2002 BELTWIDE COTTON CONFERENCES, ATLANTA, GA – JANUARY 8-12

TRANSGENIC EXPRESSION OF YEAST CASEIN KINASE 1 ISOFORM YCK2 AS A MEANS OF CONFERRING SALT TOLERANCE TO COTTON

Michael J. Wheeler, Dalton R. Gossett , Lucy C. Robinson and Stephen W. Banks
Department of Biological Sciences
Louisiana State University in Shreveport
Shreveport, LA
Department of Biochemistry
Louisiana State University Health Sciences Center
Shreveport, LA

Abstract

The yeast casein kinase 1 isoform Yck2 is an enzyme that, when overexpressed, acts as a suppressor of salinity intolerance. Although there is ample evidence that salt stress resistance in plants is multifactoral, we advanced the hypothesis that expression of Yck2 could confer a degree of salt tolerance in recipient plants. To test this hypothesis, we have genetically transformed cotton callus with a plasmid encoding a GFP fusion to Yck2. Transformed cotton callus tissue shows increased salt tolerance when grown on media containing 150mM NaCl, and transformed cells show the presence of a GFP-Yck2 fusion protein at the cell periphery. We are currently examining the growth performance of transformed tissue on salt-amended growth media.

Introduction

Plants are directly influenced by changes in their immediate environmental surroundings. Changes in both the internal and external environment can create stresses that affect the health of plants. Internal stress can be due to events such as chloroplast electron leakage (Asada and Takahashi, 1987) or actions of plant hormones. External stress results from biotic factors such as pathogens and from abiotic factors, including temperature extremes, nutrition deficiency, drought, ozone, soil pH, and salinity (Allen, 1995). By far the two most important factors limiting commercial plant productivity are salinity and drought (Boyer, 1992). In an evolutionary context, stresses are abiotic environmental conditions that substantially reduce the fitness of non-motile organisms and their productivity (Stanton, et al., 2000). The majority of crop plants have a finite capacity for stress tolerance, and increasing this tolerance is a high priority in modern agriculture due to the increasing world population and decreasing availability of arable land. Salinity is one of the main factors limiting the productivity of agricultural crops (Apse, *et al.,* 1999). Salinity affects more than forty percent of currently irrigated land, especially in the Mediterranean basin, California, Southern Louisiana and Southern Asia (Serrano and Gaxiola, 1994). Worldwide some 3 x 10^6 km^2 of saline soil, excluding major deserts, have been identified. (Flowers, *et al.,* 1977). Salt stress is significant in countries such as India, with a large amount of coastal land too saline for the majority of crops. Furthermore, many regions are affected by the application of irrigation water with a high saline content (Flowers, *et al.,* 1977; Fowler, 1986). These factors lend significance to the study of salinity and its effect upon crop plants. Enhancement of saline tolerance in cotton and other crops may ultimately result in extending the range of soils that can support useful agriculture.

Cotton does not exhibit decreases in yields until approximately 90mM NaCl stress is applied, whereas tomato exhibits a significant decrease in yields with the application of only 50mM NaCl (Fowler, 1986). NaCl levels at 188mM have been reported to result in a 50% reduction in cotton yields. Therefore, cotton is considered to be salt-tolerant when compared to other crop plants such as tomato, but is not classified as a halophyte (Ayers and Westcot, 1977). It is not uncommon to find irrigated agricultural soils with NaCl in excess of 100mM. NaCl has been shown to reduce stomatal conductance, stomatal resistance, and transpiration rates in cotton (Gossett, *et al.,* 1991). In general, the detrimental effects of salt on plants are due to both water deficiency, resulting in osmotic stress, and the effects of excess sodium ions on critical biochemical processes (Wyn-Jones, 1981). Data indicate that salt stress elicits an oxidative response in cotton, generating reactive oxygen intermediates (Gossett, *et al.,* 1996: Bellaire, *et al.,* 2000). In recent years the techniques of biotechnology offer the possibility to increase the saline tolerance of cotton. Identification of genes that can improve upon salt stress resistance may be expected to open the way for the production of genetically engineered cotton plants that can be productive under saline conditions.

Many specific genes have been shown individually to convey marked degrees of salinity tolerance. The study of these genes has included several functional strategies such as overexpression of vacuolar Na^+/H^+ antiporters (Apse, *et al.,* 1999); expression of catalytic and regulatory subunits of yeast calcineurin (PP2B) in tobacco (Pardo, *et al.,* 1998); overexpression of a plant homologue of yeast Dbf2 kinase, a transcriptional regulator in general stress tolerance (Lee, *et al.,* 1999); and overexpression of SOS1, a novel plant Na^+/H^+ cell membrane antiporter (Wu, *et al.,* 1996). It is quite clear that salt tolerance

is due to gene expression of a multifactoral nature, and these different genetic approaches have yielded results that make plants more tolerant to salinity.

Our strategy to confer salt tolerance in cotton is to overexpress a gene encoding a yeast casein kinase 1 isoform (*YCK2*). The rationale behind the use of this gene is its isolation from yeast as a multi-copy suppressor of high salinity intolerance (Robinson, *et al.,* 1992). The *YCK2* gene, when overexpressed, allows yeast cells to forego prior conditioning at 0.4-0.6 M NaCl to enable growth at >1M NaCl (Robinson, *et al.,* 1992). The protein encoded by the *YCK2* gene is found in plasma membrane fractions (Wang, et al., 1992; Vancura, et al., 1993). Therefore, the hypothesis may be advanced that Yck2 has a regulatory role in ion transport at the plasma membrane.

The *YCK2* gene is a member of an essential gene pair, with *YCK1* its functional homologue (Robinson, *et al.,* 1992; Wang, et al., 1992). Thus, with the loss of one of these genes in yeast, cells are viable, but the loss of both is lethal (Robinson*, et al.,* 1992). *YCK2* is one of four isoforms of casein kinase I found in yeast (Hoekstra et al., 1991; Robinson, *et al.,* 1992; Wang, *et al.,* 1996). Casein kinase 1 (CK1) is ubiquitous in eukaryotic cells, and can phosphorylate serine and threonine residues in the context of clusters of neighboring acidic sites (Tuazon and Traugh, 1991). The specificity of this enzyme is such that phosphoamino acids can substitute for acidic residues, thus, other protein kinases can "prime" phosphorylation by CK1 (Hardie, 2000). The cellular functions of CK1 kinases remain somewhat enigmatic. There is currently a lack of evidence for acute regulation *in vivo* (Hardie, 2000), although studies of the mammalian CK1 enzyme and Yck2 indicate that changes in localization occur during the cell cycle (Brockman*, et al.,* 1992; Robinson, *et al.,* 1999) and could provide one level of regulation.

Plant cells overexpressing *YCK2* could potentially have altered ion transport. The plasma membrane H^+ATPase of yeast (Pma1) has been found*, in vitro*, to be subject to CK1-dependent phosphorylation (Estrada, *et al.,* 1996). This phosphorylation appeared to be inhibitory (Estrada*, et al.,* 1996). Yck2 could also affect plant Pma1-homologous H^+ATPases. It has been shown that endogenous plant casein kinase 1 enzymes share little homology to that of yeast casein kinase 1 enzymes (Klimczak, *et al.,* 1995), however, the H^+ATPases of plants are very similar to those of yeast. Pma1 is a plasma membrane proton pump driven by the hydrolysis of ATP and found exclusively in the plasma membrane of plants and fungi (Palmgren, 2001). The role of this ATPase is to provide the driving force for transport of ions into and out of the cell. The plasma membrane H^+ATPase is an electrogenic enzyme, since it extrudes protons, and thus produces a negative membrane potential (Palmgren, 2001). In plant cells the membrane potential may exceed –200mV (Hirsch*, et al.,* 1998). This potential is crucial, since cations, anions, and neutral solutes cross the membrane via specific carrier proteins (*i.e.* ENA1) through which transport is energized by concomitant H^+ uptake (Palmgren, 2001). In plant salinity research, several studies have focused on the H^+ATPase homologs to the yeast *PMA1* gene (Moriau, *et al.,* 1999; Maudoux, *et al.* 2000). H^+ATPases (PMA1) from plants have been shown to complement *pma1* in yeast (Palmgren and Christensen, 1993). The PMA2 plant H^+ATPase, when expressed in yeast, becomes phosphorylated at its penultimate residue, leading to an increase in activity (Maudoux, *et al.,* 2000). Since P-type H^+ATPases of yeast and plants are functionally similar, it is possible that if the salinity tolerance induced by overexpression of the Yck2 kinase in yeast is due to an effect on Pma1 activity, this effect may also be exhibited in a transgenic cotton plant. An important question regarding this approach is whether an increase in the activity of PMA1 will lead to increased NaCl tolerance. Currently, there are no data directly supporting this hypothesis. However, whether the effect is due to a direct or an indirect effect on ion transport, we considered it worth testing whether Yck2 overexpression can confer salt tolerance in plants as it does in yeast.

Materials and Methods

Production of cDNA Expression Constructs

Two constructs that contain variants of the *YCK2* gene served as the starting material for this work. One plasmid, designated pUC19ΔEYCK2, contains the full length *YCK2* gene on an *Xba*I-*Sac*I restriction fragment in a pUC19 vector with the *Eco*RI MCS restriction site deleted (Robinson, *et al*., 1999). The other plasmid contained the *YCK2* open reading frame (ORF) cloned into the *Bam*HI and *Sal*I restriction sites of the pUC19 multi-cloning site (Robinson, *et al*., 1999). The *YCK2* ORF in the latter plasmid is fused at the 5' end to the gene encoding green fluorescent protein (GFP). The GFP variant used was a double mutant containing mutations S65T (Heim *et al*., 1995) and F64L, resulting in an eightfold brighter fluorescence than the parent protein (Robinson, *et al*., 1999). This *YCK2* gene fusion was designated GFPY2orf (Robinson *et al*., 1999). These plasmids were amplified in *E. coli* (Maniatis, *et al*., 1982), and the GFP:*YCK2* fusion was obtained on a *Bam*H1-*Sal*I fragment. This fragment was ligated into the yeast vector pGBD-C3. The fusion gene was digested from pGBDc3 at the *Bam*HI and *Bgl*II sites and was ligated into the binary plant expression vector pBI121 (Clontech) at the single *Bam*HI site. The GFP:*YCK2* fusion gene insert was non-directional in its insertion into the single *Bam*HI site of the pBI121 vector, so we obtained both sense and anti-sense clones. To facilitate cloning, the ends of the pBI121 vector were dephosphorylated with alkaline phosphatase (Boehringer-Mannheim).

pBI121 GFPY2$_{orf}$ constructs with the gene in both sense and antisense orientations were electroporated into *Agrobacterium tumefaciens* strain EHA105 using techniques described previously (Mersereau, *et al*., 1990; Mattanovich, *et al*., 1989). The sense and antisense constructs were isolated and observed with one *Hind*III insert fragment appearing on a 0.7% agarose gel at ~3100 base pairs (bp) for the sense, and ~ 600 bp for the antisense construct. These sizes correspond well with the predicted sizes of 3040 bp for the sense insert and 574 bp for the antisense insert fragment. A separate GFP construct (pBIN19 GFP) was obtained from Dr. Satyendra Nath Rajguru (University of Arkansas) to use as an internal control in cotton for the expression of GFP.

A second construct, based on the pEGAD vector, (Cutler, *et al*., 2000) was constructed to eliminate the complication of splicing of GFP message in plants. The *YCK2* gene was inserted into this plant expression vector to produce a GFP fusion (Cutler, *et al*., 2000). The enhanced GFP (eGFP) used in this construct does not generate the splicing error specific to some plants, thus, the intact protein should be translated in all plant tissues (Haseloff, *et al*., 1996). The eGFP cDNA has a multicloning site directly following the GFP ORF to allow fusion to the N-terminus of the protein of interest. The pEGAD vector construct was created at Stanford University as a plant expression vector for library screening using eGFP as the reporter gene (Cutler, *et al*., 2000), and confers resistance to both the antibiotic kanamycin and the herbicide Basta in transformed plant tissues. The *YCK2* gene was cloned into pEGAD on an *Eco*RI-*Pst*I fragment from the pUC19ΔE:*YCK2* plasmid. The *Eco*RI site directly follows the ATG of the *YCK2* ORF, allowing fusion to the eGFP coding sequence. The *YCK2* fragment was gel purified using a Quiex II kit (Qiagen Inc.) and was ligated into the *Eco*RI and *Pst*I sites of the yeast pRS314 vector. A restriction digest with *Eco*RI and *Bam*HI confirmed the presence of the insert. The *YCK2* insert was shown to be 1640 bp in length. The *Eco*RI and *Bam*HI insert was gel purified and ligated into the pEGAD vector. Candidate plasmids were digested with *Eco*RI and *Bam*H I to verify the presence of the *YCK2* 1640 bp insert. The resulting pEGAD:*YCK2* clone was electroporated into *Agrobacterium tumefaciens* strain EHA 105 (Mersereau, *et al*., 1990; Mattanovich, *et al*., 1989).

Plant Transformation

The EHA 105 *Agrobacterium tumefaciens* cells containing the *YCK2* plasmids were used to transform eight day old cotton hypocotyls grown on Stewarts media (Gossett, *et al* 1994b). The sense and antisense GFPY2$_{orf}$ in EHA 105, pBI121in EHA105, and the non-transformed EHA105 strains were inoculated into hypocotyl segments of the cotton cultivar Coker 312. These strains were maintained on modified (Banks *et al.,* 1994) Luria Bertani media (Maniatis, *et al* 1982) containing tryptone 10g/L, yeast extract 5g/L and NaCl 5g/L (pH adjusted to 7.5 with NaOH). Liquid media of this composition was used to prepare plant transformation cultures. The newly inoculated cotton tissue was transferred to plates that contained Murashige and Skoog media with Gamborg's B5 vitamins, (Dixon, 1985), with 30 g/L of glucose and 0.75 g/L of $MgCl_2$. After three days of co-cultivation the hypocotyls were transferred to plates containing media amended with the antibiotics kanamycin (50μg/ml) to select for transformed plant tissue, and amoxicillin (1000 μg/ml), potassium clavulanate at 500 μg/ml in the form of the antibiotic, Augmentin^{TM} (Smith-Kline Beecham) to kill the *Agrobacterium*. The cotton callus was subcultured to fresh media every 5 weeks. Simultaneously, the *Arabidopsis thaliana* ecotype Columbia was used to produce callus transformed with the same cDNA constructs as was the cotton by leaf disc transformation of the excised leaf tissue (Van der Graff and Hooykaas, 1998). *Arabidopsis* was transformed with EHA105 strains either without plasmid or carrying the sense or antisense GFPY2$_{orf}$ constructs or pBI121. Excised leaves were incubated on C1 (callus induction) media with α-naphthalene acetic acid (NAA) at 1mg/L and benzylaminopurine (BAP) at 0.1 mg/L (Van der Graff and Hooykaas, 1998). After five days under white light conditions, the *Arabidopsis* leaf discs were inoculated with the four EHA105 cultures in individual groups. After two days under white light conditions the leaf discs were washed in liquid C1 media supplemented with Augmentin^{TM}, and kanamycin, as above. Washed leaf discs were transferred to fresh C2 media differing from C1 only in that the hormone concentrations were 0.2 mg/L NAA and 1 mg/L BAP (Van der Graff and Hooykaas, 1998). Excised *Arabidopsis* leaves were transferred to C2 media for callus growth under kanamycin selection (50μg/ml) for the transformed phenotype (Van der Graff and Hooykaas, 1998).

In addition to cotton and *Arabidopsis*, tobacco was also used to attempt transgenic expression of the GFP:*YCK2* gene. The tobacco variety used in this experiment was *Nicotiana tabacum cv.*xanthium. Tobacco seed was surface sterilized in a 20% sodium hypochlorite, 5% Tween-20 solution and washed five times with sterile water. Tobacco seeds were deposited on Stewart's media in Magenta boxes (Dixon, 1985). After four weeks, the seeds germinated and grew leaves. Leaves were excised and washed with 5% sodium hypochlorite (Schuler and Zielinski, 1989), then were sectioned with a sterile scalpel and placed on C1 medium with 1 mg/L NAA and 0.1 mg/L BAP (Schuler and Zielinski, 1989). After 3 days on C1 media, the excised leaf tissue thickened. At this point, transformation was performed by immersion into modified LB media containing *Agrobacterium* EHA105 strains, untransformed or carrying each of the specific constructs (Schuler and Zielinski, 1989). The cDNA constructs used in these experiments were pBI121, GFPY2$_{orf}$ sense, pBIN19 GFP. Leaf tissue was dried on sterile filter paper and placed on N-media plates. N-media were composed of MS media with B5 vitamins, 3% sucrose, 3% Mannitol, and the hormones NAA at 0.1μg/ml and BAP at 0.1 μg/ml. N-media was amended with antibiotics as described above. Transformed callus was selected using kanamycin (50 μg/ml).

Western Blotting

SDS-polyacrylamide gels were made with a resolving gel of 9% (Ausubel, *et al*., 1999). Concentrations of total protein extracts were determined using the Pierce BCA assay. 100 µg and 200 µg samples of protein extract were mixed with 5X sample buffer, heated to 70°C, loaded onto the gel and electrophoresed. The separated proteins were transferred to an equilibrated PVDF membrane by liquid transfer in a blotting apparatus (Ausubel, *et al*., 1999). The PVDF membrane was blocked with 5% nonfat dry milk in Tris-buffered saline and then probed with the appropriate primary antibody. The primary antibody was either rabbit anti-β-glucuronidase or anti-green fluorescent protein. Binding of primary antibody was detected with goat anti-rabbit antibodies and a biotinylated streptavidin-based alkaline phosphatase (Bio-Rad). The immunoblot was photographed with an Ultra-Lum gel documentation device.

Protein Extraction

Cotton callus samples were prepared for catalase, peroxidase, glutathione reductase (GR), and ascorbate peroxidase (APX) analyses (Anderson, *et al*., 1992, Gossett, *et al*., 1994). Approximately two grams of callus were added to a series of culture tubes on ice containing 0.25 g of insoluble PVP and one drop of anti-foam A in 2.5 ml of 50mM PIPES buffer pH 6.8. This also contained 6mM cysteine hydrochloride, 10mM D-isoascorbate, 1mM EDTA, 1% PVP-10, and 0.3% Triton X-100. The homogenate was centrifuged for 20 min at 4°C at 10,395 rcf. Following centrifugation, 1 ml of the supernatant was centrifuge-desalted through a 10 ml bed of Sephadex G-25 (Anderson, *et al*., 1992; Gossett*, et al.,* 1994).

Antioxidant Enzyme Determination

Catalase activity was determined by measurement of a decrease in absorbance at 240 nm in a reaction mixture: 1.9 ml of H_2O, 1 ml 0.059 M H_2O_2 in KPO_4 buffer (pH 7.0), and 100 µl plant extract (Beers and Sizer, 1952). Peroxidase activity was measured by monitoring the oxidation of reduced 2,3',6-trichloroindophenol at 675 nm in a reaction mixture containing 120 mM H_2O_2 , 17 mM $Na_2S_2O_3$, and 0.3 mM 2,3', 6-trichloroindophenol in 40 mM $NaPO_4$ buffer (pH 6.0), and 50 µl of plant extract (Nickel and Cunningham, 1969). Ascorbate peroxidase (APX) activity was assayed at 265 nm by monitoring the ascorbate-dependent reduction of H_2O_2 in a reaction mixture containing 500 µl 166 mM HEPES-KOH (pH 7.0) and 100 µl each 1 mM EDTA, 1.5 mM Na ascorbate, 1 mM H_2O_2, and plant extract (Anderson, *et al*., 1992). One unit of enzyme for catalase, peroxidase, and APX was defined as the amount necessary to decompose 1µmole of substrate/min at 25°C. GR activity was determined by monitoring glutathione-dependent oxidation of NADPH in a reaction mixture containing 0.15 mM NADPH, 0.5 mM GSSG, and 3 mM $MgCl_2$ in 50 mM Tris (pH 7.5) and 50 µl of plant extract (Schaedle and Bassham, 1977). One unit of GR was defined as the amount of enzyme required to reduce 1 nmol of substrate/min at 25°C (Gossett *et al*., 1994).

Data Analysis

The data shown are the mean values for five replicates. All data were subjected to a one-way analysis of variance, and the significance was determined at the 95% confidence limit.

Microscopy

The expression of the GFP-Yck2 fusion protein in live cells was monitored using an Olympus AX70 Provis microscope with differential interference contrast (DIC) optics and epifluorescence. GFP fluorescence was monitored using a filter set optimized for the emission spectra of the S65T GFP variant (Chroma Technology Inc.). The *Arabidopsis* cells were untransformed or from callus transformed with the pEGAD vector. The callus tissue was observed after 3 weeks of growth. DIC and fluorescence images were acquired with a Photometrics cooled CCD camera. The images were transferred to a Macintosh G4 computer and processed using IPLab *Spectrum* software.

Results

The original GFP-YCK2 fusion gene as $GFPY2_{orf}$ was inserted into the pBI121 vector (Clontech Inc.) in both the sense ($GFPY2_{orf}$) and antisense ($GFPY2_{orf}$) orientations. These vectors contain the *YCK2* gene under the control of the Cauliflower Mosaic Virus 35S promoter (**Figure 1**). The selectable marker for these vectors was the neomycin phosphotransferase (NPT II) gene under the control of the nopaline synthase (NOS) promoter. This cassette confers kanamycin resistance to transformed plant cells. Following *Hin*dIII digestion of the sense $GFPY2_{orf}$, and analysis of the restriction digest using agarose gel electrophoresis the GFP-YCK2 insert was visible at *ca.* 3040 bp confirming sense orientation of the GFP-Yck2 in pBI121**.**

Figure 2 shows the pEGAD vector containing the *YCK2* gene ligated in the sense orientation. (Cutler, *et al*., 2000). A restriction digest with *Eco*RI and *Bam*HI of the pEGAD with the *YCK2* insert followed by analysis of the restriction digest using agarose gel electrophoresis reveals the *YCK2* insert fragment migrating at *ca.*1600 base pairs. This sense pEGAD-YCK construct was used to transform *Agrobacterium* strain EHA105, and the resulting strain was used to transform cotton (Co312), tobacco, and *Arabidopsis*. Growth of kanamycin resistant callus tissue was achieved with all three plant species. Control callus was generated by exposing plant tissue to EHA105 that contained no plasmid constructs followed by growth of the callus on media lacking kanamycin.

Confirmation of Presence of Plasmids in Transformed Plant Tissue

Expression of the pBI 121 based constructs in plant tissue was confirmed by Western blotting using antibodies to the β–glucuronidase protein. **Figure 3.** shows the β-glucuronidase Western blot. Lane $^{\#}1$, shows the positive control for β-GUS at *ca.*74,000 Daltons. Lanes $^{\#}2,^{\#}3,^{\#}4$ contain protein from control cotton callus and lanes $^{\#}6,^{\#}7,^{\#}8$ contains protein from experimental callus, (*i.e.* transformed with GFPYCK-2_{orf}) The amounts of total cellular protein loaded in lanes $^{\#}2,^{\#}3,^{\#}4$ and in lanes $^{\#}6,^{\#}7,^{\#}8$ were 200 µg, 100 µg, and 50 µg respectively. This immunoblot clearly shows the presence of the β-GUS protein in transformed plant tissues at *ca.* 68,000 Daltons in lanes $^{\#}6,^{\#}7,^{\#}8$ and the absence of this protein in the control callus, lanes $^{\#}2,^{\#}3,^{\#}4$. Immunoblots for GFP in transformed tissue did not show clearly the presence of GFP or GFP-Yck2**.**

Cotton Cells Carrying the GFP:YCK2 Plasmid Tolerate High NaCl Media Concentration

Figure 4 shows photographs of callus grown on Murashige and Skoog media amended with 150mM NaCl. Transformed and non-transformed callus tissue of cotton c.v. Coker 312 were placed on this media. These pictures show the callus transformed with GFP:YCK2 sense in a viable state on the 150 mM NaCl media, while the non-transformed callus is necrotic. The results of the enzyme assays performed with basal non-stressed transformed and control tissue indicate that in a non-stressed, *i.e.* 0 mM NaCl, environment the tissues transformed with GFPY2_{orf} sense exhibited a 43% reduction in peroxidase activity but no significant changes in ascorbate peroxidase, glutathione reductase or catalase activities were observed.

Arabidopsis Callus Cells Carrying the eGFP:YCK2 Fusion Show Peripheral Green Fluorescence

In Figures 5 and 6, the results of the pEGAD fluorescence experiments are shown. Control callus is shown in **Figure 5** shows the normal distribution of chlorophyll fluorescence. In **Figure 6** however, callus transformed with the pEGAD-YCK2 construct, is very clearly fluorescent around the periphery of the cell. Figures of 5 and 6 feature both differential interference contrast (DIC) illumination and fluorescent illumination through a Green Fluorescent Protein filter system.

Discussion

The rationale for transforming plant tissue with *YCK2* was promoted by the observation that this gene when overexpressed in yeast leads to suppression of salt intolerance (Robinson*, et al*., 1992), which led to the hypothesis that plant tissue transformed with *YCK2* could also exhibit increased salt tolerance. This investigation was initiated by constructing GFP:*YCK2* chimeric genes and inserting these into *Agrobacterium* transmission vectors. Two constructs were made, GFPYCK2_{orf} sense and pEGAD-*YCK2*. These were used to transform cotton, tobacco and *Arabidopsis* tissues. The inability to unambiguously detect the expression of the GFP in tissues transformed with GFPYCK2_{orf} by immunoblotting together with the clear presence of β-glucuronidase in transformed tissue suggested that there may be a problem with the expression of the GFP-Yck2 fusion protein in plant tissue. One explanation for this phenomenon is the activity of an apparent cryptic intron in the GFP gene, which is spliced at the hn-RNA stage of transcription. The final mRNA is missing this piece of the encoded message (Haseloff, *et al*., 1997; Chiu, *et al*., 1996; Reichel, *et al*., 1996). The cryptic intron is removed in frame so that a frameshift mutation does not occur, there is only a deletion of 84 nucleotides. However, the deletion is in a critical section of the chromophore of the GFP molecule (Haseloff, *et al*., 1997). This is important because although the GFP is not functional as a reporter in some plants, the gene of interest should still be expressed. This splicing does not occur in all plant species. Maize and tobacco do not splice out wild-type GFP, but cotton and *Arabidopsis* do.

The results of the antioxidant enzyme assay indicate that in unstressed cotton callus tissue, the baseline enzymatic activities of catalase, ascorbate peroxidase (APX) and glutathione reductase (GR) are not significantly different between the non-transformed callus and the callus expressing GFPY2_{orf} sense. However, the peroxidase activity in callus tissue that expressed the GFPY2_{orf} sense gene was much lower than that of the untransformed callus. These data may indicate that while the basal level of stress in the control and transformed callus has similar effects on the catalase, APX, and GR enzymes, a much lower level of superoxide or H_2O_2 indigenous to the plant may be the reason for the low peroxidase level. If the basal level of superoxide and/or H_2O_2 was lower in the transformed cotton callus, the plant would experience a much lower level of initial stress in its environment.

In a series of tests designed to determine the effects of NaCl on callus growth, the standard Murashige and Skoog media with Gamborg's vitamins (Gossett *et al.,* 1994b) was supplemented with 150mM NaCl. Callus tissue was placed on this media and allowed to grow for several months. After four months, it was clear in that the non-transformed callus was dead, while the transformed GFPY2_{orf} callus was still viable. The transformed callus growth was not rapid on the salt media, but the callus tissue did not become desiccated. Hence, the transformed callus did appear to exhibit some degree of salt tolerance, by effectively resisting desiccation under these conditions.

An effort was made to correct the problem with the previously mentioned cryptic intron of the initially constructed GFP:*YCK2* fusion. This was done using the pEGAD vector, which as previously mentioned (Methods), forms a fusion when the gene of interest is ligated into the multicloning site. The *YCK2* gene was ligated into pEGAD to make pEGAD:YCK2.

After three weeks *Arabidopsis* callus transformed with this construct was tested for fluorescence. Both transformed and control callus tissues appeared to have fluorescence, however the quality of this fluorescence was different. The untransformed callus revealed a lower and more general cytosolic distribution of the fluorescence. This was possibly due to internal chlorophyll autofluorescence. Callus tissue transformed with pEGAD:YCK2 showed a much brighter and more intense pattern around the periphery of the cell. This could be due to the presence of GFP-Yck2 at the plasma membrane. However, without a Western blot for the presence of GFP, this fluorescence cannot be directly attributed to GFP-Yck2.

Future directions for this work include a GFP immunoblot of the cells transformed with pEGAD-YCK2 to confirm the presence of the fusion protein. We are currently testing pEGAD-YCK2 transformed cotton and *Arabidopsis* callus for increased salt tolerance.

Acknowledgements

The authors gratefully acknowledge the financial support of Cotton Incorporated and the Louisiana Board of Regents Support Fund.

References

Allen, R.D. 1995. Dissection of oxidative stress tolerance using transgenic plants. Plant Physiol. 107:1049-1054

Anderson, J.V., B.I. Chevone, and J.L. Hess. 1992 Seasonal variation in the antioxidant system of eastern white pine needles. Plant Physiol. 98:501-508.

Apse, M.P., G.S. Aharon, W.A. Snedden, E. Blumwald. 1999. Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis* Science. 285:1256-1258.

Asada, J., M. Takahashi. 1987. Production and scavenging of active oxygen radicals in photosynthesis. In: D.J. Kyle, C.B. Osmond, and C.J. Arntzen (eds.), Photoinhibition. Elsevier, Amsterdam. p. 227-297.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1999. "Short Protocols in Molecular Biology", John Wiley & Sons, New York, NY.

Ayers, R.S., D.W. Westcot. 1977. Water quality for agriculture. Proc. Int. Conf. On managing saline water for irrigation: planning for the future. p. 400-430.

Banks, S.W., Gossett, D. R., Lucas, M.C., Millhollon, E. P., LaCelle, M.G., (1993) "*Agrobacterium*-Mediated Transformation of Kenaf (*Hibiscus cannabinus*) with the β-Glucuronidase Gene". Plant Molecular Biology Reporter 11: (2)101-104.

Bellaire, B., J. Carmody, J. Braud, D.R. Gossett, S.W. Banks, M.C. Lucas, and T.E. Fowler. 2000. Involvement of abscisic acid-dependent and independent pathways in the upregulation of antioxidant enzyme activity during NaCl stress in cotton callus tissue. Free Rad. Res. 33:531-545.

Beers, R. R., and I. Sizer. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195: 133.

Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. Nucl. Acids Res. 12:8711-8721.

Boyer, J.S. 1982. Plant productivity and the environment. Science. 218:443-448.

Bressan, R.A., P.M. Hasegawa, J.M. Pardo. 1998. Plants use calcium to resolve salt stress. Trends Plant Sci. 3:411-412.

Brockman, J.L., S.D. Gross, M.R. Sussman, R.A. Anderson. 1992. Cell cycle-dependent localization of casein kinase I to mitotic spindles. Proc. Natl. Acad. Sci. USA. 89:9454-9458.

Chang, A., and C.W. Slayman. 1991. Maturation of the yeast plasma membrane $[H^+]$ ATPase involves phosphorylation during intracellular transport. J. Cell Biol. 115:289-295.

Chiu, W., Y. Niwa, W. Zeng, T. Hirano, J. Sheen. 1996. Engineered GFP as a vital reporter in plants. Current Biology 6:325-330.

Cutler, S.R., D.W. Ehrhardt, J.S. Griffits and C.R. Somerville. 2000. Random GFP::cDNA fusions enable visualization of subcellular cells of Arabidopsis at a high frequency. Proc. Natl. Acad. Sci. USA 97:3718-3723.

Dhinsa, R.S., and W. Matowe. 1981. Drought tolerance in two mosses: correlated with enzymatic defense against lipid peroxidation. J. Exp. Bot. 32:79-91.

Dixon, R.A., 1985 (Ed) Plant cell culture a practical approach IRL Press, Oxford

Estrada, E., P. Agostinis, J.R. Vandenheede, J. Goris, W. Merlevede, J. François, A. Goffeau, and M. Ghislan. 1996. Phosphorylation of the Yeast Plasma Membrane H^+ ATPase by casein kinase I. Jour. of Biol. Chem. 271:32064-32072.

Flowers, T.J., P.F. Troke, A.R. Yeo. 1977. The mechanism of tolerance in halophytes. Ann. Rev. Plant Physiol. 28:89-121.

Fowler, J.L. 1986. Salinity and fruiting. In: J.R. Mauney and J.R. Stewart (eds.) Cotton Physiology. The Cotton Foundation, Memphis, TN. p. 107-111.

Foyer, C.H., H. Lopez-Delgado, J.F. Dat, and I.M. Scott. 1997. Hydrogen peroxide and glutathione-associated mechanisms of acclimatory stress tolerance and signaling. Physiologia Plantarum. 100:241-254.

Garciadeblas, B., F. Rubio, F.J. Quintero, M.A. Banuelos, R. Haro, A. Rodriguez-Navarro. 1993. Differential expression of two genes encoding isoform of the ATPase involved in sodium efflux in *Saccharomyces cerevisae*. Mol. Gen. Genet. 236:363-368.

Gossett, D.R., E.P. Millholon, W.D. Caldwell, S. Munday. 1991. Isozyme variation among salt-tolerant and salt-sensitive cultivars of cotton. Crop. Sci. 34:706-714.

Gossett, D.R., E.P. Millhollon, and M.C. Lucas. 1994a. Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. Crop Sci. 34:706-714.

Gossett, D.R., E.P. Millholon, M.C. Lucas, S.W. Banks, and M.M. Marney. 1994b. The effects of NaCl on antioxidant enzyme activites in callus tissue of salt-tolerant and salt-sensitive cultivars of cotton. Plant Cell Rep. 13:498-503.

Gossett, D.R., S.W. Banks, E.P. Millhollon, M.C. Lucas. 1996. Antioxidant response to NaCl in a control and an NaCl tolerant cell line grown in the presence of paraquat, buthionine sulfoxime, and exogenous glutathione. Plant Physiol. 112:803-809.

Hardie, D.G. 1995. Cellular functions of protein kinases. In: "The Protein Kinase FactsBook". (G. Hardie and S. Hanks, eds.) London, Academic Press. p. 48-56.

Hardie, D.G. 2000. Plant protein-serine/threonine kinases: classification into subfamilies and overview of function. In: "Advances in Botanical Research, Plant Protein Kinases". M. Kreis, J.C. Walker, and J. A. Callow (eds.) London, Academic Press. 32:1-44.

Haseloff, J., K.R. Siemering, D. Prasher, S. Hodge. 1997. Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. Proc. Natl. Acad. Sci. USA. 94:2122-2127.

Heim, R., Cubitt, A.B. Cubitt and R.Y. Tsein. 1995. Improved green fluorescence. Nature. 373:663-664.

Hirsch, R.E., B.D. Lewis, E.P. Spalding, M.R. Sussman. 1998. A role for the AKT1 potassium channel in plant nutrition. Science. 280:918-921

Hoekstra, M.F., R.M. Liskay, A.C. Ou, A.J. DeMaggio, D.J. Burbee, F. Heffron. 1991. HRR25, a putative protein kinase from budding yeast: association with repair of damaged DNA. Science. 253:1031-1034.

Jefferson, R.A., S.M. Burgess, and D. Hirsh. 1986 β -Glucuronidase form *Escherichia c*oli as a gene-fusion marker. Proc. Natl. Acad. Sci. USA 83:8447-8451.

Jefferson, R.A., T.A. Kavanagh and M.W. Bevan. 1987. GUS fusions: β--glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO J. 6:3901-3907.

Klimczak, L.J., D. Farini, C. Lin, D. Ponti, A.R. Cashmore, G. Giuliano. 1995. Multiple isoforms of *Arabidopsis* casein kinase I combine conserved catalytic domains with variable carboxyl-terminal extensions. Plant Physiology. 109:687-696.

Lee, J.H., M.V. Montagu, N. Verbruggen. 1999. A highly conserved kinase is an essential component for stress tolerance in yeast and plant cells. Proc. Natl. Acad. Sci. USA. 96:5873-5877.

Lerner, H.R. 1999. Introduction to the response of plants to environmental stresses. In: Plant Responses to Environmental Stresses, (ed.) H.R. Lerner New York/Basel: Marcel Dekker Pub. p. 1-26.

Maniatis, T., E.F. Fritsch, J. Sambrook. 1982. Molecular Cloning-A Laboratory Manual. Cold Spring Harbor Laboratory.

Mattanovich, D., F. Ruker, A. da Camara Machado, M. Laimer, F. Regner, H. Steinkellner, G. Himmler, and H. Katinger. 1989. Efficient transformation of *Agrobacterium* spp. by electroporation. Nucleic Acids Res. 17:6747.

Maudoux, O., H. Batoko, C. Oecking, K. Gevaert, J. Vandekerckhove, M. Boutry, P. Morsomme. 2000. A plant plasma membrane H^+-ATPase expressed in yeast is activated by phosphorylation at its penultimate residue and binding of 14-3-3 regulatory proteins in absence of fusicoccin. J. of Biol. Chem. 275:17762-17770.

Mersereau, M., G. Pazour , and A. Das. 1990. Efficient transformation of *Agrobacterium tumefaciens* by electroporation. Gene. 90:149-151.

Moriau, L., B. Michelet, P. Bogaerts, L. Lambert, A. Michel, M. Oufattole, M. Boutry. 1999. Expression analysis of two gene subfamilies encoding the plasma membrane H^+-ATPase in *Nicotiana plumbaginifolia* reveals the major transport functions of this enzyme. The Plant J. 19:31-41.

Nickel, R.S. and B.A. Cunningham. 1969. Improved peroxidase assay method using leuco 2,3',6-trichloroindophenol and application to comparative measurements of peroxide catalysis. Ann. Biochem. 27:292-299.

Palmgren, M.G. and G. Christenson. 1993. Complementation *in situ* of the yeast plasma membrane $H^{(+)}$-ATPase gene *PMA1* by an $H^{(+)}$-ATPase gene from a heterologous species. FEBS Lett. 317:216-222.

Palmgren, M.G. 2001. Plant plasma membrane H^+ATPases: powerhouses for nutrient uptake. In: "Annu. Rev. Plant Physiol. Plant Mol. Biol.". 52:817-845.

Pardo, J.M., M.P. Reddy, S. Yang, A. Maggio, G.-H. Huh, T. Matsumoto, M.A. Coca, M. Paino-D'Urzo, H. Koiwa, D.-J. Yun, A.A. Watad, R.A. Bressan, and P.M. Hasegawa. 1998. Stress signaling through Ca^{2+}/calmodulin dependent protein phosphatase calcineurin mediates salt adaptation in plants. Proc. Natl. Acad. Sci. USA. 95:9681-9686.

Robinson, L.C., E.J.A. Hubbard, P.R. Graves, A.A. DePaoli-Roach, P.J. Roach, C. Kung, D.W. Haas, C.H. Hagedorn, M. Goebl, M.R. Culbertson and M. Carlson. 1992. Yeast casein kinase I homologues: An essential gene pair. Proc. Natl. Acad. Sci. USA. 89:28-32.

Robinson, L.C., C. Bradley, J.D. Bryan, A. Jerome, Y. Kweon, H.R. Panek. 1999. The YCK2 yeast casein kinase 1 isoform shows cell cycle-specific localization to sites of polarized growth and is required for proper septin organization. Mol. Biol. of the Cell. 10:1077-1092.

Serrano, R., R. Gaxiola. 1994. Crit. Rev. Plant Sci. 13:121-138

Serrano, R. 1996. Salt tolerance in plants and microorganisms: toxicity targets and defense responses. Int. Rev. Cytol. 165:1-52.

Schaedle, M., and J. A. Bassham. 1977. Chloroplast glutathione reductase. Plant Physiol. 59:1011-1012.

Stanton, M.L., B.A. Roy, D.A. Thiede. 2000. Evolution in stressful environments. I. phenotypic variability, phenotypic selection, and response to selection in five distinct environmental stresses. Evolution. 54:93-111.

Tuazon, P.T. and J.A. Traugh. 1991. Casein kinase I and II – multi-potential serine kinases: structure, function and regulation. Adv. Second Messenger Phosphoprotein Res. 23:123-124.

Van der Graaf, E. and P.J.J. Hooykas, 1998. "Transformation of *Arabidopsis thaliana* C24 Leaf Discs by *Agrobacterium tumefaciens*." In: *Arabidopsis* Protocols, J.M. Martinez-Zapater and J. Salinas, (Eds.) Humana Press, Totowa, N.J. p. 245-258

Vancura, A., A. O'Connor, S.D. Patterson, U. Mirza, B.T. Chait, J. Kuret. 1993. Isolation and properties of YCK2, a *Saccharomyces cerevisae* homolog of casein kinase I in budding yeast. J. Biol. Chem. 269:19271-19273.

Wang, X., M.F. Hoekstra, A.J. DeMaggio, N. Dhillon, A. Vancura, J. Kuret, G.C. Johnston, and R.A. Singer. 1996. Prenylated isoforms of Yeast Casein Kinase I, including the novel YCK3p, suppress the gcs1 blockage of cell proliferation from stationary phase. Molecular and Cellular Biology. 16:5375-5385.

Withee, J.L., R. Sen, M. Cyert. 1998. Ion tolerance of *Saccharomyces cerevisae* lacking the Ca^{2+}/CaM-dependent phosphatase (Calcineurin) is improved by mutations in *URE2* or *PMA1*. Genetics. 149: v865-878.

Wu, S., Ding, L., Zhu, J.-K. 1996. SOS1, a genetic locus essential for salt tolerance and potassium acquisition. Plant Cell. 8:617-627.

Wyn Jones, R.G. 1981. In: Physiological processes limiting plant productivity, C.B. Johnson, (Ed.) Butterworths, London. p. 271-292.

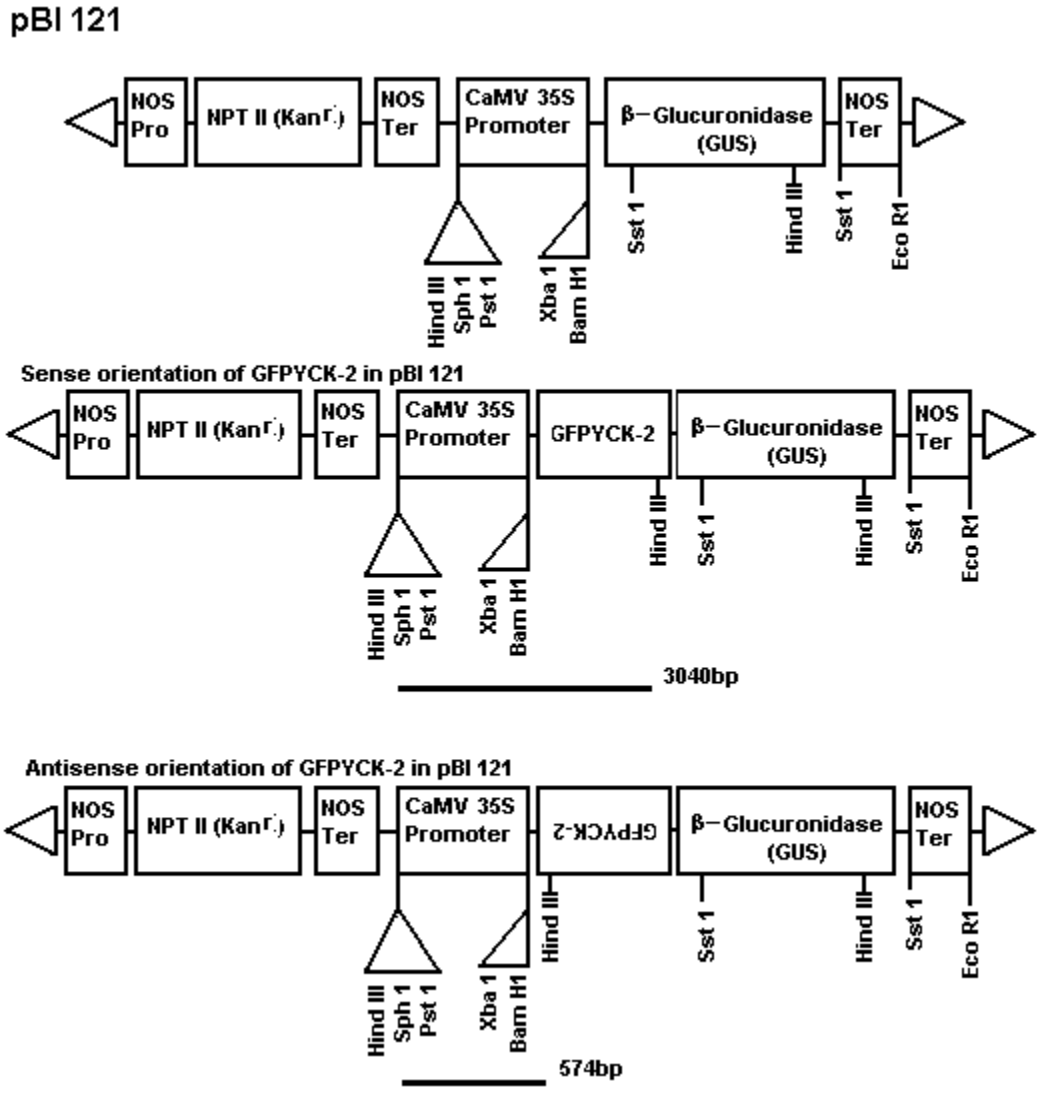


Figure 1. Map of pBI 121, $GFPYCK_{orf}$ sense orientation and $GFPYCK_{orf}$ antisense orientation

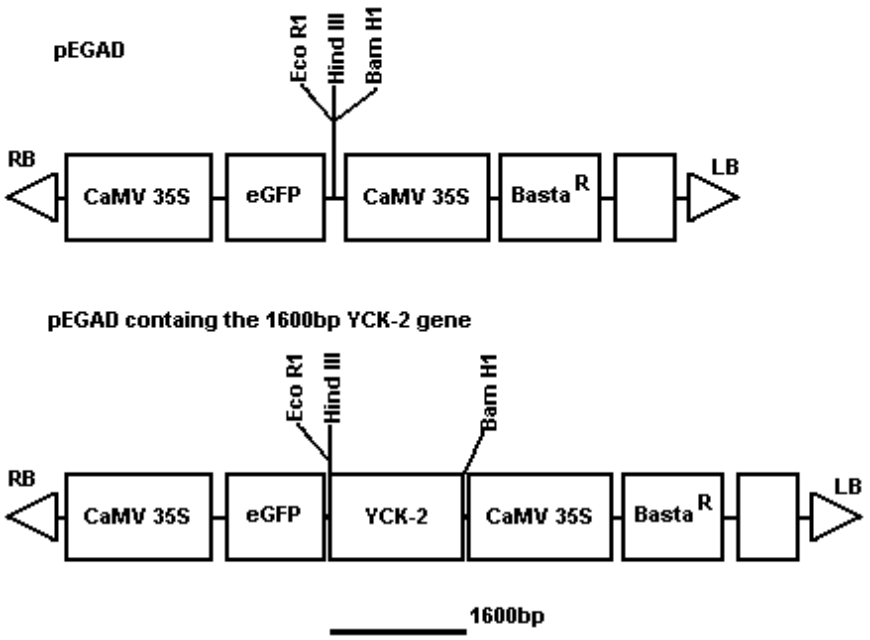
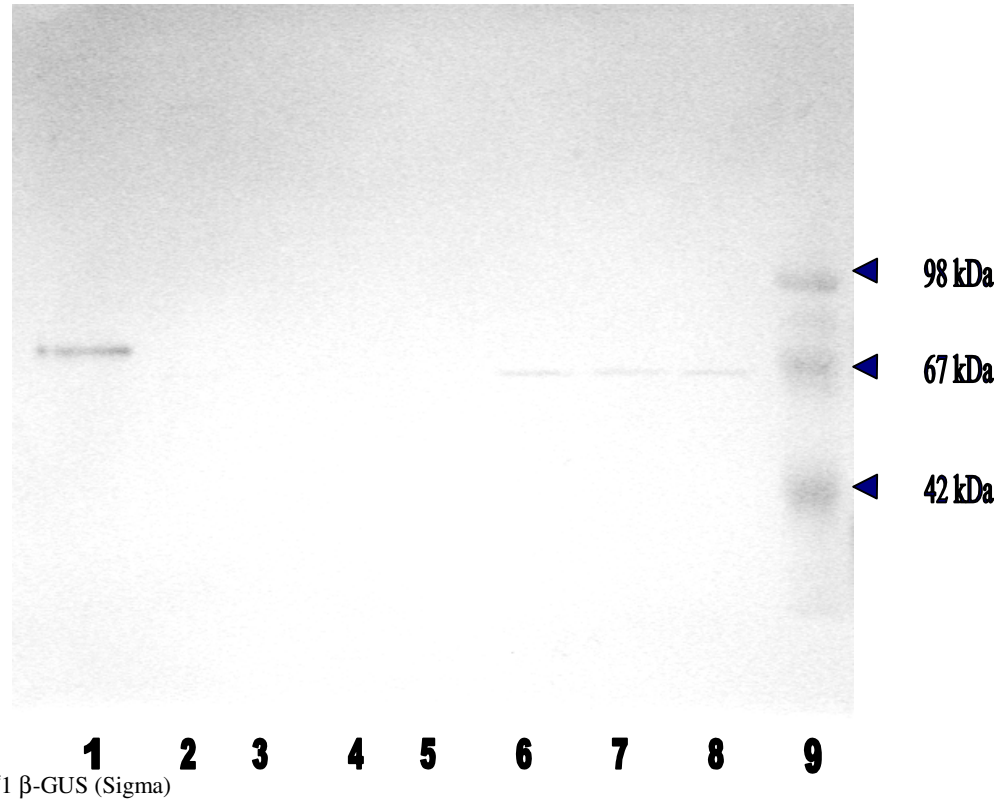
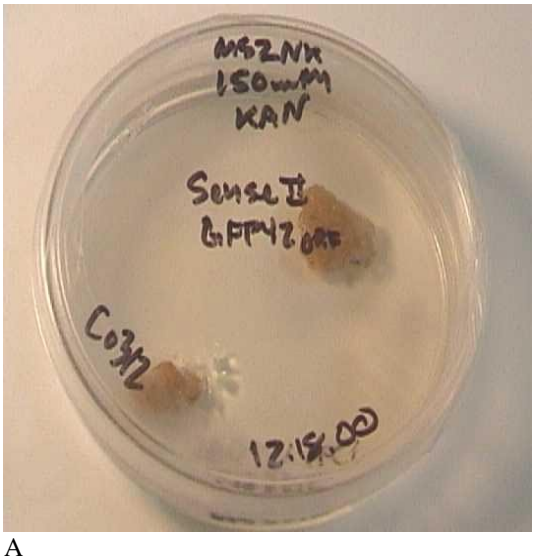


Figure 2. Map of pEGAD and pEGAD-YCK2. The YCK gene has been inserted into the EcoR1/BamH1 site

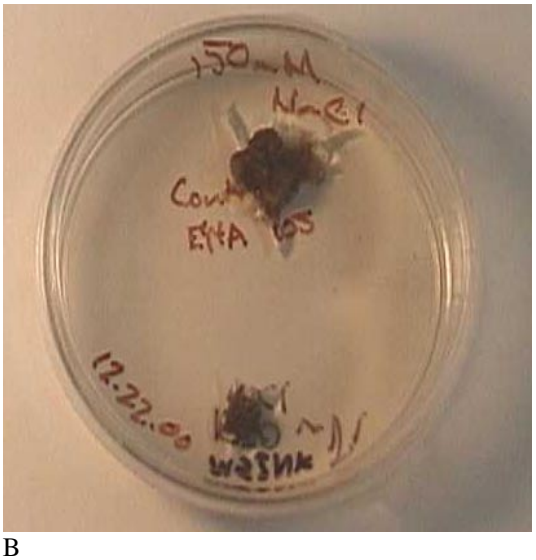


#1 β-GUS (Sigma)
#2 200µg total protein from control tissue
#3 100µg total protein from control tissue
#4 50 µg total protein from control tissue
#5 Blank lane
#6 200µg total protein from plant tissue transformed with (GFP*YCK2*$_{orf}$)
#7 100µg total protein from plant tissue transformed with (GFP*YCK2*$_{orf}$)
#8 50µg total protein from plant tissue transformed with (GFP*YCK2*$_{orf}$)
#9 Gibco/BRL prestained marker

Figure 3. Immunoblot for β-glucuronidase in transformed and control plant tissue.



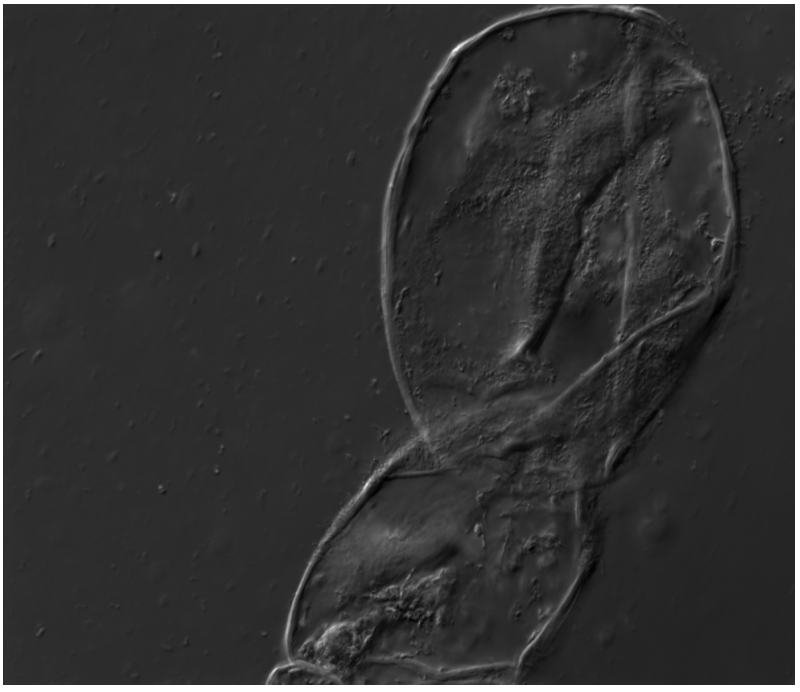
A



B

Figure 4. (A) Control Callus on Murashige and Skoog media containing 150mM NaCl. (B) Callus transformed with GFPYCK-2 in pBI 121 on Murshige and Skoog media containing 150mM NaCl and 50µg ml^{-1}kanamycin.

DIC
Optics



Fluorescent
Light

GFP filter

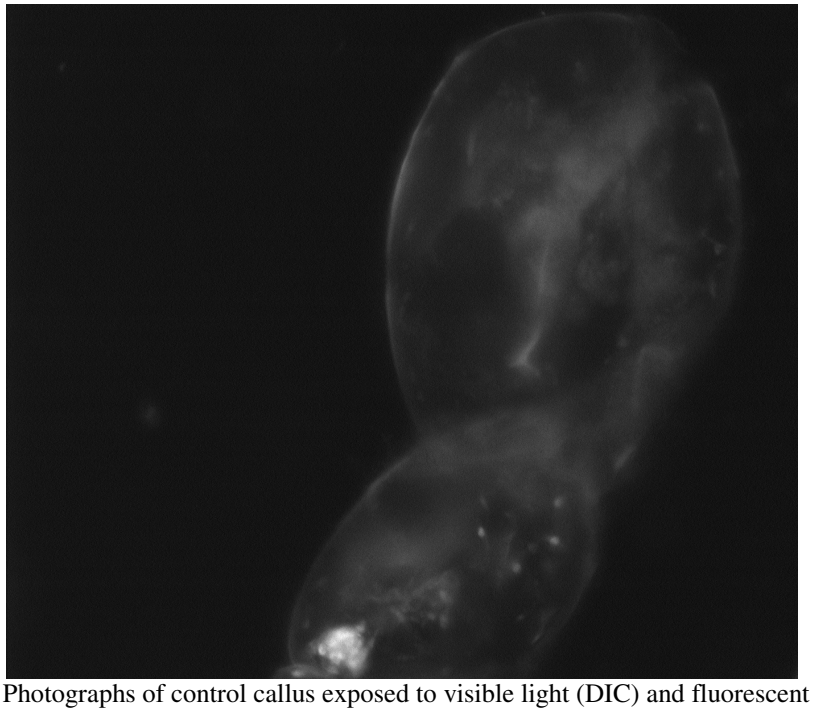
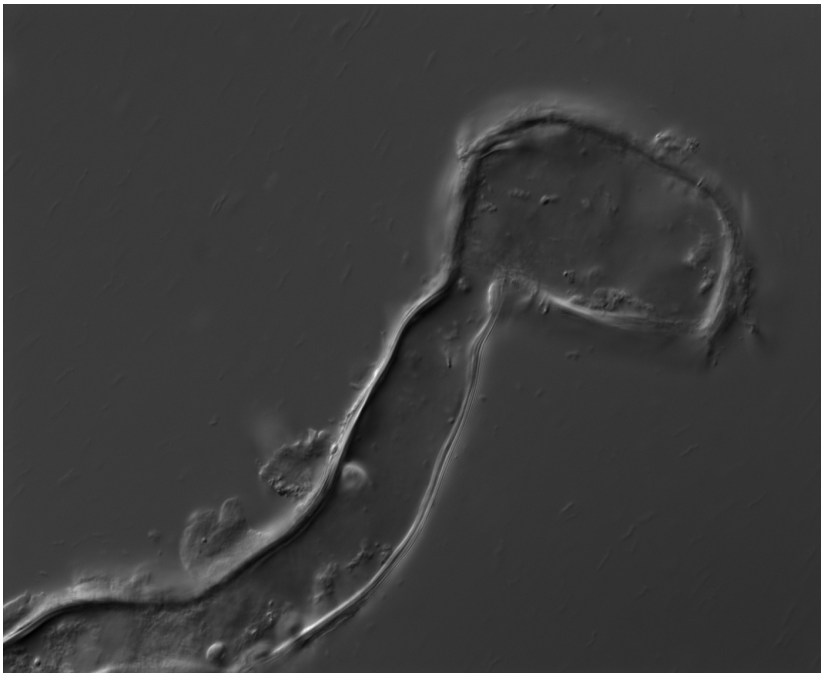


Figure 5. Photographs of control callus exposed to visible light (DIC) and fluorescent light.

DIC
Optics



Fluorescent
Light

GFP filter

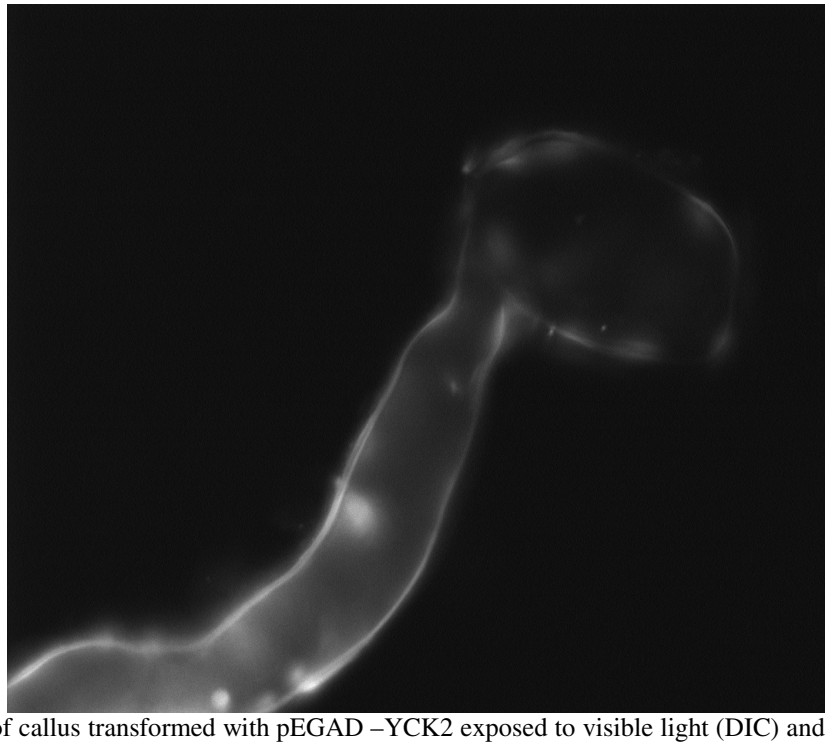


Figure 6. Photographs of callus transformed with pEGAD –YCK2 exposed to visible light (DIC) and fluorescent light.