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The Maize *Crinkly4* Gene is Expressed Spatially in Vegetative and Floral Organs

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The maize (*Zea mays*) *crinkly4* (*cr4*) gene encodes a receptor-like kinase (RLK). Its mutants show irregularities in the epidermal cells of crinkled and severely adherent leaves, resulting in dwarf characteristics and delayed growth. We successfully grew *cr4* mutants up to the reproductive stage, where we noted both pistillate and staminate spikelets in the tassels. This suggests that the *cr4* gene may be involved in the sex-determination process during bisexual floral-organ development. We found no remarkable abnormalities in the roots. Northern-blot analysis showed that *cr4* gene transcripts were abundant in the leaves, weak in the stems, tassels, and ears, and hardly measurable in the roots. Likewise, transcripts were not detected in the leaves of dark-grown seedlings, but were greatly induced after 24 h of light exposure, indicating that expression of the *cr4* gene is regulated by light. However, transcripts were not inducible in the roots of seedlings either when grown in the dark or following exposure to light treatment, which suggests that this gene is expressed only in aerial-specific organs.

Keywords: crinkled leaves, epidermis, floral-organ development, maize, receptor-like kinase

Successful plant growth and development require that proper systems be in place for transport and acceptance of environmental stimuli and endogenous signaling molecules. Cell-surface receptors in the protein kinase family play a fundamental role in these signal-transduction pathways (Stone and Walker, 1995; Becraft, 1998; Sheen, 2001). These receptor-like protein kinases (RLK) are numerous in cases of pollen self-incompatibility, as well as during cellular processes such as meristem development, hormone perception, cell morphogenesis and differentiation, organ formation and abscission, somatic embryogenesis, and gametophyte development (Mu et al., 1994; Clouse, 1996; Clark et al., 1997; Li and Chory, 1997; Becraft, 1998; Lease et al., 1998; Gomez-Gomez and Boller, 2000). In several previous functional analyses of plant RLKs, researchers demonstrated that 1) the S-locus receptor kinase (*SRK*) of *Brassica* is specifically involved in pollen self-incompatibility (Goring et al., 1993; Dwyer et al., 1994); 2) the maize *crinkly4* (*cr4*) gene is active in cellular responses to differentiation of the leaf-cell epidermis (Becraft et al., 1996); 3) *Arabidopsis* BRI1 mediates brassinosteroid hormones (Clouse, 1996; Li and Chory, 1997); 4) *CLAVATA1* (*CLV1*) of *Arabidopsis* is necessary for maintaining the balance between cell proliferation and differentiation in the shoot apical meristem (Clark et al., 1997); 5) the *Arabidopsis* leucine-rich repeat receptor kinase (*HAESA*) controls abscission of floral organs (Jinn et al., 2000); 6) the *Arabidopsis cr4* homologue (*ACR4*) is involved in proper embryogenesis (Tanaka et al., 2002); 7) wall-associated kinase 1 (*WAK1*) may function in linking cell walls to the cytoplasm (Kohorn et al., 1992; He et al., 1996) and might be involved in plant defense (He et al., 1998, 1999); and, finally, 8) *Arabidopsis* somatic embryogenesis receptor-like kinase (*SERK*) promotes embryogenesis (Schmidt et al., 1997) and is required for the development of male and female gametophytes (Mu et al., 1994).

The maize *cr4* gene encodes a receptor-like kinase (RLK), and the extracellular domain of the CR4 protein contains a cysteine-rich region similar to the repeats of tumor necrosis factor receptors (TNFRs) (Becraft et al., 1996). Moreover, this protein is autophosphorylated on serine and threonine residues (Jin et al., 2000). The typical phenotype of *cr4* mutants consists of crinkled leaves and mosaic aleurones (Becraft et al., 1996, 2001; Becraft and Asuncion-Crabb, 2000; Jin et al., 2000). These observations suggest that the maize CR4 is involved in differentiation of the epidermis and

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Abbreviations: *cr4*, *crinkly4*; TNFR, tumor necrosis factor receptor; RLK, receptor-like kinase.

acquisition of the aleurone-cell fate (Becraft et al., 1996), as mediated by a signaling from the receptor kinase that is similar to the growth factor-like differentiation response in animals (Jin et al., 2000).

Although the phenotype produced by the maize *cr4* gene has been intensively characterized, little is known about its expression pattern during maize development. Therefore, our objectives were to: 1) successfully grow *cr4* mutants to the reproductive stage, 2) describe any new phenotypes specific to mature mutant plants, and 3) measure levels of *cr4* transcripts as they related to floral organs and roots.

MATERIALS AND METHODS

Plant Material

Wild-type maize (*Zea mays* L.) plants (WT-22) were grown at 20°C under short days (8-h photoperiod). To isolate RNA, we sampled leaves, stems, and roots from two-week-old seedlings, and collected tassels and ears from mature plants. For our light treatments, the plants were grown in the dark at 20°C for 7 d on 3MM filter paper placed in 15-cm Petri dishes. Some were then exposed to light for 6, 12, 24, or 48 h.

Two strong crinkly-mutant alleles, *cr4-60* and *cr4-651*, of maize were also used. The *cr4* mutants were obtained from Dr. Philip W. Becraft, Iowa State University, Ames, IA, USA; the *cr4-60* mutant, induced by the transposon-tagging method; and the *cr4-651* mutant, achieved via the chemical mutagen EMS (Becraft et al., 1996; Jin et al., 2000).

Northern Blot Analysis

Total RNA was isolated from harvested tissues using a phenol/chloroform extraction method; northern blot analysis was carried out as described by An and An (2000). Briefly, 15 μg of total RNA was electrophoretically separated on 1.4% agarose gel using 1X MOPS [3-(N-morpholino)-propanesulfonic acid] buffer, then transferred for 12 h to membranes (Schleicher and Schuell, Keene, NH, USA) in 25 mM phosphate buffer (pH 7.0). Total RNA was cross-linked on the membranes under UV cross-linker (Startagen, La Jolla, CA, USA). The RNA probes were synthesized and labeled with α^{32}P-UTP, as described in the procedures for the Riboprobe® System (Promega Co., Madison, WI, USA). Hybridization was performed with α^{32}P-UTP-labeled probes for 14 h at 62°C in buffer [50% formamide, 1.0 M NaCl, 10% (w/v) dextran sulfate, 1.0% SDS, and 0.1 mg/mL denatured salmon sperm DNA]. Each membrane was washed twice in 2X SSC for 5 min at room temperature (RT), once in 1X SSC for 10 min (RT), once in 0.1X SSC for 20 min (RT), and once in 0.1X SSC for 5 min at 65°C. All SSC washing solutions contained 0.1% SDS. The washed membranes were exposed for 24 h to X-ray film for autoradiography. Experiments were duplicated to verify the results of the light treatments and northern analysis.

Scanning Electron Microscopy

Samples were pre-fixed in 2.5% glutaraldehyde for 4 h, and washed in phosphate buffer overnight at 4°C. Afterward, the samples were fixed in 1% O_5O_4 for 1 h, then washed in phosphate buffer. Dehydration was performed in an ethanol series, transposed twice in isoamyl acetate for 20 min. The samples were mounted on double-faced tape, gold-coated in an ion coater, and viewed with a Hitachi Scanning Electron Microscope (SEM).

RESULTS AND DISCUSSION

Expression of the *cr4* Mutant Phenotype in Developing Leaves and Floral Organs

Phenotypes of the recessive *cr4* mutants were observed in both seedlings and mature plants (Fig. 1), including the typical crinkled and adhered leaves and dwarfing that are characteristic of *cr4-60* and *cr4-651* mutants (Becraft et al., 1996). The latter had even more severe leaf distortion. However, roots from the two mutants at the seedling stage showed normal growth (Fig. 1A).

Our *cr4* mutant plants presented pistillate tassels (Fig. 1D), which indicates that the *cr4* gene interrupted the pattern of formation for sexual-organ determination, and suggests that proper functioning of the CR4 protein may be required in maize. Female and male sexual organs are spatially separated, and develop into either tassels or ears in a single monoecious plant. If the normal pistil-abortion process fails and concomitant stamen abortion is induced, staminate-to-pistillate conversion may occur in the tassel (Dellaporta and Calderon-Urrea, 1994; Ainsworth et al., 1998). The *tasselseed* genes (*Ts1*, *Ts2*, and *Ts5*) normally are involved in masculinization (Irish et al., 1994; Calderon-Urrea and Dellaporta, 1999). However, mutants of several feminization genes, *d1*, *d2*, *d3*, and *d5*, in dwarfed plants can block GA biosynthesis, resulting in stamen abortion (Phinney, 1961). Mutant pistillate-tassel organs are also induced

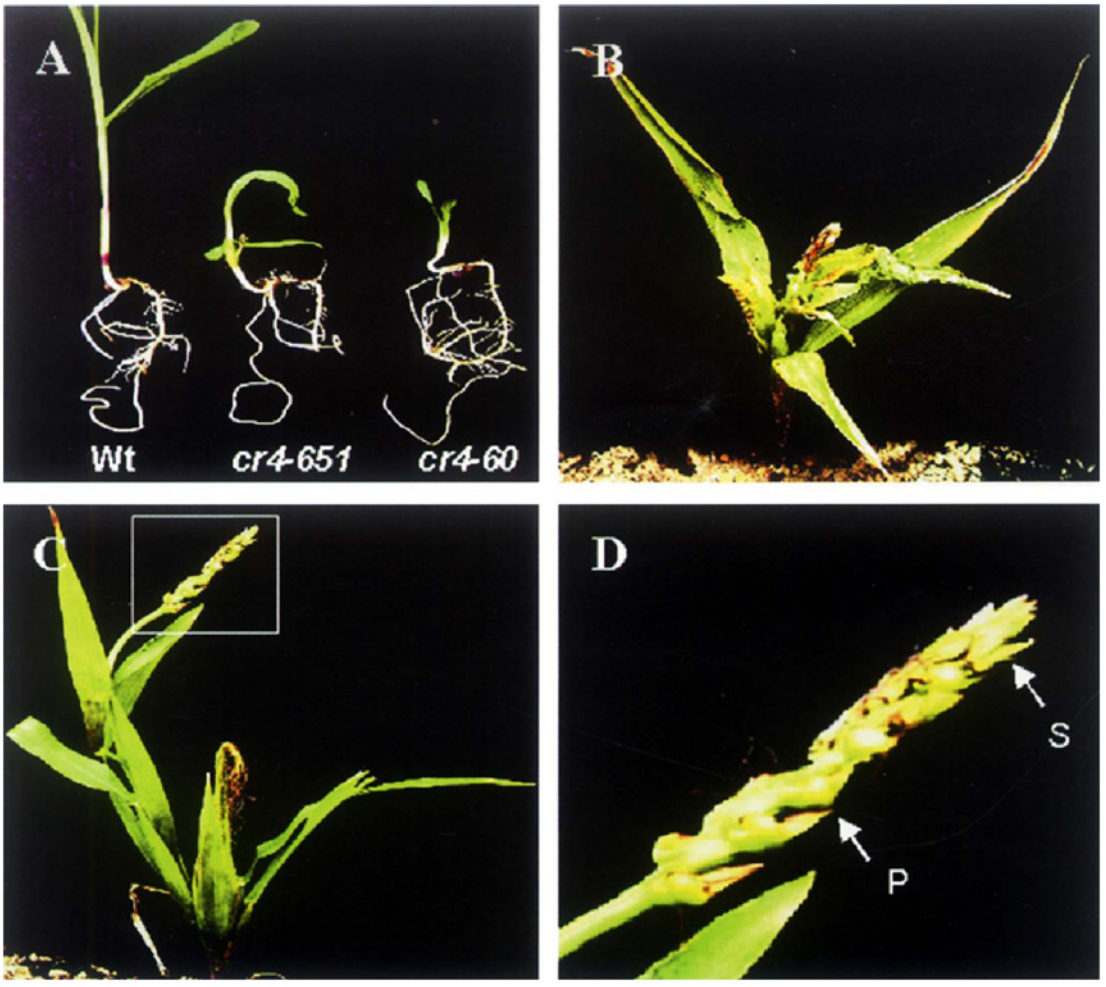

Figure 1. Phenotype of *cr4* mutants: (A) maize seedlings of mutants *cr4-651* and *cr4-60*, and wild-type W22; (B) mature *cr4-60* plant; (C) mature *cr4-651* plant; (D) *cr4-651* mutant tassel with pistillate spikelets. Staminate spikelet (S), and pistillate spikelet (P) with a silk.

by the plant hormone GA during stamen development. We observed that *cr4-60* and *cr4-651* had the typical dwarf phenotype and mutant pistillate tassels (Fig. 1D). Based on these observations, we speculate that *CR4* may be directly or indirectly involved in GA signaling or biosynthesis.

cr4 Expression in Leaf Epidermal Cells and Roots

The *cr4* mutant plants showed severe epidermal distortions in the aerial organs, an observation similar to those of Becraft et al. (1996), who had reported clearly mutated morphologies in the leaf epidermis. Cells in the wild-type leaf tissues were aligned longitudinally, having a rectangular shape and smooth surfaces (Fig. 2, A and B). However, leaves from the mutant *cr4* were crinkled and folded (Fig. 2C), and their irregular epidermal cells were roughly shaped with crevices (Fig. 2D). These typical mutant phenotypes resulted from the loss of *cr4* functions during epidermal-cell differentiation (Becraft et al., 1996). In contrast, we found no defective growth during our SEM study of root structures in those mutants. In fact, the epidermal morphology of the *cr4-651* mutant roots was not much different from that of the wild-type WT22 (Fig. 2). As with the wild type, the epidermis of the mutant was smooth and the root hairs well-developed (Fig. 2, E and H), all indicating that *cr4* does not affect epidermal-cell differentiation in the underground portions.

Expression Patterns of *cr4* Transcripts

We examined expression patterns of the *cr4* gene in organs to determine whether distribution and transcript levels in the wild-type plants were correlated with those in tissues showing abnormal phenotypes.

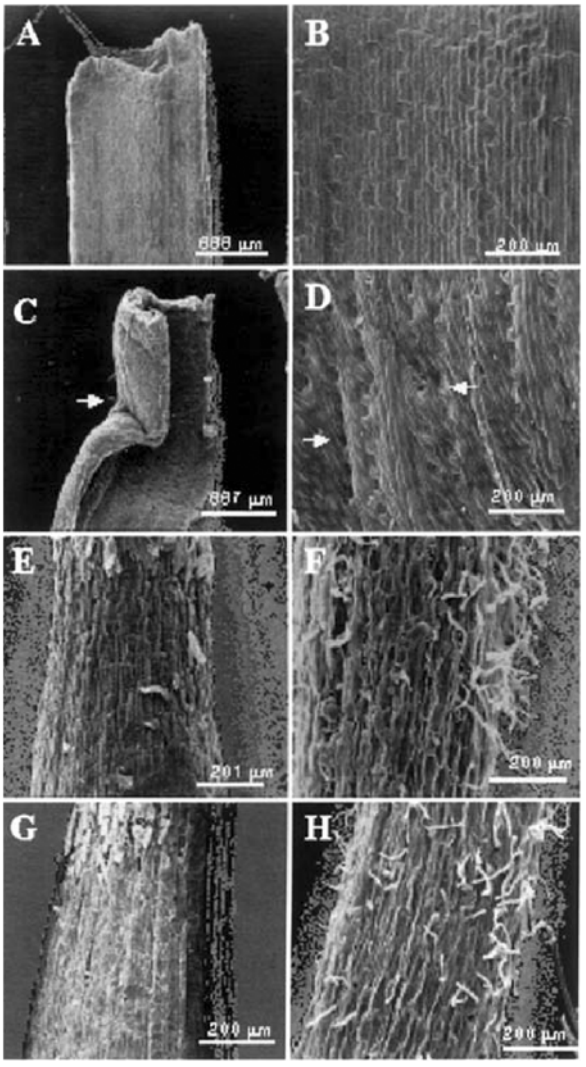

Figure 2. Scanning electron microscope (SEM) images of leaves and roots. Wild-type leaf (A, B) and root (E, F); *Crinkly4*-mutant leaf (C, D) and root (G, H). Wild-type leaf epidermal cells are rectangular, aligned longitudinally, and smooth (B); in the mutant, epidermal cells are irregular, rough-shaped, and folded (arrow in C); guard cells are placed in a crevice (at arrows) formed by irregular epidermal-cell proliferation (D); mutant root phenotype (G, H) is not different from that of the wild-type plant (E, F).

Northern blot analysis was performed with a ^{32}P-UTP-labeled *cr4* RNA probe. Transcripts were accumulated at higher levels in the leaves than in seedling stems and floral organs (Fig. 3), but were not present in the roots of either seedlings or mature plants. This lack of transcript perhaps explains why root growth was not defective in *cr4* mutants.

The floral organs in a single maize plant are partitioned

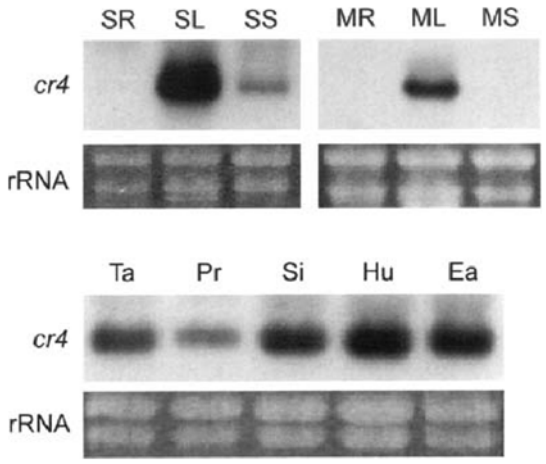

Figure 3. RNA blot analysis of *cr4* gene expression in wild-type maize. In each lane, 15 μg of total RNA was blotted and hybridized with 520 bp of the radioactive-labeled *cr4* RNA probe (cr4). Subjected total RNA was isolated from seedling roots (SR), seedling leaves (SL), seedling stems (SS), mature roots (MR), mature leaves (ML), and mature stems (MS). Total RNAs were isolated from tassels (Ta), prophyll (Pr), silks (Si), husks (Hu), and ears (Ea). Ribosomal RNA (rRNA) bands are shown as a loading control.

into separate pistilate (ear) and staminate (tassel) inflorescences. However, both *cr4* mutants produced bisexual spikelets in their mutant pistillate tassels, rather than normal staminate-tassel florets in the primary shoot. We observed that high transcript levels of *cr4* gene were detected in all reproductive organs (Fig. 3). In the ear, *cr4* transcripts were most abundant in the prophyll, silks, and husks. The phenotype of staminate spikelets in the pistillate tassel was very similar to that of *tassel-seed (ts)* mutants reported by DeLong et al. (1993). Therefore, we propose that the CR4 protein may be involved in the mechanism for sex determination in maize.

Effect of Light on *cr4* Gene Expression

From the northern blot analysis, we could see that *cr4* transcripts were abundant in aerial organs, but hardly noticeable in the underground tissues (Fig. 3). Therefore, we examined the role of light in *cr4* gene expression. After the seeds were germinated in the dark for 7 d, the seedlings were transferred to continuous light. Although *cr4* transcript levels were weak and undetectable in leaves after only 12 h of light exposure (Fig. 4), they rapidly accumulated in leaf tissues treated for 24 h, a result that indicates the *cr4* gene is light-inducible. Moreover, transcripts were not induced in the leaves of

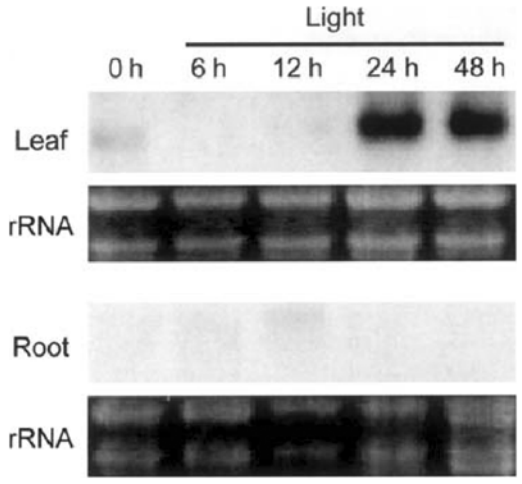

Figure 4. Accumulation of *cr4* transcripts induced by light. Fifteen micrograms of total RNA was loaded and blotted in each lane. Seedlings were germinated in the dark and transferred to the light. Samples were harvested after light exposure for 0, 6, 12, 24, or 48 h. The 520-bp radioactive-labeled *cr4* RNA probe was used for hybridization. Ribosomal RNA bands (rRNA) are shown as a loading control.

seedlings grown in the dark for 48 h (data not shown).

In parallel observations, we noted that *cr4* transcript levels in the roots of the same light-treated seedlings were not detectable (Fig. 4), which indicates that light does not induce *cr4* gene expression in roots. Tanaka et al. (2002) also reported no expression of the maize *cr4* homologue *ACR4* gene in roots. In proposing that *cr4* can be induced in the light only for aerial organs, we can also explain that this is why root morphology varied little between our wild and mutant types.

In summary, we have demonstrated here that expression of the *cr4* gene is organ-specific in maize, and that this gene may be involved in critical functions of plant morphogenesis, such as when determining the patterns of development during the formation of sexual organs.

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LITERATURE CITED

Ainsworth C, Parker J, Buchanam-Wollaston V (1998) Sex determination in plants. Curr Top Dev Biol 38: 167-223

An K, An G (2000) Overriding photoperiod sensitivity of flowering time by constitutive expression of a MADS box gene. J Plant Biol 43: 28-32

Becraft PW (1998) Receptor kinases in plant development. Trends Plant Sci 3: 384-388

Becraft PW, Asuncion-Crabb Y (2000) Positional cues specify and maintain aleurone cell fate in maize endosperm development. Development 127: 4039-4048

Becraft PW, Kang SH, Suh SG (2001) The maize *CRINKLY4* receptor kinase controls a cell-autonomous differentiation response. Plant Physiol 127: 486-496

Becraft PW, Stinard PS, McCarty DR (1996) *CRINKLY4*: A TNFR-like receptor kinase involved in maize epidermal differentiation. Science 273: 1406-1409

Calderon-Urrea A, Dellaporta SL (1999) Cell death and cell protection genes determine the fate of pistils in maize. Development 126: 435-441

Clark SE, Williams RW, Meyerowitz EM (1997) The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. Cell 89: 575-585

Clouse SD (1996) Molecular genetic studies confirm the role of brassinosteroids in plant growth and development. Plant J 10: 1-8

Dellaporta SL, Calderon-Urrea A (1994) The sex determination process in maize. Science 266: 1501-1505

DeLong A, Calderon-Urrea A, Dellaporta SL (1993) Sex determination gene *TASSELSEED2* of maize encodes a short-chain alcohol dehydrogenase required for stage-specific floral organ abortion. Cell 74: 757-768

Dwyer KG, Kandasamy MK, Mahosky DI, Acciai J, Kudish BI, Miller JE, Nasrallah ME, Nasrallah JB (1994) A superfamily of S locus-related sequences in *Arabidopsis*: Diverse structures and expression patterns. Plant Cell 6: 1829-1843

Gomez-Gomez L, Boller T (2000) FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol Cell 5: 1003-1011

Goring DR, Glavin TL, Schafer U, Rothstein SJ (1993) An S receptor kinase gene in self-compatible *Brassica napus* has a 1-bp deletion. Plant Cell 5: 531-539

He ZH, Cheeseman I, He D, Kohorn BD (1999) A cluster of five cell wall-associated receptor kinase genes, *Wak1-5*, is expressed in specific organs of *Arabidopsis*. Plant Mol Biol 39: 1189-1196

He ZH, Fujiki M, Kohorn BD (1996) A cell wall-associated, receptor-like protein kinase. J Biol Chem 271: 19789-19793

He ZH, He D, Kohorn BD (1998) Requirement for the induced expression of a cell wall associated receptor

kinase for survival during the pathogen response. Plant J 14: 55-63

Irish EE, Langdale JA, Nelson TM (1994) Interactions between tassel seed genes and other sex determining genes in maize. Dev Genet 15: 155-171

Jin P, Guo T, Becraft PW (2000) The maize *CR4* receptor-like kinase mediates a growth factor-like differentiation response. Genesis 27: 104-116

Jinn TL, Stone JM, Walker JC (2000) *HAESA*, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission. Genes Dev 14: 108-117

Kohorn BD, Lane S, Smith TA (1992) An *Arabidopsis* serine/threonine kinase homologue with an epidermal growth factor repeat selected in yeast for its specificity for a thylakoid membrane protein. Proc Natl Acad Sci USA 89: 10989-10992

Lease K, Ingham E, Walker JC (1998) Challenges in understanding RLK function. Curr Opin Plant Biol 1: 388-392

Li J, Chory J (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90: 929-938

Mu JH, Lee HS, Kao TH (1994) Characterization of a pollen-expressed receptor-like kinase gene of *Petunia inflata* and the activity of its encoded kinase. Plant Cell 6: 709-721

Phinney BO (1961) Plant Growth Regulation, *In* RM Kein, ed, Iowa State Univ Press, Ames, IA, USA, pp 489-501

Schmidt ED, Guzzo F, Toonen MA, de Vries SC (1997) A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development 124: 2049-2062

Sheen J (2001) Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. Plant Physiol 127: 1466-1475

Stone JM, Walker JC (1995) Plant protein kinase families and signal transduction. Plant Physiol 108: 451-457

Tanaka H, Watanabe M, Watanabe D, Tanaka T, Machida C, Machida Y (2002) *ACR4*, a putative receptor kinase gene of *Arabidopsis thaliana*, that is expressed in the outer cell layers of embryos and plants, is involved in proper embryogenesis. Plant Cell Physiol 43: 419-428