“XCT regulates ethylene responses in Arabidopsis”

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“XAP5 CIRCADIAN TIMEKEEPER regulates ethylene responses in aerial tissues of Arabidopsis”

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ABSTRACT

The phytohormone ethylene differentially regulates plant architecture and growth in both a light- and nutrient-dependent fashion. The modulation of plant development by ethylene in response to both external and internal signals can also generate tissue-specific differential responses. Here we report that \textit{XAP5 CIRCADIAN TIMEKEEPER (XCT)} is involved in blue light-dependent ethylene responses in the aerial tissues of \textit{Arabidopsis thaliana} seedlings. \textit{XCT} was first identified as a circadian clock mutant with a short free-running period. The \textit{xct} mutation also causes sugar-specific hypocotyl growth defects, in which mutants are short in blue light when grown on a sucrose-rich medium, but tall when grown on sucrose-deficient media. Our data suggest that the hypocotyl defects in blue light are not directly caused by defects in clock or light signaling, but rather by enhanced ethylene responses. In blue light, \textit{xct} mutants have a more active ethylene response pathway and exhibit growth phenotypes similar to the constitutive ethylene signaling mutant \textit{ctr1}. \textit{xct} mutants also have reduced ethylene emission, analogous to plants that have lost CTR1 function. Genetic analysis suggests that \textit{XCT} negatively regulates ethylene responses downstream of \textit{EIN3} in aerial tissues. However, \textit{XCT} is not required for all ethylene-mediated processes, such as the inhibition of root growth. Thus, \textit{XCT} acts downstream of a major transcriptional regulator in an organ-specific manner, playing an environment-dependent role in the regulation of plant growth.
INTRODUCTION

As sessile organisms, plants must tightly control their growth in order to optimize and complete their life cycle. To this end, plants have evolved sophisticated mechanisms to modulate their growth in response to various internal and external stimuli. Studies in growth control of the hypocotyl, the plant embryonic stem, have revealed diverse molecular players involved in this process (Jimenez-Gomez and Maloof, 2009). The circadian clock, light signaling, and various phytohormones act to control growth of the hypocotyl as well as other organs (Vandenbussche et al., 2005, Nozue and Maloof, 2006). An emerging understanding of these pathways is beginning to shed light onto how each acts independently and cooperatively to impact growth.

Light regulates plant growth and development in a process called photomorphogenesis. Plants perceive different qualities and quantities of light using a variety of photoreceptors. The phytochrome family in Arabidopsis contains five photoreceptors, phyA-phyE, that perceive and respond to red and far-red light (Montgomery and Lagarias, 2002). The cryptochrome family, consisting of CRY1 and CRY2, perceive and respond to blue light (Jiao et al., 2007). Both phytochromes and cryptochromes act as negative regulators of hypocotyl elongation and positive regulators of the photomorphogenic response (Holm et al., 2002). In this sense light signaling, regardless of the quality of light perceived, generally acts to inhibit elongation growth.

Phytohormones affect virtually every aspect of plant growth and development, including regulation of hypocotyl growth (Vandenbussche et al., 2005). One phytohormone involved in the development of a wide variety of plant organs is ethylene, a simple gaseous hydrocarbon that impacts every stage of plant growth and development (Kieber and Ecker, 1993; De Paepe and Van Der Straeten, 2005). Ethylene has differential effects on the growth of different plant organs (Vandenbussche et al., 2007). In dark-grown plants, ethylene initiates the classic triple response, which consists of the radial swelling and shortening of the hypocotyl, the inhibition of root growth, and the formation of an exaggerated apical hook (Kieber et al., 1993).

As well as being organ-specific, ethylene responses are also influenced by growth conditions. For example, light quality affects ethylene responses: in monochromatic blue or white light, ethylene promotes hypocotyl elongation; in contrast, in monochromatic red light, ethylene inhibits hypocotyl elongation (Khanna et al., 2007; Vandenbussche et al., 2007). A further complication is that in light-grown plants ethylene action is also dependent on the
nutrient status of the growth media (Smalle et al., 1997; Collett et al., 2000). Ethylene signaling promotes hypocotyl elongation when light-grown plants are maintained on minimal media, but this effect is partially or totally masked on rich media (Smalle et al., 1997). Other external factors, such as temperature, have also been reported to modulate the action of ethylene on hypocotyl growth (Collett et al., 2000). Therefore, the role of ethylene in regulating growth of the hypocotyl is extremely dependent on environmental conditions.

The biosynthesis of ethylene in planta begins with the stepwise conversion of L-methionine to S-adenosyl-L-methionine (AdoMet) to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS). The conversion of AdoMet to ACC is the rate-limiting step in the catalytic pathway to produce ethylene. ACC oxidase (ACO) then converts ACC into ethylene by an oxidation reaction (Chae and Kieber, 2005). Both ACS and ACO are present in multigene families in the Arabidopsis genome (as well as in other plant species), are regulated at the transcriptional and posttranscriptional level, and contain a high degree of functional specificity (Tsuchisaka et al., 2009). Several characterized ethylene biosynthesis mutants fail to properly regulate ACS stability, which in general are short-lived proteins. For example, the ethylene overproducing mutant eto1 confers increased stability to the ACS5 protein, resulting in increased ethylene production (Chae et al., 2003; Chae and Kieber, 2005).

After ethylene is synthesized in the cytosol, it is perceived by ER membrane-localized ethylene receptors, of which there are five in Arabidopsis (Lin et al., 2009). In the absence of ethylene, the receptors are bound to a MAPKKK protein called CTR1. CTR1 is a negative regulator of the entire pathway, thus ethylene signaling is constitutively active in loss-of-function ctr1 mutants (Kieber et al., 1993). The interaction of ethylene with the ethylene receptors alters receptor activity, allowing the inactivation of CTR1 and thus relieving its repression of the pathway (Chen et al., 2007).

Perception of ethylene and subsequent inactivation of CTR1 activates a protein called EIN2 through an unknown mechanism. EIN2 is an ER-localized transmembrane protein with unidentified function that is essential for the activation of EIN3 activity. EIN3 is a nuclear-localized transcription factor with several homologs, termed EIN3-like proteins, or EILs (Chao et al., 1997; Solano et al., 1998; Lin et al., 2009). Loss of EIN3 results in full or partial ethylene-insensitivity depending on the environmental condition, as EIN3 and the EILs have partially overlapping functions (Binder et al., 2007). EIN3 binds to the promoters of target genes called
ERFs, or ethylene response factors. Despite an abundance of data supporting the molecular function of EIN3, very few ERFs have been functionally characterized and can have roles in other signaling pathways, such as ABA signaling (Nakano et al., 2006). There are over 100 ERF or ERF-like genes in the Arabidopsis genome, and they themselves encode transcription factors. ERF transcription factors bind to the promoters of ethylene-regulated genes at an element containing a GCC motif and initiate the ethylene response at the molecular level, which includes both activation and repression of target genes (Guo and Ecker, 2004; Gutterson and Reuber, 2004; Nakano et al., 2006).

Ethylene signaling is tightly regulated at every level, ranging from ethylene biosynthesis to downstream ethylene-mediated transcriptional regulation. ACS proteins function as both homo- and heterodimers, providing some spatial and temporal specificity to ethylene production (Tsuchisaka et al., 2009). Ethylene receptors also function as protein complexes and this may influence how and when ethylene can be perceived (Lin et al., 2009). CTR1 is involved in the activation of a MAP kinase signaling cascade involving MAPKK9 and MPK3/6, which activate EIN3 independently of EIN2 (Yoo et al., 2008). EIN3 is also regulated by additional regulatory proteins, such as EBF1/2, which destabilize EIN3 (Binder et al., 2007), as well as in response to different environmental conditions such as sugar availability and light (Yanagisawa et al., 2003) (Lee et al., 2006). Downstream of EIN3, little is known about how ERF proteins are regulated or how their target genes might be additionally regulated.

Another important regulator of plant development is the circadian clock, which influences growth via multiple pathways. For example, it regulates the production of auxin and ethylene and modulates plant sensitivity to multiple hormones (Thain et al., 2004; Covington and Harmer, 2007; Covington et al., 2008; Michael et al., 2008; Legnaioli et al., 2009; Rawat et al., 2009). In addition, the circadian clock directly controls expression of growth-promoting transcription factors (Nozue et al., 2007). It is thus not surprising that mutations in central clock genes such as CCA1, LHY, and TOC1 also cause growth-related phenotypes (Nozue and Maloof, 2006).

Previously, we reported the molecular identification of XAP5 CIRCADIAN TIMEKEEPER (XCT), a novel gene with pleiotropic effects on plant growth and development (Martin-Tryon and Harmer, 2008). xct mutants have delayed greening, short-period circadian rhythms, and altered regulation of hypocotyl elongation. XCT is a nuclear-localized protein that
is highly conserved across eukaryotes and yet has no known molecular function in any organism. Here, we report that XCT is a negative regulator of the blue-light mediated ethylene response in aerial tissues of Arabidopsis. Loss-of-function xct mutants have phenotypes qualitatively similar to but less severe than the strong constitutive ethylene signaling mutant ctr1-3. Our data strongly suggest that XCT does not act within the ethylene signaling pathway itself, but rather acts as an organ-specific regulator of the ethylene response downstream of the transcription factor EIN3, perhaps introducing additional specificity to the ethylene response. Our data therefore suggest that XCT acts in the nucleus to modulate the activity of one or more transcription factors, providing important clues regarding the molecular function of XCT-like proteins in plants and other eukaryotes.
RESULTS

XCT differentially regulates hypocotyl elongation in a sugar- and light-specific manner

Since the molecular function of XCT is unknown, we decided to examine its biological role in control of hypocotyl elongation, a well-studied model for growth control in plants. We previously reported that xct-2 mutants have a short hypocotyl in blue light but have a tall hypocotyl in red light when grown on MS media supplemented with 3% sucrose, a standard condition for circadian experiments (Martin-Tryon and Harmer, 2008). To investigate whether the presence of sucrose in the media might affect the growth phenotype, we assayed hypocotyl elongation in xct-2 grown on media not supplemented with sucrose. Surprisingly, when grown in blue light without the addition of exogenous sucrose, xct-2 mutants had a tall hypocotyl relative to wild type (Figure 1A). We did not observe any significant difference in root growth between wild type and xct mutants grown either plus or minus sucrose (Figure 1C and 1D). XCT is expressed and produces a stable protein in the roots (Martin-Tryon and Harmer, 2008), so the lack of a root phenotype in xct mutants is not due to hypocotyl-specific expression. Therefore, XCT plays a sugar- and organ-dependent role in growth control in blue light.

Given this surprising phenotype in blue light, we examined whether media composition could alter the growth phenotype of xct mutants in red light. In this condition, xct mutants had elongated hypocotyls relative to wild type regardless of the presence or absence of sucrose in the growth media (Figure 1E and 1F). Thus the hypocotyl phenotype of xct mutants is sugar-dependent in blue, but not red light.

Since the circadian clock regulates the rhythmic growth of the hypocotyl (Nozue and Maloof, 2006), we hypothesized that the sugar-specific hypocotyl growth defect in xct-2 in blue light might be due to differential clock defects in this mutant when grown in the presence or absence of sucrose. However, xct-2 plants grown in blue light had the same short period phenotype both in the presence and absence of exogenous sucrose (Figure 1G). Since the presence or absence of sugar had no effect on the circadian phenotype but completely changed the nature of the hypocotyl phenotype, the growth phenotypes in xct-2 are likely not directly caused by the circadian defect.
Since the \textit{xct} hypocotyl and clock phenotypes seemed independent, we reasoned that XCT might act in a light-signaling pathway to control hypocotyl elongation. An important mediator of both blue- and red-light signaling is HY5, a bZIP transcription factor (Oyama et al., 1997; Nozue and Maloof, 2006). To determine whether XCT acts in HY5 signaling, we examined epistatic interactions between \textit{xct-2} and \textit{hy5-215}. We found that the \textit{xct-2} and \textit{hy5-215} phenotypes were additive when plants were grown either in the presence or absence of sucrose (Supplemental Figure 1). This suggests that XCT does not act primarily in a HY5-regulated light signaling pathway, a conclusion supported by the normal sensitivity of \textit{xct} mutants to increasing intensities of light (Martin-Tryon et al. 2008).

We next hypothesized that the hypocotyl phenotypes might be related to an altered hormone-signaling pathway. It has been previously reported that ethylene regulates hypocotyl elongation in a differential manner, depending on the light environment and nutrient status of the growth media (Pierik et al., 2006). Indeed, \textit{ctr1-3} mutants have a constitutively active ethylene signaling pathway and demonstrate both light quality- and nutrient-specific growth phenotypes (Vandenbussche et al., 2007) similar to \textit{xct-2}. We therefore reasoned that the blue light hypocotyl phenotypes in \textit{xct-2} might be due to an over-active ethylene pathway.

\textbf{XCT plays a role in ethylene signaling}

To determine whether XCT is involved in ethylene signaling, we analyzed \textit{xct-2} hypocotyl growth in blue light in the presence of an ethylene signaling inhibitor. Plants were grown under blue light for six days on MS media containing sucrose or media containing both sucrose and silver nitrate (AgNO$_3$), an antagonist of the ethylene signaling pathway thought to act at the level of the ethylene receptors. The addition of 50 $\mu$M silver nitrate to the growth media resulted in an increase of the hypocotyl height in wild type plants (Figure 2A), suggesting that ethylene inhibits hypocotyl growth in blue light in a sucrose-rich media. \textit{xct-2}, like \textit{ctr1-3}, exhibited a short hypocotyl relative to wild type both in the presence and absence of silver nitrate. In contrast, the short hypocotyl phenotype of the ethylene overproducing mutant \textit{eto1-1} became indistinguishable from wild type upon the addition of silver nitrate to the media. This suggested that the \textit{xct} phenotype was not due to ethylene overproduction.
When grown on MS media without added sucrose, \textit{xct-2}, \textit{eto1-1}, and \textit{ctr1-3} were all significantly taller than wild type (Supplementary Figure S2) and the addition of silver to the media did not have any significant effect on the hypocotyl height in any of the genotypes. Consistent with a previous study (Vandenbussche et al., 2007), this suggests that ethylene signaling was not limiting for hypocotyl growth under these conditions.

Since the inability of silver nitrate to rescue the \textit{xct-2} phenotype suggested that these mutants might have some sort of ethylene signaling (as opposed to biosynthesis-related) defect, we measured ethylene emission levels in \textit{xct-2} in both the presence and absence of exogenous sucrose in blue light. \textit{xct-2} mutants produced significantly less ethylene than wild type when grown in either media (Figure 2B). This decrease in ethylene emission was completely rescued by introduction of a genomic copy of \textit{XCT} driven by its own promoter into the \textit{xct-2} mutant background (\textit{xct-2}/\textit{XCT}), indicating that the ethylene production defect was indeed solely due to loss of \textit{XCT} function. Similarly to \textit{xct-2}, \textit{ctr1-3} mutants displayed reduced ethylene emission levels both in the presence and absence of sucrose (Figure 2B), consistent with previous studies of dark-grown \textit{ctr1-3} plants (Kieber et al., 1993). This decrease in ethylene emission in mutants such as \textit{ctr1-3} that have increased ethylene signaling is likely due to negative feedback regulation on the production of ethylene. This is corroborated by the fact that ethylene insensitive mutants actually produce more ethylene than wild type plants (Thain et al., 2004). Taken together, these data suggested that \textit{XCT} negatively regulates some aspect of the ethylene signaling pathway.

\textbf{xct-2 mutants are not hypersensitive to ethylene}

The similarity between the \textit{xct-2} and \textit{ctr1-3} phenotypes suggested that \textit{xct-2} mutants might have altered responsiveness to ethylene. We therefore examined the sensitivity of \textit{xct} and ethylene signaling mutants to ACC, the precursor molecule to ethylene in the biosynthetic pathway. In the absence of ACC, the hypocotyls of \textit{xct-2}, \textit{ctr1-3}, and \textit{eto1-1} mutants grown in blue light on rich medium were all shorter than wild type (Figure 3A). When grown on plates containing the ethylene precursor, the ethylene overproducing mutant \textit{eto1-1} was indistinguishable from wild type, showing maximal growth inhibition at 5 μM ACC (Figure 3A). In contrast, the lowest concentration of ACC tested (0.1 μM) was sufficient to cause maximal
inhibition of hypocotyl growth in the constitutive ethylene signaling mutant ctr1-3. Hypocotyl elongation in xct-2 mutants, like wild type, was maximally inhibited by 5 μM ACC (Figure 3A-B). When the hypocotyl lengths of ACC-treated plants are graphed as a percentage of control plants, it is clear that xct-2 mutants show reduced responsiveness to ACC (Figure 3B).

xct-2 are therefore not hypersensitive to ethylene-induced inhibition of hypocotyl elongation in blue light. The observed reduced responsiveness could arise from a more active basal ethylene signaling pathway in xct-2, analogous to ctr1-3. However, the basal ethylene pathway in xct-2 is not as activated as in ctr1-3 given that ethylene-mediated inhibition of growth in xct-2 only saturated at 5 μM ACC. These data suggest that xct-2 mutants have an ethylene signaling defect in blue light unrelated to alterations in sensitivity to the hormone.

**Genes positively regulated by ethylene have increased expression in xct-2**

If basal ethylene signaling is increased in xct, we would expect genes positively regulated by ethylene to have higher expression levels even in the absence of exogenous ACC. To investigate this, we used quantitative PCR (qRT-PCR) to examine mRNA levels of various ethylene responsive genes in the presence or absence of ACC. In xct-2 mutants grown on MS sucrose media without exogenous ACC, the expression levels of ACO2 and ETR2 were indeed higher in xct-2 relative to wild type (Figure 4A and 4B). Similarly, the expression of these genes was elevated in eto1-1 relative to wild type. We observed similar gene expression patterns in these genotypes when grown on MS media without added sucrose (Supplemental Figure S3). These data strongly suggest that basal activity of the ethylene pathway is elevated in xct-2 mutants.

We next examined the effect of chronic ACC treatment on the expression of these genes in both wild type and mutant backgrounds. For this assay, plants were grown and harvested as previously described, but 20 μM ACC was added to the media. Growth in the presence of ACC increased the expression of ethylene-induced genes in all genotypes analyzed (Figure 4A and 4B). For eto1-1, the fold induction of ethylene-regulated genes in response to ACC was reduced relative to wild type. In xct-2, the chronic ethylene treatment resulted in a higher expression of these genes relative to wild type, but with a similar fold-change given the higher basal level of expression. This strong induction of ethylene-induced genes in xct-2 is very different from the
constitutively highly expressed and ethylene unresponsive regulation reported in *ctr1* mutants (Kieber et al., 1993). When wild type, *xct-2* and *eto1-1* were grown on media without added sucrose, ACC treatment caused induction of *ACO2* and *ETR2* expression (Supplemental Figure S3). Interestingly, although *xct-2* mutants grown on sucrose media supplemented with saturating levels of ACC had higher levels of both *ACO2* and *ETR2* expression than wild type (Figure 4), this enhanced expression was not observed in the absence of sucrose (Supplemental Figure 3). This enhanced response to ACC only in the presence of sucrose suggests that XCT may be involved in both sugar response and ethylene signaling pathways.

**XCT functions downstream of EIN3 to regulate ethylene responses in aerial tissues**

Our data suggested that *XCT* normally functions to repress the ethylene pathway somewhere downstream of the ethylene receptors. To further delineate where *XCT* acts within this pathway, we examined the genetic interactions between *XCT* and *EIN3*, an important transcriptional regulator of the ethylene response. We first characterized the hypocotyl response of *ein3-1 xct-2* double mutants grown with different concentrations of ACC on MS sucrose media. As previously seen in Figure 3, hypocotyl elongation in wild type and *xct-2* plants was inhibited by ACC under these conditions (Figure 5A). *ein3-1* single mutants lacked a strong hypocotyl phenotype in the absence of exogenous ACC and showed normal responsiveness to low concentrations of ACC. However, unlike wild type, they were fairly insensitive to increasing concentrations of ACC. The residual response to ethylene in this mutant background is likely attributed to the activity of *EIN3*-like proteins, known as the *EILs*. Interestingly, the *ein3-1 xct-2* double mutant was indistinguishable from *xct-2* single mutants at all concentrations of ACC, including its complete absence. Thus the loss of *XCT* in an *ein3-1* mutant restored ethylene responsiveness, genetically placing *XCT* downstream of *EIN3*.

We next examined the effect of exogenous ACC on additional traits in these genotypes. In wild type plants, the addition of ACC induced radial hypocotyl thickening, a hallmark of the ethylene response (Figure 5B). *xct-2* had a hypocotyl diameter similar to wild type both in the presence and absence of exogenous ACC while *ein3-1* was almost completely insensitive to the ethylene-induced radial expansion of the hypocotyl. However, the *ein3-1 xct-2* double mutant had a normal response to ethylene-induced hypocotyl thickening, further demonstrating that loss
of XCT restored ethylene sensitivity to ein3-1 mutants. Another phenotype strongly affected by ethylene is cotyledon expansion and unfolding. We found that ACC inhibited these processes in wild type, as expected, but that these responses were exaggerated in xct-2 mutants (Figure 5D and 5E). Although the ein3-1 single mutant was largely insensitive to this response, the ein3-1 xct-2 double mutants treated with ACC behaved more similarly to wild type, suggesting loss of XCT partially rescued this ein3-1 mutant phenotype. Altogether, these data indicated that loss of XCT restores ethylene responsiveness to ein3 mutants in aerial tissues.

Another aspect of the ethylene response is the inhibition of root growth. Inhibition of root growth in response to ACC was robust in both wild type and xct-2 plants (Figure 5C). In contrast, ein3-1 mutants showed reduced responsiveness to ACC in this assay, similar to the effects of ACC on hypocotyl elongation in this mutant. The ein3-1 xct-2 double mutants also showed reduced responsiveness to ACC, with the root length after ACC treatment being indistinguishable from the ein3-1 single mutant. These data, along with the lack of a root phenotype in xct-2 single mutants grown in the presence or absence of ACC, suggest that XCT is not involved in the ethylene response in root tissue despite its expression in this organ (Martin-Tryon and Harmer, 2008).

We also examined these seedling phenotypes on media not supplemented with exogenous sucrose. In the absence of ACC, xct-2 mutants had longer hypocotyls than wild type or ein3-1 (Supplementary Figure S4A). Similar to their growth patterns on sucrose media (Figure 5A), wild type and ein3-1 were not significantly different from each other on sucrose-deficient media. Low doses of ACC promoted hypocotyl elongation in wild type and xct-2, but ein3-1 showed little response under these conditions. ein3-1 xct-2 double mutants had an unexpected phenotype: in the absence of ACC they were significantly taller than xct-2 mutants, indicating a synergistic genetic interaction between these two loci (Supplementary Figure S4A). Addition of ACC caused inhibition of hypocotyl elongation in ein3-1 xct-2, rather than the promotion of growth seen in wild type and xct-2 or the unresponsiveness seen in ein3-1. This response to ACC in ein3-1 xct-2 was similar to that of wild type plants grown on MS media containing sucrose (Figure 5A). Thus, loss of XCT function restored ethylene responsiveness to ein3-1 seedlings in this assay as well, albeit in an unexpected manner.

We next monitored other seedling phenotypes in plants grown on MS without exogenous sucrose. Unlike plants grown on MS supplemented with sucrose, the ein3-1 xct-2 double
mutants had thicker hypocotyls than either single mutant or wild type even without the addition of ACC, a synergistic effect similar to their hypocotyl phenotype (Supplemental Figure S4B and S4A). Inhibition of root growth in response to ACC was impaired in both ein3-1 single and ein3-1 xct-2 double mutants (Supplemental Figure S4C), similar to the phenotypes seen in plants grown on MS plus sucrose (Figure 5C). ein3-1 xct-2 double mutants showed a partial restoration of ACC effects on cotyledons relative to the unresponsive ein3-1 single mutants, with ACC treatment causing a small reduction in cotyledon expansion and unfolding in ein3-1 xct-2 (Supplemental Figure S4D and S4E). Although the genetic interactions between xct-2 and ein3-1 are more complex in plants grown on MS without sucrose than on plants grown on MS with sucrose, in general xct-2 was epistatic to ein3-1 in aerial tissues but not in roots, as loss of XCT fails to suppress the ein3-1 phenotype in roots.

We next wanted to determine the nature of the genetic interaction between ein3-1 and xct-2 in plants grown in more natural conditions and at different life stages. We therefore examined the adult morphology of ein3-1 xct-2 double mutants and respective controls in 5-week old plants grown in soil in long days. As seen in Figure 5F, xct-2 single mutants were smaller than wild type plants, and conversely, ein3-1 mutants were larger than wild type. However, the ein3-1 xct-2 double mutants were indistinguishable from xct-2 single mutants. Thus, we found that xct-2 was epistatic to ein3-1, at least in aerial tissues, in adult plants as well as in seedlings. Taken together, the data indicate that XCT acts in the ethylene pathway downstream of the major transcription factor EIN3.
DISCUSSION

This study was prompted by the unexpected finding that xct-2 plants grown in blue light had a sugar-dependent phenotype, displaying long hypocotyls when grown in the absence of sucrose but short hypocotyls when grown in its presence (Figure 1A and 1B). This sugar-dependence is very reminiscent of the nutrient-dependent effects that ethylene has on blue light-grown plants, promoting hypocotyl elongation in plants grown in minimal media (Smalle et al., 1997) (Supplementary Figure S4A) but inhibiting elongation in plants grown on rich media (Collett et al., 2000) (Figure 3 and 5A). Since regulation of hypocotyl elongation by ethylene is more pronounced in plants grown in blue light than red light (Vandenbussche et al., 2007), we focused our attention on a potential role for XCT in the regulation of ethylene signaling in plants grown in blue light.

XCT specifically regulates ethylene responses in aerial tissues

Examining ethylene-responsive phenotypes in seedlings, we found that XCT normally represses ethylene responses in aerial but not root tissues (Figure 5). This specificity is not unique, as mutations in many other genes affect either a subset of ethylene responses or cause light- or tissue-specific ethylene-related defects. Two genes, WEI2 and WEI7, which encode different subunits of a tryptophan biosynthesis enzyme, regulate ethylene responses specifically in the roots (Stepanova et al., 2005). The eer1 mutant, which may modulate the activity of CTR1, shows altered ethylene signaling specifically in the hypocotyl of etiolated seedlings (Larsen and Chang, 2001; Larsen and Cancel, 2003). eer2 mutants show plant-wide enhanced ethylene responses, but only when grown in the light (De Paepe et al., 2005).

Specificity of the ethylene response can also arise at the level of ERF activity. When plants overexpressing ERF1, a direct target of EIN3, are grown in the dark, they exhibit a classic constitutive ethylene response but do not form an exaggerated apical hook (Solano et al., 1998). This suggests that distinct molecular players downstream of EIN3 control different physiological aspects of ethylene action. Our own data indicate that XCT modulates ethylene responses in both a tissue- and light-specific manner. We have previously reported that etiolated xct mutants lack noticeable phenotypes (Martin-Tryon and Harmer, 2008), unlike many other ethylene
signaling mutants that show obvious phenotypes as dark-grown seedlings (Guzman and Ecker, 1990). We now demonstrate that XCT affects ethylene responses in shoots but not roots (Figures 1 and 5).

In addition, we found that XCT differentially affects ethylene responses within a single organ, the hypocotyl. Hypocotyl length in both the absence and presence of exogenous ACC is altered in xct-2 mutants relative to wild type (Figure 5A and Supplemental Figure S4A). In contrast, hypocotyl diameter is normal in both untreated and ACC-treated xct-2 seedlings (Figure 5B and Supplemental Figure S4B). Therefore, xct-2 mutants appear to have specific alterations in ethylene signaling that affect the elongation but not radial expansion of hypocotyls. Hypocotyl elongation may be primarily controlled by epidermal cells (Savaldi-Goldstein et al., 2007), whereas changes in hypocotyl diameter in response to ethylene may be primarily regulated by vascular and cortical cells (Sanchez-Bravo et al., 1992). This suggests that downstream components of the ethylene signaling pathway, such as the ERFs, may be differentially required in these cell types and that XCT likely affects only a subset of these signaling molecules.

Early characterization and cloning of ethylene mutants in Arabidopsis revealed a relatively linear pathway from ethylene perception to ethylene response (Chao et al., 1997). As more data has become available, it is now clear that the ethylene signaling pathway at all levels, ranging from biosynthesis to induction of far-downstream genes, incorporates multiple levels of regulation and specificity (Lin et al., 2009). As phytohormones affect every aspect of plant growth and development, it is not surprising that their signaling pathways must be tightly regulated in order to achieve the appropriate biological response. XCT may now be added to the list of regulators of ethylene signaling that act in a complex and tissue-specific manner.

**XCT is a pleiotropic protein with separable functions in the circadian clock and ethylene response**

Although the hypocotyl phenotypes of xct mutants are sugar- and light-dependent (Figure 1A-B and 1E-F), the short-period circadian phenotype in these plants is not altered by the growth media or light quality (Figure 1G) (Martin-Tryon and Harmer, 2008). This strongly suggests the hypocotyl phenotypes are not a consequence of altered clock function. It has been previously
reported that wild type plants grown in either the presence or absence of sucrose had subtle but significant differences in the free-running period of leaf movement rhythms when assayed in constant light (Knight et al., 2008). We did not observe any significant differences in free-running period of Pro\textit{CCR2}:\textit{LUC} in wild type plants grown either on sucrose-rich or sucrose-deficient media when assayed under constant blue light (Figure 1G). It is possible that we did not detect the subtle sucrose-mediated changes in free-running period due to differences in growth media, temperature, or light intensity. Alternatively, it may be that sucrose affects the period of leaf movement rhythms but does not affect the period of rhythmic \textit{CCR2} expression.

Although there is a connection between ethylene signaling and the circadian clock, the roles for \textit{XCT} in each pathway appear molecularly separable. Ethylene emission in Arabidopsis is regulated by the circadian clock (Thain et al., 2004). However, ethylene insensitive mutants have normal circadian rhythms (Thain et al., 2004) and treatment of plants with ACC causes only minor changes in a subset of clock outputs (Hanano et al., 2006), indicating that ethylene emission is a clock output that does not feed back to affect the central clock. Mutation of clock genes may cause changes in both rhythms of ethylene emission and ethylene levels: ethylene emissions in the clock mutant \textit{CCA1-OX} are both arrhythmic and elevated, while in contrast \textit{toc1-2} mutants emit ethylene with a short period but at normal levels (Thain et al., 2004). \textit{xct} mutants have a short period similar to \textit{toc1}, yet emit reduced levels of ethylene compared to wild type (Figure 2B). Our data therefore suggest that the mechanism responsible for the ethylene and circadian defects in \textit{xct} mutants are molecularly distinct, suggesting that XCT functions in more than one biological pathway.

Genetic data also support the idea that XCT has separable molecular functions. We have previously reported that \textit{xct-1}, an EMS allele of \textit{XCT}, and \textit{xct-2}, a putative null allele, have very similar circadian phenotypes. However, \textit{xct-2} but not \textit{xct-1}, exhibits delayed greening (Martin-Tryon and Harmer, 2008), indicating that the clock and greening phenotypes are also separable. The \textit{xct-1} mutation is predicted to cause a loss of three amino acids from the XCT protein, suggesting that different regions of the XCT protein could have unique molecular functions.

\textbf{XCT function may be linked to transcriptional regulation}
We have shown that ein3-1 phenotypes in aerial tissues require functional XCT (Figure 5 and Supplemental Figure S4), effectively placing XCT downstream of EIN3 in the ethylene signaling pathway. An alternative explanation might be that XCT normally restricts the function of genes with analogous functions to EIN3, such as the EIL transcription factors. In total, the phenotypic data suggest that XCT, like CTR1, negatively regulates ethylene signaling but that, unlike CTR1, XCT acts downstream of EIN3 or related transcription factors. XCT thus may be thought of more specifically as a regulator of ethylene responses.

XCT may act in other signaling pathways as well: notably, ACC inhibits hypocotyl elongation in xct-2 ein3-1 double mutants grown on MS media without sucrose but does not have this effect in wild type or the single mutants (Supplemental Figure S4A). In contrast, ACC causes inhibition of hypocotyl elongation in all genotypes grown on MS plus sucrose (Figure 5A). This suggests there may be functional connections between XCT, EIN3, and sugar signaling. Indeed, EIN3 protein stability is negatively regulated by sugar and these signaling pathways are known to have cross-talk (Leon and Sheen, 2003; Yanagisawa et al., 2003).

Given the genetic interactions between XCT and EIN3 and that they both encode nuclear-localized proteins, it is reasonable to assume that XCT acts within the nucleus. It is unlikely that XCT directly regulates the stability of EIN3. If this were the case, we would expect to see more global and drastic effects on ethylene signaling than are present in xct-2 single mutants. Indeed, loss-of-function mutants for EBF1 and EBF2, which directly negatively regulate the stability of EIN3, have severe constitutive ethylene activity phenotypes analogous to ctr1 mutants (Binder et al., 2007). Similarly, given that overexpression of EIL1 confers global constitutive ethylene responses (Chao et al., 1997), it is unlikely that XCT directly regulates EIL1 or similar proteins. However, XCT might affect the stability of a subset of the ERFs, thus indirectly altering transcriptional regulation. The cloning and characterization of eer5 has revealed an important role for the COP9 signalosome, which regulates protein stability, in the resetting and maintenance of the ethylene signaling pathway without altering EIN3 stability (Christians et al., 2008).

Another possible biochemical function of XCT could be as a transcriptional co-regulator of EIN3-regulated genes, either by itself or as part of a larger complex. Additional transcriptional regulation of ethylene-responsive genes downstream of or in concert with EIN3 is not unprecedented. EER4, which encodes a TFIID-interacting transcription factor, is required
for the accurate induction of ERF1 transcript and thus is important for the canonical ethylene response (Robles et al., 2007). We have shown that ethylene regulated genes are mis-regulated in xct mutants (Figure 4 and Supplemental Figure 3), although it is unknown whether this effect is direct or indirect.

CONCLUSIONS

In summary, we have shown that XCT functions in the ethylene signaling pathway downstream of EIN3, a major transcriptional regulator of ethylene responses. XCT and EIN3 are both nuclear-localized proteins. However, unlike EIN3, XCT is well-conserved across eukaryotes, suggesting it may act in processes essential for normal growth and development. Indeed, knock-down of the XAP5-like gene in Caenorhabditis elegans is embryo lethal (Piano et al., 2002) and the human homolog may be involved in disease states (Chiurazzi et al., 2001). Although nothing is currently known about the biological role of XCT-like proteins in other organisms, we now demonstrate that XCT functions genetically downstream of a transcription factor in a signaling pathway that impacts every stage of plant growth and development. In future work, it will be interesting to determine how XCT separately affects the function of at least two separate and distinct plant signaling networks: the circadian clock and ethylene signaling.
MATERIALS AND METHODS

Plant Growth Conditions for Hypocotyl and Root Growth Assays

Arabidopsis seeds were surface sterilized by incubation in 70% ethanol for 5 minutes, followed by incubation in 100% ethanol for 10 minutes and finally a thorough water wash of the seeds to eliminate residual ethanol. Sterile seeds were plated on 1X Murashige and Skoog (MS) growth medium (RPI Research Products International) pH 5.7 containing 0.7% (w/v) agar (Sigma Aldrich) supplemented with or without 3% sucrose (w/v) (EMD chemicals). Plates were wrapped in aluminum foil and transferred to 4°C for 3-4 days to cold stratify the seeds. Following cold stratification, plates were moved to white light (cool-white fluorescent bulbs, 55 μmol m⁻² s⁻¹) for 6 hours to induce germination. Following a 6 hour white light treatment, plates were wrapped with aluminum foil and kept in constant darkness for 18 hours. At the end of the dark treatment, plates were transferred to their respective light quality conditions for five days of growth in constant monochromatic light. Hypocotyls were harvested on the sixth day (the light/dark pulse is counted as day one of experiment) and measured. Both monochromatic blue and red light were achieved using LED SnapLites (Quantum Devices) with a fluence rate of 3 μmol m⁻² s⁻¹ for blue light and 20 μmol m⁻² s⁻¹ for red light. All experiments were done at room temperature. Hypocotyls were harvested onto a transparency, scanned, and measured using the analysis software ImageJ (NIH). All hypocotyl growth data presented are representative of at least three independent biological replicates. Error bars are the standard error of the mean (SEM).

For the hypocotyl growth assays involving silver nitrate as the pharmacological ethylene signaling inhibitor, silver nitrate (AgNO₃) (Sigma Aldrich) was dissolved in water, filter sterilized, and added to a final concentration of 50 μM per MS plate. For ACC dose response assays, aminocyclopropane (ACC) (Sigma Aldrich) was dissolved in water, filter sterilized, and added to respective final concentration in the MS plates. Hypocotyl growth and analysis in these assays is the same as described above.

Experiments for root growth assays were performed similarly to those for hypocotyl assays. Seeds were placed on square plates and seedlings were allowed to grow vertically in constant blue light for 6 days. All experiments were performed at 22-24°C.
Mutant Alleles and Genotyping

All Arabidopsis wild type and mutant seeds used are of ecotype Columbia (Col-0). The xct-2 mutants are SALK T-DNA insertion mutants and were genotyped as previously described (Martin-Tryon et al., 2008). The hy5-215 allele is an EMS allele and was previously described (Oyama et al, 1997). The ein3-1 mutation was genotyped using the CAPS method to detect the single nucleotide change in this allele as previously described (Binder et al., 2007). The eto1-1 mutants are previously described EMS mutants (Guzman and Ecker, 1990) and were obtained through the ABRC. We genotyped this allele using the dCAPS method beginning with PCR of genomic DNA using the primers 5’ GCAACACAACCTGACCCTCTT 3’ and 5’ GGGAGAATCCCTCAGAAAGG 3’. The resulting PCR product was subjected to restriction digestion using TaqI. An induced mutation in the first primer creates a TaqI recognition site in the wild type product, but this site is absent in the eto1-1 allele. The 162-bp ETO1 product from the wild type background was cut by TaqI, resulting in fragments of 140-bp and 22-bp, whereas this enzyme did not cut the eto1-1 product. The ctr1-3 mutant has been previously described (Kieber et al, 1993). We genotyped this allele using the dCAPS method using the following primers: 5’ AATTGATTTACCTGTCGAA 3’ and 5’ GACTGGCTATCGGAGAAATA 3’. Following PCR of genomic DNA and digestion of product with the restriction enzyme NlaIII, the wild type CTR1 produces a fragment of 402 bp, while the ctr1-3 product is cut by NlaIII, producing bands of 333 bp and 69 bp (Anandkumar Surendrarao, Caren Chang, personal communication). All double mutants were obtained via genetic crossing and identified by PCR screening of F2 progeny following F1 self-fertilization.

Ethylene Emission Measurements

Ethylene measurements were essentially performed as in Thair et al. (2004), with modifications. 200-400 seeds were surface sterilized and sown in a 10 ml chromatography vial containing 5 ml of ½ MS (Duchefa, Haarlem, The Netherlands) with 0% or 3% sucrose (VWR, Leuven, Belgium) and 0.8% plant tissue culture agar (LabM, Bury, Lancashire, UK). The vial was kept 2 days at 4°C in darkness and subsequently exposed to white light for 6 h at 21°C to
stimulate germination. Seedlings were allowed to grow for 3-4 days in 3 μmoles.m⁻².s⁻¹ blue light (470nm Dragontape LEDs, Osram, Capelle a/d Ijssel, The Netherlands). The vials were capped, left in blue light for another 24h, and subsequently flushed with hydrocarbon free air (Air Liquide, Herenthout, Belgium). Ethylene in the headspace was detected with an ETD-300 photo-acoustic ethylene detector (Sensor Sense, Nijmegen, The Netherlands). Three independent sets of biological material were used for calculating mean values. The experiments were done twice with highly similar results.

RNA Extraction and Quantitative Real-Time PCR

For gene expression analysis, seedling germination and growth conditions were as described for a blue light hypocotyl experiment. Seedlings were grown in 3 μmol m⁻² s⁻¹ blue light either on MS or MS + 3% sucrose plates, or on these plates supplemented with 20 μM ACC. On the fifth day of growth, approximately 30-40 seedlings of each genotype were harvested together during subjective afternoon. Total RNA was isolated from 5 day-old seedlings grown in monochromatic blue light using TRizol reagent (Invitrogen) according to manufacturer’s protocol and cleaned by sodium acetate/ethanol precipitation. 500 ng-1 μg of total RNA from each sample was used in first-strand cDNA synthesis using an oligo(dT)₁₈ primer and SuperScriptII (Invitrogen) reverse transcriptase per manufacturer’s instructions. The cDNA was diluted 1:5 and 2 μL of this working cDNA was used as template in a 20 μL qRT-PCR reaction. The reagents and their concentrations used in the qRT-PCR master mix are as previously described (Martin-Tryon et al., 2008). Each cDNA sample was run in triplicate using an iCycler iQ (Bio-Rad) and data was analyzed with iCycler iQ Optical Systems Software v3.1 (Bio-Rad). Relative expression values for experimental genes are presented normalized to PP2A expression level. Melt curve analysis was done following product amplification to confirm that cDNA product was specifically and solely amplified. Error bars are the standard deviation of relative expression level calculated using the standard curve method (ABI Prism). Expression data presented are representative of at least three independent biological replicates.

Sequence of the PP2A primers is as previously described (Martin-Tryon et al, 2008). For ACO2, we designed and used 5’ CTCCTCTCAAACCACTCTATTGTATC 3’ and 5’
GGCTCCTTGGGCTGAAACTTG 3’. For ETR2, we designed and used 5’
CGGCGGCTATGGGTTAGG 3’ and 5’ GAGCGTGGTGGTGAGG 3’.

Circadian Period Assay and Quantification

Arabidopsis seeds were sterilized and plated as described for hypocotyl growth assays. Following cold stratification, plates were released into 12:12 white light/dark cycles (cool-white fluorescent bulbs, 55 μmol m⁻² s⁻¹) for 7 days at constant 22°C. After 7 days in light/dark cycles, plants were sprayed with 3mM D-Luciferin (Biosynth AD) and then sent into an ORCA II ER CCD camera (Hamamatsu) for imaging of luciferase bioluminescence at constant 20 μmol m⁻² s⁻¹ blue light (LED SnapLite-Quantum Devices). All seeds for this assay harbor a proCCR2::LUC reporter construct, allowing for the CCD camera to monitor the real-time expression of luciferase under the control of the CCR2 promoter in each genetic background analyzed. After five days of luciferase imaging in constant blue light, images were analyzed using the MetaMorph software (Molecular Devices) to produce quantitative luciferase bioluminescence data. This bioluminescence data over time was then fit to a cosine wave using the Fourier Fast Transform-Non-Linear Least Squares program (Plautz et al., 1997), allowing for estimations of various circadian parameters, including free-running period.

Seedling Imaging

6-day old blue light-grown seedlings were imaged using Zeiss Stemi SV 11 dissecting microscope. Images were taken with the use of a QImaging camera and processed using the software QCapture Pro.
SUPPLEMENTAL MATERIAL

Supplemental Figure S1. Analysis of hy5-215 xct-2 double mutant growth patterns on various media conditions.

Supplemental Figure S2. Hypocotyl response to silver nitrate when grown on MS media.

Supplemental Figure S3. Ethylene-regulated gene expression in plants grown on MS media.

Supplemental Figure S4. Analysis of ein3-1 xct-2 double mutant growth patterns on MS media.
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FIGURE LEGENDS

Figure 1-Phenotypic analysis of \textit{xct-2} growth patterns in the presence or absence of sucrose

(A) and (B) Hypocotyl length of wild type and \textit{xct-2} (n=35-40) grown under 3 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) constant monochromatic blue light for six days on MS media in the presence or absence of 3% sucrose.  

(C) and (D) Root length of wild type and \textit{xct-2} (n=25-30) grown under 3 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) monochromatic blue light for 6 days on MS media in the presence or absence of 3% sucrose.  

(E) and (F) are similar to (A) and (B), but were performed in 20 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) monochromatic red light.  

(G) Average free-running period, assayed by monitoring bioluminescence of a Pro\textit{CCR2::LUC} reporter construct in each genotype (n=12-15) grown under blue light on different media conditions.  

All error bars represent standard error.  Asterisks (*) denote a significant difference from wild type under the same condition based on Student’s \(t\)-test (p < 0.05).  All data presented are representative of at least three independent experiments.

Figure 2-\textit{xct-2} and \textit{ctr1-3} have similar phenotypes

(A) Hypocotyl length of various genotypes (n=35-40) grown on MS sucrose media in the presence or absence of 50 \(\mu\text{M} \text{AgNO}_3\) (silver nitrate) for six days under constant monochromatic blue light.  

(B) Ethylene emission levels (n=200-400) of various genotypes grown in the presence or absence of 3% sucrose.  

Seedlings were grown under blue light for 3-4 days, capped and allowed to accumulate ethylene over 24 hours in blue light, then sent for ethylene detection.  

All error bars represent standard error.  Asterisks denote a significant difference from wild type under the same condition based on Student’s \(t\)-test (p < 0.05).  All data presented are representative of at least three independent experiments.

Figure 3-Inhibition of hypocotyl elongation by ACC

(A) Hypocotyl length of various genotypes (n=35-40) grown on MS sucrose media in constant blue light with the addition of various concentrations (0-50 \(\mu\text{M}\)) of ACC to the growth media.  

All error bars represent standard error.  

(B) Change in hypocotyl length in response to ACC as viewed in (A), represented as the percentage of the hypocotyl length relative to untreated hypocotyls.  All data presented are representative of at least three independent experiments.
**Figure 4—Analysis of ethylene-regulated gene expression**

Quantitative RT-PCR analysis of *ACO2* (A) and *ETR2* (B) in various genotypes in response to ACC treatment. Plants were grown under constant blue light for five days on MS sucrose supplemented with or without 20 μM ACC. Plants were harvested on the fifth day and RNA was extracted with subsequent cDNA synthesis and analysis. All error bars represent standard error. All data presented are representative of at least two independent experiments, with each sample in each experiment containing three technical replicates for analysis.

**Figure 5—*XCT* is epistatic to *EIN3***

(A) Hypocotyl lengths of various genotypes (n=35-40) grown under blue light for six days on MS sucrose supplemented with increasing concentrations of ACC. (B) Hypocotyl diameter of various genotypes (n=35-40) grown under blue light for six days on MS sucrose supplemented with or without 10 μM ACC. (C) Root length of various genotypes (n=25-30) grown under blue light for six days on MS sucrose supplemented with or without 20 μM ACC. (D) and (E) Six day old plants grown under constant blue light on MS sucrose supplemented with (E) or without (D) 10 μM ACC. (F) 5-week-old plants grown under long day conditions (18 h light : 6 h dark) in soil under 40 μmol m⁻² s⁻¹ white light. All error bars represent standard error. Asterisks denote a significant difference from wild type under the same condition based on Student’s *t*-test (p < 0.05). All data presented are representative of at least three independent experiments.

**Supplemental Figure S1—Analysis of *hy5-215 xct-2* double mutant growth patterns on various media conditions**

Hypocotyl length of various genotypes (n=35-40) grown on MS Suc media (A) or MS media (B). Plants were grown for six days under constant monochromatic blue light. All error bars represent standard error. Asterisks denote a significant difference from wild type under the same condition based on Student’s *t*-test (p < 0.05). All data presented are representative of at least two independent experiments.

**Supplemental Figure S2—Hypocotyl response to silver nitrate when grown on MS media**

Hypocotyl length of various genotypes (n=35-40) grown on MS media in the presence or absence of 50 μM AgNO₃ (silver nitrate) for six days under constant monochromatic blue light.
All error bars represent standard error. Asterisks denote a significant difference from wild type under the same condition based on Student’s *t*-test (p < 0.05). All data presented are representative of at least three independent experiments.

**Supplemental Figure S3**-Ethylene-regulated gene expression in plants grown on MS media
Quantitative RT-PCR analysis of *ACO2* (A) and *ETR2* (B) in various genotypes in response to ACC treatment. Plants were grown under constant blue light for five days on MS media supplemented with or without 20 μM ACC. Plants were harvested on the fifth day and RNA was extracted with subsequent cDNA synthesis and analysis. All data presented are representative of at least two independent experiments, with each sample in each experiment containing three technical replicates for analysis.

**Supplemental Figure S4**- Analysis of *ein3-1 xct-2* double mutant growth patterns on MS media
(A) Hypocotyl lengths of various genotypes (n=35-40) grown under blue light for six days on MS media supplemented with increasing concentrations of ACC. (B) Hypocotyl diameter of various genotypes (n=35-40) grown under blue light for six days on MS media supplemented with or without 10 μM ACC. (C) Root length of various genotypes (n=25-30) grown under blue light for six days on MS media supplemented with or without 20 μM ACC. (D) and (E) Six day old plants grown under constant blue light on MS media supplemented with (E) or without (D) 10 μM ACC. All error bars represent standard error. Asterisks denote a significant difference from wild type under the same condition based on Student’s *t*-test (p < 0.05). All data presented are representative of at least three independent experiments.
Figure 1. Phenotypic analysis of xct-2 growth patterns in the presence or absence of sucrose

(A) and (B) Hypocotyl length of wild type and xct-2 (n=35-40) grown under 3 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) constant monochromatic blue light for six days on MS media in the presence or absence of 3% sucrose. (C) and (D) Root length of wild type and xct-2 (n=25-30) grown under 3 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) monochromatic blue light for 6 days on MS media in the presence or absence of 3% sucrose. (E) and (F) are similar to (A) and (B), but were performed in 20 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) monochromatic red light. (G) Average free-running period, assayed by monitoring bioluminescence of a ProCCR2::LUC reporter construct in each genotype (n=12-15) grown under blue light on different media conditions. All error bars represent standard error. Asterisks (*) denote a significant difference from wild type under the same condition based on Student’s \( t \)-test (p < 0.05). All data presented are representative of at least three independent experiments.
Figure 2. *xct-2* and *ctr1-3* have similar phenotypes

(A) Hypocotyl length of various genotypes (n=35-40) grown on MS sucrose media in the presence or absence of 50 μM AgNO3 (silver nitrate) for six days under constant monochromatic blue light. (B) Ethylene emission levels (n=200-400) of various genotypes grown in the presence or absence of 3% sucrose. Seedlings were grown under blue light for 3-4 days, capped and allowed to accumulate ethylene over 24 hours in blue light, then sent for ethylene detection. All error bars represent standard error. Asterisks denote a significant difference from wild type under the same condition based on Student’s *t*-test (p < 0.05). All data presented are representative of at least three independent experiments.
Figure 3. Inhibition of hypocotyl elongation by ACC

(A) Hypocotyl length of various genotypes (n=35-40) grown on MS sucrose media in constant blue light with the addition of various concentrations (0-50 μM) of ACC to the growth media. All error bars represent standard error. (B) Change in hypocotyl length in response to ACC as viewed in (A), represented as the percentage of the hypocotyl length relative to untreated hypocotyls. All data presented are representative of at least three independent experiments.
Figure 4. Analysis of ethylene-regulated gene expression

Quantitative RT-PCR analysis of *ACO2* (A) and *ETR2* (B) in various genotypes in response to ACC treatment. Plants were grown under constant blue light for five days on MS sucrose supplemented with or without 20 μM ACC. Plants were harvested on the fifth day and RNA was extracted with subsequent cDNA synthesis and analysis. All error bars represent standard error. All data presented are representative of at least two independent experiments, with each sample in each experiment containing three technical replicates for analysis.
Figure 5. XCT is epistatic to EIN3
(A) Hypocotyl lengths of various genotypes (n=35-40) grown under blue light for six days on MS sucrose supplemented with increasing concentrations of ACC. (B) Hypocotyl diameter of various genotypes (n=35-40) grown under blue light for six days on MS sucrose supplemented with or without 10 μM ACC. (C) Root length of various genotypes (n=25-30) grown under blue light for six days on MS sucrose supplemented with or without 20 μM ACC. (D) and (E) Six day old plants grown under constant blue light on MS sucrose supplemented with (E) or without (D) 10 μM ACC. (F) 5-week-old plants grown under long day conditions (18 h light : 6 h dark) in soil under 40 μmol m⁻² s⁻¹ white light. All error bars represent standard error. Asterisks denote a significant difference from wild type under the same condition based on Student’s t-test (p < 0.05). All data presented are representative of at least three independent experiments.