primary and secondary structure comparisons we have shown that these proteins all belong to a large protein superfamily present in diverse phyla. The mRNA expression pattern of the Zm-HIR3 gene in the Les9 genetic background associates the Zm-HIR3 gene with maize defense responses. These results are therefore compatible with the tobacco HR study (4), and the Arabidopsis systemic acquired resistance study (5), implicating HIR gene involvement in plant defense. They further suggest that a death/disease response function of these HIR genes is conserved in diverse monocot and dicot plants.
The sequence and structural similarities of plant HIR proteins with prohibitins, stomatins and other integral membrane proteins, some of which, in particular stomatins, are known to regulate ion channel function, suggests that the HIR proteins are involved in hypersensitive reaction and cell death through the regulation of ion channel activity. The C-terminal region of stomatin is very rich in alpha-helical content and has been postulated to act as a plug to regulate potassium ion channels (17,32). The HIR and prohibitin proteins are also predicted to have helical content near their C-termini (Fig. 2), suggesting there may be a similar structure and function of this region to that of the stomatin C-terminus.

Studies indicate that in animal models, such as tumor cell lines, potassium plays a significant role in the maintenance of membrane potential and integrity, and controls apoptosis (33), and energy conservation across membranes (34). In *Drosophila*, apoptotic proteins, *Rpr* and *grim*, have been shown to stably block shaker-type K+ channels to induce apoptosis (35). In plants, potassium is also involved in control of cellular homeostasis and cytoplasmic pH (36). In plants attacked by pathogens there is often an efflux of cellular potassium causing cellular acidification and extracellular alkalinization (36). Potassium levels in animals can activate several enzymes, including caspases, which are involved in apoptosis (37,38). Although caspases or their functional equivalents are poorly understood in plants, a recent study indicated that caspase inhibitors blocked plant HR and cell death (39). In the same study, the amount of cell death was correlated to leakage of ions from leaf discs.

Tumor suppressor genes are known to regulate both cell proliferation and cell death (9,40). Prohibitins act as negative regulators of cell proliferation in mammals and are implicated in tumor suppression (7). Some tumor suppressor genes when over-expressed are known to cause cell death (41,42). The HIR genes when overexpressed also cause cell death (4). Given the fact that HIR proteins are structurally related to prohibitins, they may represent a novel class of plant tumor suppressors. Prohibitins have been shown to suppress the G1 to S phase transition in the cell cycle. If HIR proteins have similar function, they likely also act at the G1 to S phase checkpoint. The Retinoblastoma (Rb) and p53 tumor suppressor genes are known to act at the G1 to S phase checkpoint. Interestingly, suppression of K+ channel activity in a tumor cell line by a potassium channel blocker blocks G1 to S transition by...
keeping Rb in a dephosphorylated state (43), indicating that potassium channels function in cellular proliferation signal transduction. There is some indication that prohibitin may be involved in ion control in mitochondria (7). However, this is the first report showing a structural relationship of prohibitins to stomatins, which are known potassium channel regulators, suggesting that their common molecular function is ion channel regulation.

In conclusion, this study demonstrates that the HR activating protein NG1 belongs to a novel gene family, which we name HIR. This family is conserved in monocot and dicot plants and appears to play a role in cell death, especially in relation to disease responses. This HIR family is in turn part of a large structurally-related superfamily of proteins widespread in the biosphere, which includes prohibitins, stomatins and other membrane proteins. Members of this PID superfamily are involved in cell proliferation, ion channel activity, and cell death. We postulate that these genes are generally involved in controlling ion channels, in particular potassium ion channels, and that through this control they affect regulation of seemingly diverse processes ranging from cell division, osmotic homeostasis, and cell death.

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References:


**Figure Legends:**

**Fig. 1.** An unrooted dendogram showing relationships among 32 members of PID superfamily. The dendogram was generated by neighbor-joining method using the PHYLIP 3.572 package (24). The dendogram clearly shows partitioning of families within the superfamily. The larger group (with branches colored in Red) is comprised of stomatins, integral membrane and hypothetical proteins that may possibly have some ion channel regulating activity in different organisms. The family containing prohibitins is in blue. The plant HIR protein family is in green. Bacterial membrane proteins HFLC and HFLK proteins involved in lysogenization form a separate group colored brown.
Fig. 2. Multiple sequence alignment, consensus, and DSC secondary structure predictions for PID superfamily. A multiple alignment of 32 amino acid sequences for representative members of the PID superfamily was constructed by ClustalW program and then manually refined. The HIR proteins (green bar vertical at left), stomatins and membrane proteins (red bar), HFLK proteins (brown bar), and prohibitins (blue bar). Highlighted are identical (red) or similar residues (blue) shared by at least three families within the PID superfamily. The alignment was used to generate a consensus sequence, which spans the region corresponding to amino acids 1 to 253 on Zm-HIR1. The consensus sequence at the bottom was based on conservation of a residue at any given position in >80% of sequences. Amino acids conserved 80% or more are capitalized in red-bold, and two 100% conserved amino acids, Asp and Ala, are capitalized and underlined in red-bold. Abbreviations for amino acid structural groups are in lower case as follows: o, alcohol [ST]; l, aliphatic [I,L,V]; a, aromatic [F,W,Y]; c, charged [D,E,H,K,R]; h, hydrophobic [A,C,F,G,H,I,K,L]; -, negative [D,E]; p, polar [C,D,E,H,K,N,Q,R,S,T]; +, positive [H,K,R]; s, small [A,S,T,V]; u, tiny [A,G,S]; t, turn-like [A,C,D,E,G,H,K,N,Q,R,S,T]; dot (.), any residue or gap. The location of the PID superfamily regular expression is identified by arrows. The location of the stomatin PROSITE signature is similarly indicated. Shown below the consensus sequence is the secondary structure predictions that were carried out using the latest version of DSC algorithm (44), the prediction accuracy of which is over 72%: DSC_ALL, DSC_HIR, DSC_PHB, DSC_STM, and DSC_HFLK represent consensus predictions for, respectively, all the PID superfamily sequences and all the members of the respective families of HIR proteins, prohibitins, stomatins and HFLK proteins. Abbreviations are: C, coil; E, beta strands; H, helix, and dots (.), gaps.

Fig. 3. Hydropathy profiles of paired members of the PID superfamily according to the Kyte-Doolittle method. Top panel, Zm-HIR1 vs. Zm-PHB4; Middle panel, Maize Zm-HIR1 vs. T. brucei Prohibitin (Accession gi|2952299); Lower panel. T. brucei Prohibitin vs. Synechocystis sp. Stomatin (Accession gi|2493271). Note the close correspondence of the hydropathy profiles.
Fig. 4. Northern blot determination of Zm-HIR3 mRNA abundance for family 3 wild-type and Les9 plants. Leaf blades that had developed Les9 lesions on the mature half of the leaf tissue were harvested and divided into basal, lesion-free zone (BL), transition zone where lesions were starting to form (TR), and leaf tip where lesions had reached a mature stage (TP). Corresponding tissue from wild-type sibs were also harvested. The probe was the Zm-HIR3 gene (EST CMSAR19R). In the upper panel, note the hybridization intensity of Zm-HIR3 mRNA is higher in Les9 than wild-type for all three portions of the leaves tested. This affirms the GeneChip® result. In the lower panel is a control showing the ethidium bromide stained gel and equality of rRNA loading per lane.
TABLE I
Zm-HIR3 mRNA expression levels in three families of Les9 vs wild-type genotypes using Affymetrix mRNA profiling Technology

<table>
<thead>
<tr>
<th>Family</th>
<th>Zm-HIR3 Est</th>
<th>Rep</th>
<th>Fold Change</th>
<th>Ave (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CBPCC63R</td>
<td>1A</td>
<td>-3.3</td>
<td>-2.7 (0.5)</td>
</tr>
<tr>
<td>1</td>
<td>CBPCC63R</td>
<td>1B</td>
<td>-2.2</td>
<td></td>
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<td>CMSAR19R</td>
<td>1A</td>
<td>-2.8</td>
<td></td>
</tr>
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Figure 1

Stomatins, Membrane Proteins

HFLK

Prohibitins
**MEME Motif 2**

**MEME Motif 3**

---

**Consensus**

---

**Figure 2**
Prohibitins, Stomatins, and Plant Disease Response Genes Comprise a Protein Superfamily that Controls Cell Proliferation, Ion Channel Regulation, and Death

Ramgopal Nadimpalli, Nasser Yalpani, Gurmukh S Johal and Carl R Simmons

J. Biol. Chem. published online June 21, 2000

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