Cell Number Regulator1 Affects Plant and Organ Size in Maize: Implications for Crop Yield Enhancement and Heterosis

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Genes involved in cell number regulation may affect plant growth and organ size and, ultimately, crop yield. The tomato (genus Solanum) fruit weight gene fw2.2, for instance, governs a quantitative trait locus that accounts for 30% of fruit size variation, with increased fruit size chiefly due to increased carpel ovary cell number. To expand investigation of how related genes may impact other crop plant or organ sizes, we identified the maize (Zea mays) gene family of putative fw2.2 orthologs, naming them Cell Number Regulator (CNR) genes. This family represents an ancient eukaryotic family of Cys-rich proteins containing the PLAC8 or DUF614 conserved motif. We focused on native expression and transgene analysis of the two maize members closest to Le-fw2.2, namely, CNR1 and CNR2. We show that CNR1 reduced overall plant size when ectopically overexpressed and that plant and organ size increased when its expression was cosuppressed or silenced. Leaf epidermal cell counts showed that the increased or decreased transgenic plant and organ size was due to changes in cell number, not cell size. CNR2 expression was found to be negatively correlated with tissue growth activity and hybrid seedling vigor. The effects of CNR1 on plant size and cell number are reminiscent of heterosis, which also increases plant size primarily through increased cell number. Regardless of whether CNRs and other cell number–influencing genes directly contribute to, or merely mimic, heterosis, they may aid generation of more vigorous and productive crop plants.

INTRODUCTION

Plant and organ size can vary in natural populations; for example, leaf size can vary as much as a thousand-fold between species and severalfold within species (Duvick, 1999; Mizukami, 2001). While there are undoubtedly functionally adapted constraints determining mature organ size (Mizukami, 2001; Weiss et al., 2005), artificial selection may also have an impact. The intrinsic mechanisms responsible for organ size variation are not well understood, but two major underlying factors determining plant and organ size are certainly cell number and cell size. It is generally believed that among equivalent organs from plant species of different size, larger organs result chiefly from increased cell number, rather than from larger cells (Mizukami, 2001). However, perturbing either cell number or size may not necessarily alter the final plant and organ size because changes in one factor can be compensated for by modifications of others. For example, in some instances, decreased cell number is compensated for by increased cell size, which consequently results in the final organ size being largely unchanged (Doonan, 2000; Inze and De Veylder, 2006). Such a mechanism of cell growth coordination suggests endogenous regulation of plant and organ size. Recent studies are now uncovering key regulatory genes and pathways that affect plant organ size by altering cell number, cell size, or both.

A key regulator of plant organ size is Arabidopsis thaliana AINTEGUMENTA (ANT), an AP2 domain–containing transcription factor (Elliott et al., 1996; Krizek, 1999). Arabidopsis AUXIN REGULATED GENE INVOLVED IN ORGAN SIZE (ARGOS) regulates organ size by functioning upstream of ANT (Hu et al., 2003). For both ANT and ARGOS, transgenic overexpression results in increased plant and organ size. Both genes promote growth through prolonging the cell proliferation period (but not the rate), and the final organ size change is due chiefly to an increase in cell number, not cell size (Mizukami and Fischer, 2000; Hu et al., 2003). Interestingly, Arabidopsis ARGOS-LIKE, which is also growth promoting, increases organ size by increasing cell size not cell number (Hu et al., 2006). EBP1, a putative Arabidopsis ortholog of human ErbB-3 epidermal growth factor receptor binding protein, is another key regulator promoting plant and organ growth (Horvath et al., 2006). In contrast with ARGOS and ANT, transgenically overexpressing EBP1 accelerates plant growth and development by simultaneously stimulating cell growth, proliferation, and development, resulting in both increased cell number and cell size (Horvath et al., 2006). For all three of these growth-promoting genes, not only does transgenically increasing expression promote plant and organ growth, but also downregulation of gene expression, or loss-of-function via mutation, reduces the plant and organ growth.

A number of additional regulators of plant and organ size have been reported and were reviewed recently (Busov et al., 2008;
Among these key players are growth repressors, one example being *Arabidopsis AUXIN RESPONSE FACTOR2 (ARF2)*. Loss of ARF2 function causes enhanced organ size, such as thicker stems, and larger seeds, floral organs, and leaves (Schruff et al., 2006). Many of these genes affecting plant and organ size function via hormone regulatory pathways, consistent with the established notion that hormones, such as auxin, play important roles in plant growth and development. Genes affecting hormonal metabolism and signaling are one of the major classes of genes involved in plant and organ size regulation (reviewed in Busov et al., 2008). For example, *Arabidopsis ARGOS* expression is auxin inducible, which in turn leads to activation of *ANT* and the cell proliferation pathway (Hu et al., 2003). *ARF2* responds to brassinosteroids through BRASSINOSTEROID-INSENSITIVE2 (Vert et al., 2008) and negatively regulates *ANT*, demonstrating integration of auxin signaling and brassinosteroid signaling pathways. The expression of *EBP1* is regulated by auxin through effects on the stability of the protein (Krizek, 2008).

Tomato (*Solanum lycopersicum*) *fw2.2* governs a quantitative trait locus involved in determining fruit weight. *fw2.2* plays important roles in domestication and agronomic improvement of tomato and is responsible for ~30% of the fruit size variation between the domesticated tomato (*Solanum lycopersicum*) and their smaller fruit relatives (Alpert and Tanksley, 1996; Frary et al., 2000). Tomato fruit size is primarily affected by the size of the carpel ovary, which in turn is correlated with the number of cells in the carpel ovary (Frary et al., 2000). The *fw2.2* allelic effects on fruit size are due to differences in the regulatory region and, thus, to altered mRNA expression rather than protein function. The allelic differences in expression involve both the level and the timing of expression. The expression of the larger fruit allele has an earlier and shorter duration, whereas the expression from the smaller fruit allele peaks later and persists for a longer period and thus produces an overall higher level of transcript during tomato fruit development (Cong et al., 2002). This indicates that higher gene expression correlates with smaller fruit and fewer cells; hence, *fw2.2* is thought to be a negative regulator of cell number. Further research indicated that the primary effect of *fw2.2* is in determining fruit size, with other associated phenotypic effects on fruit number and photosynthetic distribution (Nesbitt and Tanksley, 2001). While the mechanism by which *FW2.2* mediates cell number or division in tomato fruit remains unclear, yeast two-hybrid screening revealed that *FW2.2* interacts with CKII kinase at or near the plasma membrane (Cong and Tanksley, 2006). CKII kinases from yeast and animals are known to be part of the cell cycle control signal transduction pathway; therefore, the gene may be involved in the cell cycle signaling and cell cycle regulation machinery.

Crop domestication has resulted in dramatic increases in yield, often through altering organ size, overall plant size or biomass, and positive agronomic traits. Heterosis increases plant and organ size as well as yield. For example, in maize (*Zea mays*), where heterosis is pronounced, the F1 hybrid plants are taller, have increased leaf area, increased biomass, and larger ear size, and can yield two to three times as much as their inbred parents (Duvick, 1999; Flint-Garcia et al., 2009). Accordingly, genes that regulate cell number and organ size in plants could potentially contribute to yield increases and to heterosis itself. In this study, we isolated the putative maize orthologs and other family members of the tomato *fw2.2* gene and named these genes as *Cell Number Regulators (CNRs)*. We characterized the maize gene family by gene structure, phylogenetic analyses, RNA expression regulation, and its correlation with tissue growth activity and hybrid vigor. Insight into their function is achieved by ectopically expressing *CNR1*, the consequences of which support the idea that CNRs function as cell number regulators in maize. This suggests potential for application to crop improvement.

### RESULTS

#### Characterization of the Maize CNR Gene Family

Using the tomato *fw2.2* gene and relatives to search maize genomic and transcript data, we identified up to 13 gene family members. Due to the putative role of at least some in regulating cell number, we named these maize genes *CNRs*, and the numerical order of *CNR 1-13* was given based upon the order in which they were discovered (Table 1). We believe that this is likely a full account of the maize gene family. The encoded proteins were predicted based on open reading frames contained in EST contigs or full insert cDNA sequences and also directly from genomic sequences with the aid of the conserved intron locations and peptide sequences of the greater gene family. All the genes except *CNR11* and *CNR12* had cDNA and massively parallel signature sequencing (MPSS) tag transcript evidence of expression. The regional genomic sequence around the *CNR12* locus indicates that it would not encode a complete gene product. We conclude that *CNR12* is likely nonfunctional. The gene structures for all except *CNR12* were determined (Figure 1). The genes are generally compact, with 9 of 12 being just over 1 kb or less for the coding region plus introns. Despite the conserved nature of the proteins, there is diversity in the gene architectures. All the genes have at least one intron in the coding portions of the gene: four have one intron, four have two, two have three, one has four, and one has six. Some of the genes also have 5’ untranslated region (UTR) introns: *CNR5* has three and *CNR13* has one.

The chromosomal locations of all the gene family members were in silico mapped using their source BAC sequence and a proprietary integrated genetic-physical map that included a curated version of the public B73 BAC FPC contig index (Table 1). The chromosome positions are expressed physically in terms of the specific BACs containing the genes and genetically in terms of the chromosome bin intervals. Interestingly, *CNR3, 9, 10, and 12,* which all come from subclade C (Figure 2), make up a gene cluster all located on the same BAC AC186166 on chromosome 5. *CNR9* and *CNR10* are in a tandem arrangement within 3 kb of each other. Both *CNR1* and *CNR2* are on chromosome 4 but are located in nonadjacent bins genetically unlinked at ~45 centimorgans apart.

#### Phylogenetic and Protein Analysis

The relationships of the maize gene family members were analyzed in the context of the greater eukaryotic gene family.
Hundreds of related proteins were found in public databases, in plant, animal, and fungal genomes, revealing that the tomato fw2.2 and maize CNR proteins are not plant specific but rather belong to a diverse and broadly distributed ancient eukaryotic gene family. A total of 136 full-length peptide sequences, including 12 maize proteins, were chosen for further analysis. A complete list of these sequences, their sources, annotation details, and various analyses can be found in Supplemental Data Set 1 online. Note that aside from maize and Arabidopsis, this list is not necessarily a complete account of all family members for the other higher plants. The proteins range in size from 108 to 557 amino acids, but most are relatively short, between 120 and 200 amino acids. The animal proteins range from 108 to 144 amino acids and the fungal members from 148 to 157. The animal orthologs are also known as PLAC8 (placenta-specific 8) or cornifelin proteins. The Cys-rich conserved portion is sometimes referred to as the PLAC8 motif; however, its function is considered unknown and is sometimes named DUF614. This protein family indeed tends to be Cys rich (averaging 8.7% per peptide). The proteins also tend to be Pro rich, and in a third of the cases there are more Pro than Cys residues. The family averages over 15.2% Cys plus Pro by amino acid count. The dipeptide sequence Pro-Cys is also common among these proteins: 12 have four, 29 have three, 58 have two, 30 have one, and just six have zero, the latter mostly are the more distal animal PLAC8-like proteins. The data set also contains diverse plant members from green algae, bryophytes, lycophytes, conifer, dicots, and monocots. All proteins longer than 157 amino acids are plant proteins, which are usually longer due to an N-terminal extension, but occasionally a C-terminal extension.

A representative dendogram of the inferred evolutionary relationships of these proteins derived from ClustalW alignment (available in Supplemental Data Set 2 online) and the neighbor-joining algorithm is shown in Figure 2. The dendogram divides the superfamily into multiple major and minor subclades. Many of the deeper phylectic divisions have weak statistical support, so the relative evolutionary relationships remain uncertain. Nonetheless, the cladogram is roughly divided into a left side
composed of members from diverse phyla, including animals, fungi, algae, lower vascular plants, but also flowering plants. These presumably represent the ancestral superclade within the family. The right side of the cladogram is a plants-only superclade, but with many subclades. Some plant-containing clades within these two superclades have diverse vascular plants, such as bryophytes, lycopodes, gymnosperms, and angiosperms. Other clades from the plant-specific superclade on the right are chiefly angiosperms, and some are exclusively or predominantly made up of dicot or monocot proteins. This suggests a plant-specific expansion and radiation within the superfamily.

This analysis also reveals that tomato FW2.2 is a member of the plant-specific superclade. The specific subclade to which it belongs, labeled clade 1 in Figure 2, contains just one maize protein, CNR1. CNR2 is a member of an adjacent subclade, labeled 2 in Figure 2. A global pairwise amino acid comparison between FW2.2 and CNR1 shows 57.6% similarity/47.1% identity. FW2.2 is slightly more like CNR2 at 60.2% similarity/50.8% identity.
identity, with CNR1 and CNR2 showing 60.2% similarity/45.0% identity to each other. However, the results of this neighbor-joining phyletic test demonstrate that CNR1 is actually closer to FW2.2 than is CNR2 to FW2.2. This greater proximity of CNR1 to FW2.2 was also revealed using three other phyletic relationship algorithms, such as maximum parsimony, minimum evolution, and UPGMA phyletic tests, with each also done using bootstrap testing. Both of the presently known cell number-regulating proteins, FW2.2 and CNR1, are members of subclade 1. Members of this subclade contain the subsequence CLXXXPCPC, which in many of the other members of this superfamily, including CNR2, is instead CXXXXXPCPC, and this is especially the case for the cadmium-resistant proteins (Song et al., 2004). For these reasons, CNR1 may be considered the best maize candidate ortholog to tomato FW2.2; therefore, it was chosen for transgenic functional testing.

Considering the overall superfamily to which CNR1 and FW2.2 proteins belong, it is apparent that they are members of a larger family that contains proteins with at least two partially known functions: (1) those that can confer cadmium resistance in plants, and that are cross-functional in transformed fungi; and (2) proteins involved in calcium influx in plant roots that also function as such in fungi (Figure 2). The study of several members from the Arabidopsis family plus rice (Orz ya sativa) PLANT CADMIUM RESISTANCE1 (PCR1) reveals that diverse proteins across the superfamily are plasma membrane proteins that can confer plant cadmium resistance. All of these cadmium resistance proteins have the CXXXPCPC motif (Song et al., 2004). In addition, Arabidopsis MCA1 is marked by distinctive long N-terminal extensions and also is an experimentally confirmed plasma membrane-localized protein, but it is instead involved in calcium influx-mediated mechanosensing (Nakagawa et al., 2007). There are closely related maize ortholog candidates to members bearing these cadmium resistance and calcium influx functions (Figure 2).

**Structural Modeling and Analysis**

There is substantial experimental and computational evidence to indicate that members of this family tend to be transmembrane domain proteins and especially plasma membrane proteins. For example, experimental evidence points to membrane locations for the Arabidopsis PCR proteins conferring cadmium resistance (Song et al., 2004), AtMCA1 involved in Ca2+ uptake and mechanosensing (Nakagawa et al., 2007) and likely for FW2.2 too (Cong and Tanksley, 2006). The conserved Cys- and Pro-rich portions often span the range of the (usually two) computationally predicted transmembrane (TM) helices. For instance, in AtPCR1 (Song et al., 2004), the locations of the two TM helices are at residues 29 to 50 and 56 to 78. Both Cys and Pro are hydrophobic and as such they are compatible with TM locations. Furthermore, the propensity for Cys to cross-link to other amino acids, and for Pro to introduce bends, can aid helical formation. Results from this analysis thus support the idea that the proteins have two TM helices, with helix 1 (H1, nearest the N terminus) being more highly conserved but also relatively hydrophilic, and TM helix 2 (H2) being both more hydrophobic and less conserved. Figure 3A shows a standard helix wheel representation of the PCR1 TM H1. The amino acid residue numbering begins with the predicted start of TM H1 (position 1 in the figure, but actually position 29 from the N terminus). The helical wheel reveals a clear tendency of the hydrophilic residues to align to one side of the helix. Because the strongly hydrophilic residues such as Glu-17 and Gln-14 (helix coordinates) are by their nature prohibited from facing the lipid membrane bilayer, they very likely face the channel lumen. The fact that TM H2 is more hydrophobic, but less conserved overall, strongly suggests that its primary role is to be anchored in the membrane and not to perform the core conserved pore-forming and presumed metal-interacting function of the protein.

A well-documented mechanism for proteins mediating metal cation transport is by forming a membrane-spanning ion channel. To gain insight into whether and how CNR and related proteins may form cation channels, we conducted structural analysis and modeling. There are several characterized metal ion channels formed by TM helices, such as a zinc transporter (Lu and Fu, 2007), copper transporter (De Feo et al., 2009), calcium ATPase (Toyoshima et al., 2000), and Mg2+ transporters (Payandeh and Pai, 2006; Hilf and Dutzler, 2008), but none of them shows strong similarity to the CNR protein family. However, like the CNR family, CorA, a Mg2+ transporter, has only two TM helical motifs. CorA, named for cobalt-resistant mutants, represents the primary Mg2+ uptake system in most prokaryotes and also mediates Mg2+ efflux in certain conditions. Similar proteins of a prokaryotic subfamily conduct Zn2+ efflux (Payandeh and Pai, 2006). The CorA transmembrane pore is formed by a homopentamer with five identical α-helices packing side-by-side along the pore circumference. In addition, the pentameric helix arrangement is one of the simplest pore topologies that can create a proper lumen size (~2.5 to 7 Å in diameter) suitable for allowing bare or partially hydrated cations to pass through the membrane while maintaining sufficient selectivity. Consequently, for these various reasons, we chose the transmembrane pore of CorA (Ther motoga maritima) as the template upon which to build a pore model for the CNR family. Because we are focusing on a transmembrane metal/cation interaction/transport protein model, we chose to build the model with AtPCR1, one of the cadmium resistance proteins of this family.

We aligned the PCR1 TM H1 to the pore-forming α-helices of CorA, with the hydrophilic side inward, facing the lumen, and we aligned TM H2 to the outside, exposed to the lipid bilayer (Figure 3). We created a pentameric model based on the CorA precedent. This results in a model with the five TM H1s forming the inner axle or pore lumen and the five TM H2 helices radiating outward like short spokes anchored in the membrane (Figure 3B). With the diameter increase to the outside, the TM H2s are unable to reach and interact with each other and are thus independently exposed to and embedded in the apolar bilayer, which is consistent with both their strongly hydrophobic nature and lack of conservation, suggesting a lack of protein–protein or protein–substrate interaction. To the inside of the resulting pore lumen, the five highly conserved H1 E17s form a negatively charged ring that could facilitate cation passing. Similarly, the side chains of Gln-14, Cys-2, Cys-6, Cys-9, and Asp-20 could also provide polar atoms (N, O, and S) that could directly interact with cations in the lumen channel. At first glance, the positively
charged side chain of R21 would seemingly repel cation transport, but in the model, its long side chain is actually located at the lipid-water interface, a position where its guanidium group sits just outside the bilayer. In this position, it could act as a collar to interact with the negatively charged lipid heads and stabilize the pore. The highly conserved P8 opposite the channel lumen cannot directly interact with passing cations, but its importance may instead be its ability to introduce a bend or kink to the helix. This model is not experimentally confirmed, but it is consistent with multiple observations of this family’s protein sequences, and we believe it is a useful guide to thinking about these proteins and interpreting the transgenic function results of maize CNR1 and CNR2 below. Importantly, note that the Leu of the CLXXXXCPC versus CCXXXXCPC comparison (corresponding to FW2.2/CNR1 versus PCR1/CNR2, respectively) corresponds to position two of the helical wheel on the pore lumen side of the helix (Figure 3). Leu, being larger and more hydrophobic than Cys, should constrain pore size and alter cation interaction, likely leading to a protein functional difference.

**Expression Patterns of the Maize CNR Gene Family in Different Tissues**

We examined the tissue expression patterns of the CNR gene family using an RNA profile database derived from MPSS. This proprietary database contains deep RNA profiles of >250 libraries and from a broad set of tissue types. The MPSS transcript profiling technology is a quantitative expression analysis that typically involves 1 to 2 million transcripts per cDNA library (Brenner et al., 2000a, 2000b). It produces a 17-base high-quality, usually gene-specific, sequence tag captured from the (usually) 3′-most DpnII restriction site in the transcript for each expressed gene. The use of these MPSS data, including statistical analyses, replications, etc., has been described previously (Guo et al., 2008). The expression patterns of the maize CNR genes across tissue types are shown in Figure 4. Eleven of the 13 CNR genes were assayed. CNR17 and CNR12 exhibited no expression in any sample nor did either have any available cDNA in the database. CNR8 had the highest and broadest expression, with the highest expression in the pericarp and stalks. CNR13 also had high relatively consistent expression but no expression in pollen. Most of the other genes had relatively low expression in multiple tissues. Two genes displayed tissue preferences: CNR3 was in pollen only, and CNR7 was found preferentially in silk. CNR1 and CNR2 had similar profiles with moderate expression in several vegetative tissues, but lower expression in meristems, immature ears and kernels. CNR1 showed higher expression in coleoptiles and silks, whereas CNR2 showed higher expression in tassel spikelets, leaves, and stalks.
Because of their putative roles as negative regulators of cell number, we examined the expression patterns of CNR1 and CNR2 in relation to tissue growth activity. CNR2 exhibited expression patterns that negatively correlated with tissues exhibiting cell division activity (Figure 5A). The MPSS database includes leaf, silk, embryo, and endosperm samples at different stages of development in a series that represent these tissues with different growth activity levels. In maize leaf development, the basal region of immature leaves has the most active cell division, whereas zones of cell expansion, transition, and maturity are found in more distal regions (Sharman, 1942; Sylvester et al., 1990). In the RNA profiles of the leaf tissues, CNR2 expression was not detected in the cell division zone, but it was detected at very low levels in the cell expansion and transition zones and at the highest level in the mature or fully grown leaf tissue (Figure 5A). Silk tissue was sampled through a time course following pollination, corresponding to a range from fast growing silks to silks with no growth activity. As shown in Figure 5A, the level of CNR2 expression was low just after pollination, but as silk growth slowed down and eventually stopped, CNR2 expression progressively increased, reaching the highest level at 72 h. CNR2 expression was not detected in the pollen samples (Figure 4), indicating that the increase of expression found in pollinated silks was not from contaminating pollen. For the embryo and endosperm tissue series, the profiled samples were collected at sequential days after pollination (DAP). As shown in Figure 5A, the level of CNR2 expression was very low in the early stages of embryo and endosperm development (before 30 DAP), when most of the cell division occurs (Sabelli and Larkins, 2009), and then increased progressively as the seed matured. The highest expression level in embryo and endosperm was at 45 DAP when the seeds were fully grown. Such patterns of expression demonstrate a negative correlation of CNR2 expression with growth activity in kernel tissues. CNR1 did not exhibit this marked growth activity–related expression pattern (see Supplemental Figure 1 online).

**RT-PCR Corroboration and Extension of CNR2 Expression Analysis**

To corroborate and build upon the CNR2 MPSS expression observations, we performed quantitative RT-PCR analysis using the leaf tissue development series from two maize genotypes, B73 and Mo17, respectively. The tissue types used were the same as those used in the MPSS experiments, consisting of leaf sections taken from cell division, cell expansion, transition, and mature zones. The RT-PCR data are consistent with the MPSS data; that is, CNR2 expression increased as the leaf tissue growth activity decreased (Figure 5B). To examine further the relationship of CNR2 expression with growth activity in various tissues, we performed RT-PCR analysis in additional tissues that are known to have no growth activity, such as the ovule before fertilization. As expected, CNR2 expression was high in these ovules compared with other immature tissues and was comparable to that found in mature leaves (Figure 5C). Apparently this CNR2 expression is turned off upon fertilization, as the early developing embryo and endosperm had little or no expression (cf. Figures 5A and 5C).

**CNR2 Expression Negatively Correlates with Hybrid Seedling Vigor**

The hybrid vigor in maize plants is obvious during seedling growth. Figure 6A shows the growth rate of hybrids and their inbred parents as measured by dry biomass during the first three weeks (V2 to V4 stage). The hybrids from both reciprocal crosses, B73/Mo17 and Mo17/B73, was reduced (Figure 6B), indicating the downregulation of CNR2 expression in the hybrids of reciprocal crosses, B73/Mo17 and Mo17/B73, was reduced (Figure 6B), indicating the downregulation of CNR2 expression in the more vigorously growing hybrid relative to the inbred parents. We performed quantitative RT-PCR analysis with fully opened V1-V3 leaves comparing two inbred and reciprocal hybrid genotypes. The level of CNR2 expression in the hybrids of reciprocal crosses, B73/Mo17 and Mo17/B73, was reduced (Figure 6B), indicating the downregulation of CNR2 expression in the more vigorously growing hybrid relative to the inbred parents. Granted, contrary to this pattern, both shoot biomass and CNR2 expression was slightly higher in inbred B73 versus Mo17, but this could of course result from other inherent differences in the inbred lines. We further examined the CNR2 expression in a hybrid (Hi-II) that has no hybrid vigor at the seedling growth stage as shown in V4 seedling dry mass (Figure 6C) even though it exhibits heterosis during later and mature stages (Armstrong...
et al., 1991). The level of CNR2 expression was not reduced in this hybrid (Figure 6D) as seen in the heterotic hybrids of B73-Mo17. The data are consistent with the negative relationship of the CNR2 expression level with hybrid seedling vigor.

**Transgenic Overexpression of CNR1 Reduces Plant and Organ Size**

To understand further the role of the CNR genes on plant growth and organ size, we ectopically expressed the CNR1 and CNR2 open reading frames in maize. Early transgenic tests of CNR2 using a constitutive promoter from maize *ubiquitin Prom*$_{ZmUBI}$ (McElroy and Brettell, 1994) did not show any obvious phenotype and were not pursued further (data not shown). Transgenic plants overexpressing CNR1 were generated using the maize *ubiquitin Prom*$_{ZmUBI}$. Seven independent, single-copy transgenic events from the T3 generation (after two more generations of backcrossing to the wild type to achieve three doses of the wild-type genetic background) were grown in the field for phenotypic evaluation. Plant height, stem diameter, and ear length were measured from five plants per replicate, and three replicates for each transgenic event (and its nontransgenic sibling control) were performed. Transgenic plants showed reduction in overall plant size and organ size, such as tassel, ear, and leaf size, compared with their nontransgenic control (Figure 7A; see Supplemental Figure 2 online). Plant height, ear length, leaf area, and estimated biomass of the transgenic plants were reduced compared with the controls with the exception of one event (Figure 7B; see Supplemental Figure 2 online). The plant height reduction was due mainly to reduced length of internode, as the number of internodes did not change (see Supplemental Figure 3 online).

We further tested the relationship between the CNR1 transgene expression and the reduction in plant and organ size in transgenic plants. From the same experiments used for the phenotypic data collection, we sampled leaf tissue from five transgenic events and performed real-time RT-PCR analysis. We found a significant correlation between the CNR1 transcript level and the phenotypic changes in plant height, ear length, and estimated biomass, respectively; that is, the higher the CNR1
transcript level, the greater the reduction in plant and organ size (Figure 7C). We also observed a strong correlation of transgene copy number with the degree of growth suppression; that is, the higher copy number of the transgene, the smaller the plants (see Supplemental Figure 4 online), presumably due to increased net CNR1 expression level (data not shown).

Cosuppression of Endogenous CNR1 Enhances Plant Growth

One exceptional event of the CNR1-overexpressing construct (event 7) did not show any reduction in plant and organ size; instead, it exhibited growth enhancement, with increases in estimated biomass, ear length, and kernel number per row (Figures 7B and 8A to 8C). We investigated the possibility of cosuppression of the endogenous CNR1 expression in this event. As shown in the top panel of Figure 8D, CNR1 expression of event 7 was indeed cosuppressed in at least nine of the 15 individual plants analyzed compared with the nontransgenic sibling controls. This was consistent with the expression data in Figure 7C, in which event 7 had the lowest data point (at the lower left corner), representing the averaged CNR1 expression from a pool of five individual plants. By contrast, the bottom panel of Figure 8D shows an example of the CNR1 expression in one of the typical overexpressing, growth-reduced events, indicating a much higher and consistent CNR1 expression across all five individual plants.

RNA Interference Downregulation of Endogenous CNR1

To confirm that downregulating CNR1 expression level enhances plant and organ growth, we created transgenic plants containing an RNA interference (RNAi) vector (PromZmUBI:CNR1RNAi). Five individual transgenic events containing a single copy of the transgene were obtained. All five events expressed the RNAi transgene, indicating the construct is working effectively in expressing the transgene. Nonetheless, the silencing of the endogenous CNR1 expression by the transgene was effective in just one event, event 5 (E.5). As shown in Figure 9A, endogenous CNR1 expression from four individual plants of this E.5 event was very low or could not be detected under 35 cycles of RT-PCR, whereas the nontransgenic control (lane to the left) showed the normal expression of the endogenous gene. Plant height of the five T1 transgenic events was measured from two rows (5 to 10 plants per row of each transgenic and control segregating in the same rows). Of the five events, only E.5, with silenced CNR1 expression, showed a plant height increase over the control, nontransgenic segregating siblings (Figures 9B and 9C). Figure 9D demonstrates that the ear size of this E.5 is also larger and had fuller kernel set than the control.
Alteration in Leaf Epidermal Cell Numbers in \textit{CNR1} Transgenic Plants

To understand whether the reduced organ size in transgenic \textit{CNR1} plants was due to changes in either cell number or cell size, we counted leaf epidermal cell number per unit area as a measure of relative cell size. We analyzed one of the ectopic overexpressing transgenic events (event 5 of the \textit{PromZmUBI:CNR1}) that showed plant size reduction and the growth-enhancing, cosuppression event (event 7). In each event, we collected data from five individual plants of each transgenic and nontransgenic sibling control. We found that the average number of cells per fixed leaf unit area (0.4 mm$^2$ field) was not different between transgenic and control, indicating that the overall cell size did not change (see Supplemental Figure 5 online). Since the overall organ size or leaf area of the transgenic plant was either smaller (in the growth suppressing event; see Supplemental Figure 2 online) or larger (in the growth enhancing event) than that of the control, the total cell number per leaf or per organ was altered. The data therefore indicate that the changes in overall plant and organ size were due chiefly to altered cell number, not cell size.

DISCUSSION

Overexpression of \textit{CNR1} Reduced Plant and Organ Size, Supporting Its Role as a Negative Regulator of Cell Number

To understand further the function of the \textit{CNR} genes, we ectopically expressed in transgenic maize \textit{CNR1}, the closest ortholog to tomato \textit{fw2.2}. The consistent reduction in overall plant and organ size across all transgenic events overexpressing \textit{CNR1} demonstrated that it functions as a negative regulator of growth. The transgene effects on both vegetative and reproductive growth were profound. The strong correlation of organ size reduction with the level of \textit{CNR1} expression (Figure 7), as well as with transgene copy number (see Supplemental Figure 4 online), provided further evidence to support this argument. These data...
that the growth-regulating function of cell size (see Supplemental Figure 5 online). The results suggest the cosuppressing event was due to changes in cell number, not altered plant organ size in dependence (Liu et al., 2003). Correlated with the level of alleles (Cong et al., 2002) and that the tissue (fruit) size was highly coding sequences, differentiated the large versus small fruit gene regulatory regions affecting expression, rather than protein expression. The fact that cosuppression or RNAi silencing of the endogenous CNR1 expression increased plant and organ size (Figures 8 and 9) also suggests that the natural expression level of the gene can be reduced, and there is thus potential to enhance plant and organ growth, and by inference possibly yield, by judiciously downregulating native gene expression. The fact that there is considerable variation in transgene expression, endogenous gene silencing, and growth enhancement among the comparatively few events studied to date suggests that the magnitude of growth enhancement may be further improved and honed by more effective downregulation of RNA levels by these or other approaches or by simply screening more events. This CNR1 transgenic data implies, as does perhaps also the native expression pattern of CNR2, that by diminishing their normally negative regulation of cell number, through various means of decreasing their net expression or function, one may create larger or more vigorous plants, or alterations in the sizes of specific organs or in tissue growth rates, to achieve more optimal plant architecture and agronomic performance.

**Heterosis, Cell Number Regulation, and the CNR Genes**

Heterosis is an important mechanism that gives rise to crop plants enhanced in yield and performance, and it is the foundation of the hybrid seed industry. The hallmark of heterosis is increased growth vigor and plant stature of the F1 hybrid compared with the inbred parents. The mechanism of heterosis is commonly thought broadly to involve complementation of numerous gene alleles, whether by protein function, by gene expression, epigenetic states, or otherwise (Birchler et al., 2003; Guo et al., 2004, 2006; Springer and Stupar, 2007; Swanson-Wagner et al., 2009). Other nonexclusive hypotheses envision a smaller set of key genes disproportionately driving the effect (Redei, 1962; Birchler et al., 2003, 2006; Semel et al., 2006), especially those whose alteration in function might result in changes in plant vigor. Heterotic effects from either individual loci corresponding to single genes (e.g., *erecta* and *angustifolia*) are consistent with the fw2.2 observations in which differences in gene regulatory regions affecting expression, rather than protein coding sequences, differentiated the large versus small fruit alleles (Cong et al., 2002) and that the tissue (fruit) size was highly correlated with the level of fw2.2 transcript in a study on dosage dependence (Liu et al., 2003).

Leaf epidermal cell counts using microscopy indicated that the altered plant organ size in CNR1-overexpressing events and in the cosuppressing event was due to changes in cell number, not cell size (see Supplemental Figure 5 online). The results suggest that the growth-regulating function of CNR1 alters plant and organ size chiefly through regulating cell proliferation and not cell expansion, which is also consistent with the cell number regulator role of fw2.2 (Frary et al., 2000; Cong et al., 2002). This demonstrates that the cell number–regulating function of this class of genes is conserved between monocots and dicots and that it functions in diverse tissues. Tomato fw2.2 affects fruit carpel cell number, whereas CNR1 affects many or most maize tissues, as the overexpressing plants in this study were smaller yet fully viable and normally proportioned. In the tomato fw2.2 study, the phenotype was caused by a change in the spatial-temporal expression of fw2.2 during fruit development (Cong et al., 2002). That study is thus not equivalent to this report wherein the CNR1 transcript was ectopically expressed with the ubiquitin promoter operating in many tissues. Nonetheless, between the fw2.2 work and our results on CNR1, it now appears that the effect of this class of genes is one of general cell number regulator because the effect persists across tissue types and major plant phyletic groups.

Importantly, by overexpressing or underexpressing this single gene CNR1, we demonstrated that maize plants can be made either smaller or larger, respectively. The fact that cosuppression or RNAi silencing of the endogenous CNR1 expression increased plant and organ size (Figures 8 and 9) also suggests that the natural expression level of the gene can be reduced, and there is thus potential to enhance plant and organ growth, and by inference possibly yield, by judiciously downregulating native gene expression. The fact that there is considerable variation in transgene expression, endogenous gene silencing, and growth enhancement among the comparatively few events studied to date suggests that the magnitude of growth enhancement may be further improved and honed by more effective downregulation of RNA levels by these or other approaches or by simply screening more events. This CNR1 transgenic data implies, as does perhaps also the native expression pattern of CNR2, that by diminishing their normally negative regulation of cell number, through various means of decreasing their net expression or function, one may create larger or more vigorous plants, or alterations in the sizes of specific organs or in tissue growth rates, to achieve more optimal plant architecture and agronomic performance.

**Figure 8. Cosuppression of Endogenous CNR1 in the Growth-Enhanced Event 7.**

(A) Ear length in the line from transgenic (TG) event 7, a line that has the transgene for overexpressing CNR1 (Pro<sub>ZmUBi</sub>:CNR1) but is growth enhanced, and the non-transgenic control. Standard error was based on three replicates and 10 ears per replicate (same as shown in Figure 7).

(B) Kernel number per row. Standard error was based on average count of three kernel rows per ear, 10 ears per replicate, and three biological replicates.

(C) Examples of ear phenotype.

(D) Top panel: CNR1 net expression (endogenous and transgene) in leaf tissue sampled from 15 individual plants of the cosuppressing event 7. Control (C) is a nontransgenic sibling. Bottom panel: CNR1 expression in 15 individual plants of a typical growth-suppressing (i.e., noncosuppressing) event. Asterisks indicate individual plants with cosuppressed CNR1 expression.

[See online article for color version of this figure.]
Redei, 1962) or finely mapped individual chromosomal regions (Semel et al., 2006) have been described. A key observation made in maize heterosis was that the greater plant size is primarily (90%) due to increases in cell number, not cell size (Kiesselbach, 1922; East, 1936). Accordingly, genes that regulate cell number could potentially be involved in heterosis and yield gain. As reported in Arabidopsis, many potential yield genes are involved in cell proliferation and determination of organ size (Gonzalez et al., 2009). In this work, we are not concluding that CNR genes have a direct connection to heterosis or yield. However, their behavior affecting plant and organ size via cell number does invite the prospect of their possible role in heterosis or in developmental processes that can lead to a mimic or analog of heterosis and yield enhancement.

Molecular Function Revisited in Context of the Superfamily

We surveyed broadly the greater gene family to which_fw2.2_and the maize CNR genes belong to gain new perspectives that may shed light upon the CNR1, CNR2, and FW2.2 protein functions. We believe the resulting 136-member data set is largely representative of this ancient eukaryotic gene family, especially among the higher plant families, in particular for maize and Arabidopsis, as this set may contain the complete gene family for these two species. The plant gene family sizes are consistently larger than nonplant families, ranging in bryophytes, lycopods, dicots, and monocots from 10 to 20 members. Fungi and animals appear to have only one or a few members. This suggests that plants have adapted this gene family to plant-specific purposes, and this is also likely indicated by the expanded N- and C-terminal extensions unique to the plant genes, notably the N-terminal extension of the Arabidopsis MCA1 calcium-influx mechanosensor signaling protein. The CNR1 and FW2.2 proteins, however, represent chiefly the core ancient conserved region without these extensions. The plant-specific subclades, plus the differing tissue expression patterns indicated by the maize genes, also strongly point to multiple physiological or organ-specific roles.

\textit{CNR1} and \textit{CNR2} are the closest \textit{fw2.2} orthologs based on protein sequence similarity. Both genes are expressed in multiple tissues. The expression patterns of \textit{CNR1}, at the current level of tissue analysis resolution, did not itself suggest a role as a negative regulator of cell number. That of \textit{CNR2} did, however, in that it showed a distinctive expression pattern remarkably anticorrelated with the growth activity of multiple diverse tissues, such as leaves, embryos, endosperm, and silks, suggesting it may have a broad and general role in determining cell number throughout the plant. That \textit{CNR2} is highly expressed in the ovule,
which is a tissue with no cell division activity until pollination, suggests this gene may place a brake on cell division activity in ovules, which is then released upon fertilization, when the CNR2 expression drops early after fertilization. CNR2 expression then climbs again as the embryos mature (Figure 5). Interestingly, CNR2 is not expressed at all in pollen, whereas CNR3 is exclusively and highly expressed in pollen. While other CNR genes showed various tissue-specific expression patterns, their relationship to tissue growth activity was not obvious and their roles in regulating cell number remains to be tested.

Although CNR1 and CNR2 are about equally related to FW2.2, the sequence identity between the two is relatively low (47.1%). CNR2 does have a key difference from CNR1 in that it contains the CXXCXCCPC motif in the first transmembrane domain, a site associated with cadmium resistance for Arabidopsis and rice PCR members of the family (Song et al., 2004). We speculate that cadmium resistance, possibly via direct cadmium chelation or interaction, may be a product of the protein Cys richness and especially this second Cys in TM1 of the presumed pore lumen. Electron D-orbitals of heavy metals such as cadmium can interact strongly with the Cys thiol groups (Kozlowski et al., 1990), and through such interactions cadmium and other heavy metals can be poisonous because they interrupt vital protein functions (Ortega-Villasante et al., 2005). In both the cell number regulating proteins FW2.2 and CNR1, this TM1 motif is instead CLXXXCP, and CNR1 is the only maize family member bearing Leu in this location. Our structural modeling suggests this Leu is located at a critical position in the presumed pore lumen and could affect protein function. Therefore, with regard to this CC versus CL difference alone, FW2.2 and CNR1 may be considered more closely orthologous and that, at some level, CNR2’s function may differ. Of possible relevance to this, transgenic maize plants overexpressing CNR2 have yet to display the cell number regulator phenotype of CNR1. Nevertheless, CNR2 exhibits a striking expression behavior, indicating a negative association with cell division. So, while CNR2 expression looks the part of a cell number regulator, CNR1 actually functions in affecting cell number in transgenic experiments. We have no further data to resolve this conundrum and simply speculate that CNR1 and CNR2 proteins may somehow cooperate directly or indirectly in cell number regulation.

Investigations of FW2.2 function immediately entertained a role in cell cycle control, since fw2.2 expression affected cell number in tomato carpels (Frary et al., 2000). A subsequent report indicated that FW2.2 interacts with the CKII kinase regulatory subunit, likely at the plasma membrane, and since CKII kinases are implicated in fungal and animal cell cycle signaling, this lent credence to the cell cycle notion (Cong and Tanksley, 2006). The effect on cell number for both FW2.2 and CNR1 does imply at some level cell cycle involvement, despite the CKII result, a direct connection to the cell cycle remains unclear, and no other proteins of the greater gene family, including those from animals and fungi, with their often well-studied cell cycle systems, is reported to be connected to the cell cycle. Previous work and the computational evidence presented here indicate that members of this protein family are membrane proteins, especially plasma membrane localized, and that they interact with metal ions, such as cadmium (Song et al., 2004) and calcium (Nakagawa et al., 2007). Taken together, this information suggests this protein family tends to form membrane-bound pore complexes that interact with metals or cations and/or regulate their uptake or signaling. In light of the plant-specific subfamily to which these proteins belong, it should be considered that FW2.2 and CNR1 may have a plant-specific cell proliferation function. Plant cell proliferation is often connected to phytohormones, such as auxin and cytokinin (Hu et al., 2003; Busov et al., 2008; Krizek, 2008; Vert et al., 2008), and as noted in the Introduction, the known plant genes affecting organ and plant size are often connected to plant hormones. While the literature on specific effects of cadmium on plant cells is not extensive, it has been observed that some auxin-regulated genes are also cadmium inducible (Hagen et al., 1988; Kusaba et al., 1996). Cadmium is thought to exert its toxicity in part by displacing divalent metal ions, such as zinc from essential proteins; Cd²⁺ is similar in size to Zn²⁺ and is from the same group in the periodic table, yet binds more strongly than Zn²⁺ to biological molecules, especially those containing sulfur atom ligands (Lohmann and Beyersmann, 1994). It is therefore intriguing that the maize auxin binding protein 1 interacts with auxin via a zinc atom (Woo et al., 2002). Could it be that fw2.2 and CNR1 regulate cell number in some manner related to phytohormones, perhaps auxin, and/or perhaps via metal cation-mediated signal transduction across the cellular membranes?

**METHODS**

**Gene Family Roundup and Analysis**

The transcripts and predicted proteins for the maize (Zea mays) CNR family were identified and curated from a mixture of public and proprietary genomic and cDNA/EST sequences. Comparisons to gene products of various species, in particular those from tomato (Solanum lycopersicum), Arabidopsis thaliana, rice (Oryza sativa), and sorghum (Sorghum bicolor), helped determine gene model completeness. The tomato FW2.2, maize CNR, and Arabidopsis PCR family member peptide sequences were used as BLAST queries against key plant genome draft gene model data sets and the National Center for Biotechnology Information nonredundant protein data sets. Hundreds of likely orthologs were identified. A total of 136 gene products from plants, animals, and fungi were curated by sequence alignment comparisons of family members across all species and were full length and correctly predicted. This full-length set does not necessarily contain complete accounts of all family members for any particular species but is close to it for Arabidopsis and maize. We believe this set is representative of the diversity in the greater gene family.

This data set was then further examined using a number of criteria, including phylogenetic and cluster analysis, presence of key conserved motifs, protein length, N- and C-terminal extensions, predicted subcellular localization, and predicted transmembrane helices. The 136 proteins were aligned by ClustalW using the Blossum62 matrix, with a Gap opening penalty of 10 and a Gap extension penalty of 0.1. The alignment is available as Supplemental Data Set 2 online. All amino acid positions containing gaps and missing data were eliminated. The cladistic or phyletic relationships of the 136 proteins were inferred using the neighbor-joining method (Saitou and Nei, 1987), with 1000 iterations of bootstrap analysis (Felsenstein, 1985), as implemented by the MEGA4 software (Tamura et al., 2007). The percentage of replicate trees in which the associated proteins clustered together in the bootstrap test (per 1000 replicates) are shown next to the branch nodes in the dendogram (Figure 2). Where there was <10% statistical support, however, the branch nodes
are not labeled and are collapsed together. The clades are rooted on the midpoint. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965), and these distances are suggested but not exactly to scale in the fixed radial plot of Figure 2.

Protein Structural Modeling Analysis

The multiple sequence alignment of the CNR family was produced by ClustalW using the default Blossorn 62 matrix and manually edited in the TM helical regions. A position-specific profile of hydrophobicity and conservation within the TM helices was generated. The position-specific conservation score was calculated by summing the amino acid substitution penalty score among all the aligned residue pairs at a given position. The amino acid substitution penalty score was obtained from a TM-specific substitution matrix PHAT (Ng et al., 2000). Conservational disparity and amphipathicity of the TM helix was analyzed with standard helix wheel representation. Secondary structure propensity was predicted by Jpred (http://www.compbio.dundee.ac.uk/www-jpred/). The Arabidopsis PCR1 TM channel topology was manually built with InsightII (Accelrys) and the Thermotoga maritima CorA structure (PDB:2hn2) was used as the template. The structural graphs were prepared with Pymol (http://pymol.sourceforge.net/).

T-DNA Constructs and Maize Transformation

T-DNA constructs and plant transformation Gateway technology (Invitrogen) were used for vector construction. For the overexpression vector, full-length cDNA sequence of CNR1 was amplified (primers are listed in Supplemental Table 1 online), integrated between the ubiquitin promoter and PinII terminators, and cointegrated with JT vectors as previously described (Unger et al., 2001; Cigan et al., 2005). The plasmid also contained a red florescent protein (RFP) (Clonetech Lab), driven by an aleuronic-specific promoter barley (Hordeum vulgare) Lipid Transfer Protein, PromHVLTPo (Opsahl-Sorteberg et al., 2004) to serve as a marker. The construct PrOuub:CNR1 was introduced into Agrobacterium tumefaciens strain LBA4404 and used to transform Hi-II maize embryos as previously described (Unger et al., 2001; Cigan et al., 2005). Typically, 20 individual events were generated. Seven independent transgenic events with a single copy of transgene were obtained, confirmed with transgene expression, and advanced for crosses to wild-type (nontransgenic) plants and further characterization.

For the RNAi construct to silence CNR1 expression, a CNR1/ADH1 intron/CNR1 (reverse complementary) hairpin composed of 102 bp of 5’ UTR with 414 bp of coding region of CNR1 was integrated between the ubiquitin promoter and PinII terminators and cointegrated with JT vectors using Gateway technology as previously described (Unger et al., 2001; Cigan et al., 2005). Typically, 20 individual events were generated. Seven independent transgenic events with a single copy of transgene were obtained, confirmed with transgene expression, and advanced for crosses to wild-type (nontransgenic) plants and further characterization.

Phenotypic Data Collection and Analysis

The seedling shoot biomass data of B73, Mo17, B73/Mo17, and Mo17/B73 were collected from three biological replicates and five plants per replicate at each developmental stage from plants grown in the greenhouse. For Pro2ub:CNR1 plants, plants from the transgene segregating populations were identified by the RFP marker as red kernels. Transgenic plants derived from the primary transformed T0 lines were backcrossed to the wild type (nontransgenic) for two generations to obtain T3 transgenic plants. Transgenic seed and their negative sib (null control) identified by the RFP maker were planted in the field in Johnston, IA, during the summer of 2006. The plants were grown in three replicates of two-row plots (20 plants per row) for each event. Phenotypic data were collected from three replicates, five plants per replicate. Plant height was measured from ground level up to the ligule of the flag leaf. Basal stem diameter was collected ~14 d after anthesis using PRO-MAX digital callipers (Fowler). Biomass was estimated based on plant height and stem diameter measurements, using the formula BM = 0.087658 × Vol 1.03518 (Vega et al., 2001), where Vol (volume) is derived from plant height and stem diameter. Approximately 45 d after anthesis, primary ears were harvested, and ear length data were collected from the same plants measured previously. An additional five ears (total 10) were measured for ear length with the cosuppressed event. As there was no RFP marker built in the Pro2ub:CNR1RNAi construct, individual plants were genotyped as transgenic and nontransgenic by molecular analysis via a molecular marker (MO-PAT) strip testing (MO-PAT strips 3000006; Strategic Diagnostics) of transgene presence and absence, respectively. Phenotypic analysis was done with plants segregating in the same rows in the field.

Biological Material, Tissue Sampling for RT-PCR Expression Analysis, and Dry Mass Measurement

For maize tissue sampling, four public inbred lines (B73, Mo17, A, and B), and three F1 hybrids (B73/Mo17, Mo17/B73, and Hi-II [A/B], first listed is the female parent for each hybrid) were grown in the greenhouse. Plants were grown in the greenhouse in 4-inch flats (for seedlings) or 9-inch pots (for fully grown plants). Plants were watered and fertilized as needed. The day temperature was ~28°C, and night temperature was ~20°C with light photoperiod of 16 h/day.

Leaf tissue samples were harvested at the V1 to V3 stages, corresponding to stages of one to three fully opened leaves. Seedling dry mass of aboveground tissue was collected from five plants per replicate and three replicates per genotype at each V stage. For the leaf tissue growth activity series, the leaf blade was divided into three sections based upon the growth activity: (1) cell division zone, 0 to 6 mm from the base; (2) cell expansion zone, 6 to 20 mm from the base; (3) transition zone, a mix of mature and expanding tissues, 20 mm from the base to the tip of the leaf; (4) mature tissue, bulked V1, V2, V3 mature, or fully opened leaves; and (5) whole V1 seedling aboveground. With the exception of the whole seedling sampling, all leaf sheaths were removed prior to sampling. Three biological replicates were harvested, each derived from three individual plants. Tissues were frozen immediately in liquid nitrogen and stored at −80°C. Tissue for transgenic gene expression analysis was sampled by leaf punches from plants at the V6 stage. Leaf punches were collected from five plants randomly selected from one row for each event. All harvested tissues were frozen immediately in liquid nitrogen and stored at −80°C.

RNA Isolation, RT-PCR, and Multiplexed RT-PCR

Tissue samples were ground and RNA was isolated using Trizol reagent (Invitrogen) following the manufacturer’s protocol, and 10 μg of each RNA sample was DNase treated using the DNA-free kit and protocol (Ambion). First-strand cDNA was made from 700 ng of DNase-treated RNA per sample using the SuperScript II kit and protocol (Invitrogen). One micro-liter of first-strand cDNA was used in 25–μL RT-PCR reaction volumes. A 33-cycle HotStarTaq (Qiagen) multiplex RT-PCR was performed using 0.5 to 1.0 μM primer concentrations for CNR2 and maize α-tubulin, respectively. The primers used are listed in Supplemental Table 1 online. Primers were designed such that they flanked introns or spanned the intron/exon junction to discriminate genomic DNA contamination. For different genotypes, primers were designed in a conserved region between the alleles for equal amplification. RT-PCR parameters (DNA quantity of templates and primers, and the number of PCR cycles) were optimized by following similar protocol as previously described (Guo et al., 2004). For the mature
tissue, due to the high level of CNR2 expression, samples were optimized separately to avoid outcompeting of CNR2 with the maize α-tubulin gene using equal (0.5 μM) concentrations of each primer set, but following the same PCR parameters. PCR products were quantified using ethidium bromide–stained gels and the Quantity One Image Quant software (Bio-Rad).

A linear range for ethidium bromide quantification is shown in Supplemental Figure 6 online. DNA Molecular Weight Marker X (Roche Applied Science) was resuspended to a concentration of 100 ng/μL. Varying amounts (10, 20, 40, and 80 μL) were loaded on the gel and in three replicates. The gels contained 1.5% Seakem GTG Agarose (Lonza), 5 μL of 10 mg/mL ethidium bromide, and 1× TAE (Omnipur; EMD Chemicals). The electrophoresis was conducted at 90 V for 1.3 h in a running buffer of 750 mL, 1× TAE. These were the same gel parameters used for the CNR2/tubulin multiplex gels. The gels were imaged on a Bio-Rad gel imaging system and quantified with Quantity One Image Quant software (Bio-Rad). The 517/506-bp band was quantified since this band is close to the 497-bp CNR2 PCR product and the tubulin 322-bp PCR product.

Real-Time PCR Analysis of CNR1 Expression

Total RNA was treated with DNase I (Invitrogen) and reverse transcribed using the Taqman reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. The final RNA concentration in the cDNA synthesis reaction was 30 ng/μL. Absence of genomic DNA contamination was confirmed by the PCR of no RT control. Primers and 6-carboxyfluorescein-labeled LNA probe were designed using Universal ProbeLibrary (Roche Diagnostics), and primers and 5’ VIC labeled MGB probe for β-actin was designed using Primer Express 2.0 software (Applied Biosystems) (see Supplemental Table 1 online). The expression of each gene was assayed in triplicate in a total volume of 20 μL PCR reaction containing 1× Taqman master mix, 300 nM forward and reverse primers, 100 nM probe, and 3 μL of the 1:4 diluted cDNA. The PCR thermal cycling parameters were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All the assays were run on an ABI 7500 real-time PCR system (Applied Biosystem). Transcript levels of CNR1 were measured relative to the endogenous reference β-actin with the ΔΔt method as described by the manufacturer. The PCR amplification efficiency was determined by measuring a series of input cDNA concentrations. The PCR efficiency for CNR1 and β-actin was 1.00 and 1.02, respectively.

Microscopy Counts of Leaf Epidermal Cells

Tissue was collected from greenhouse grown plants at flowering stage. A 2-cm² section was cut from the midpoint of leaf number eight, counting up from the plant base with the first true leaf as leaf one, and fixed in 70% ethanol. From this fixed tissue, a 1-cm² section was removed, cleared in a saturated solution of chloral hydrate, and mounted on a glass slide in Hoyer’s medium (Dhingra and Sinclair, 1985) under a cover glass. Images were collected with a Zeiss LSM510 microscope using Nomarski differential interference contrast optics and a ×20 Plan-Apochromat (0.75 numerical aperture) objective lens. Images of three nonoverlapping fields of view (0.4 mm²) that did not include a major or minor leaf vein were recorded for each sample. All whole cells captured in the field were counted.

Accession Numbers

Accession numbers for the maize CNR genes are listed in Table 1. Accession numbers for sequences used in the phylogenetic analysis are given in Supplemental Data Set 1 online. Additional sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: maize α-tubulin, X53178; maize β-actin, J01238. The Protein Data Bank accession for the T. maritima CorA structure is 2 hn2.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. CNR1 Expression Patterns in Different Tissues.

Supplemental Figure 2. Leaf Area of Transgenic Plants Overexpressing CNR1 (ProZmUBI::CNR1) and Nontransgenic Controls.

Supplemental Figure 3. Internode Length of Transgenic Plants Overexpressing CNR1 (ProZmUBI::CNR1) and Nontransgenic Controls.

Supplemental Figure 4. Transgenic Plants with Different Copy Numbers of the Transgene for Overexpressing CNR1 (ProZmUBI::CNR1).

Supplemental Figure 5. Leaf Epidermal Cell Number Counts.

Supplemental Figure 6. A Linear Range for Ethidium Bromide Quantification.

Supplemental Table 1. RT-PCR Primers.

Supplemental Data Set 1. The 136 CNR and Related Eukaryotic Sequences Used in This Study, Including in Figure 2.

Supplemental Data Set 2. Text File of the Alignment Used for the Phylogenetic Analysis Shown in Figure 2.

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