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ANNUAL PROGRESS REPORT

Bermudagrass Cold Hardiness: Characterization of Plants for Freeze Tolerance and Characterization of Low Temperature-Induced Genes

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Executive Summary

Injury to bermudagrass turf caused by freezing temperatures during winter is a persistent problem over much of its geographic area of use in the USA. This research seeks to reduce risk of freeze injury to bermudagrass grown in temperate regions. The research focuses on accurately assessing the freeze tolerance of bermudagrass cultivars, isolating genes responsible for enhanced freeze tolerance, and enhancing knowledge of the fundamental mechanisms associated with cold hardiness. Specific objectives are to: 1) quantify cold-hardiness of advanced breeding lines, recently released varieties, and established standard varieties and 2) isolate and characterize cold regulated (*Cor)* genes responsible for conferring freeze tolerance.

The low temperature tolerance (LT_{50}) of 11 turf bermudagrasses was evaluated. LT_{50} values (°C) for clonal varieties were: GN-1 =-5.8, Baby = -6.1, Tifway = -6.6, Tifton 94, Quickstand = -8.0, and Midlawn = -8.4. LT_{50} values for seeded varieties were: Arizona Common = -5.6, Mirage = -6.1, Jackpot = -6.3, Guymon = -7.4, and OKS 91-11 = -7.6. These evaluations will continue with selected varieties from: 1) vegetatively-propagated fairway types, 2) seeded fairway types, 3) vegetatively-propagated putting green types, and 4) experimental fairway breeding lines.

The primary structure of the preprotein encoded by the bermudagrass chitinase genes (CynCht1, CynCht2) were analyzed. Both chitinase genes encode low molecular weight hydrophilic (secreted) proteins, which can be structurally classified as Class II chitinases. The mature polypeptide of CynCht1 is composed of 227 amino acid residues with a molecular weight of 25 kDa and calculated pI of 8.10. CynCht2 mature polypeptide, on the other hand, consists of 229 with a molecular weight of 25.5 kDa and calculated pI of 8.82.

Alignment of the amino acid sequences of the mature polypeptides encoded by the two genes revealed significant homology with a number of known chitinases from higher plants. Both chitinases are most closely related to the Class II chitinases from peanut and tomato.

Functional analysis on the products of the low temperature-inducible CynCht1 gene via *Agrobacterium* – mediated transformation of *Arabidopsis thaliana* is underway. Selection of a suitable *Arabidopsis* ecotype has been conducted by determining the cold hardiness of 10 ecotypes collected from a variety of cold and warm habitats. Results suggested that the Tmid values (using electrolyte leakage test) slightly differ between the non-acclimated and cold acclimated plants and there is no significant difference in the cold hardiness of the 10 ecotypes evaluated so far.

The plasmid that will be used for *Agrobacterium*-mediated transformation (via the binary system) is being constructed. The coding region (1.2 kb) of the CynCht1 from the main clone Stul 456-1 was PCR amplified using forward and reverse primers. Procedures for inserting the CynCht1 gene into the binary T- DNA vector (pBECKS), recently provided by Dr. Alex McCormac from the University of Southampton, UK , are being optimized.

Introduction

Bermudagrass, *Cynodon* sp., is one of the most important turf species in the southern USA and throughout much of the world. Injury due to freezing temperatures during winter is a persistent problem throughout much of the geographic area of use of the species in the USA (Anderson, et al. 1997). For example, extensive winter injury was experienced in the winters of 1977- 78, 1978-79, 1989-90, 1993-94 and 1995-96, with many areas requiring re-establishment (Anderson et al., 1997; Gatschet et al., 1994; O'Brien, 1994; O' Brien, 1996). Bermudagrass winter injury is unsightly, it disrupts turfgrass use during repair, and it is costly. The economic loss from even a few thousand acres of bermudagrass winterkill can be in the millions of dollars (Anderson et al., 1997). Reducing the risk of freeze injury to bermudagrass grown in temperate regions can be accomplished by a combination of actions. These include: 1) identifying and using best adapted varieties and 2) following management practices that mitigate freeze injury, and 3) developing more cold tolerant varieties. Our research focuses on accurately assessing freeze tolerance of bermudagrasses and identifying genes involved in cold tolerance. Both are fundamental to the breeding improvement of turf bermudagrasses for freeze tolerance.

Cold Hardiness Evaluations

Bermudagrasses grown in the transition zone between warm- and cool-season grasses are subject to winter kill. Bermudagrass germplasm improvement programs have identified improved winter survival as a priority. Breeding programs require a rapid, reproducible means to quantitatively evaluate cold hardiness. Although test winters probably supply the best indication of winter survivability, their occurrence is unpredictable and not reproducible. Therefore, our objective is to quantify cold hardiness of advanced lines, recently released varieties, and established standards using laboratory-based methodology. Standardized, quantitative information on tissue cold tolerance is vital to scientists to track their progress in developing new varieties. Cold tolerance is also one of the most important pieces of information for turf managers selecting bermudagrasses for the transition zone.

Cold hardiness evaluations have been divided into four groups, three based on intended use and the fourth comprising advanced selections from the OSU breeding program. The vegetatively propagated fairway types include Baby, Midlawn (standard), Tifway, GN-1, Tifton 94, and Quickstand Common. The second set of bermudagrasses comprises seeded varieties from the last NTEP trial: Jackpot, Mirage, OKS 91-11, Guymon, and Arizona Common (standard). The third series of plants represent bermudagrasses used for putting greens: Floradwarf, Champions, Tifeagle, MS Supreme, Miniverde, Tifdwarf, and Tifgreen (standard). The final set of cold hardiness determinations will examine advanced selections from the OSU breeding program including OKS 95-1 and 18-4. Experiments with fairway and seeded bermudas have been completed. Putting green varieties are currently being evaluated and studies with advanced selections are planned for late 2000. Plans call for repeating experiments for each use type on three dates.

All plants were clonally propagated in cone-tainers except for the seeded group. After plants were established at 28/24 C day/night temperatures, they were acclimated at 8/2 C day/night temperatures for 4 weeks. The 10-hour photoperiod had a light intensity of 400 $E \cdot m^{-2} \cdot s^{-1}$. Tmid values (midpoint of survival vs temperature response curve) for each genotype were determined as previously described (Anderson et al., 1993). Significant differences in Tmid means from the seeded study were determined following ANOVA. Since one of the replications of the fairway study became infested with insects, it was discarded and mean separation could not be performed.

Fairway Bermudas

Genotype	Tmid (°C)
GN-1	-5.8
Baby	-6.1
Tifway	-6.6
Tifton 94	-7.4
Quickstand	-8.0
Midlawn	-8.4

Seeded Bermudas

Genotype	Tmid (°C)
Arizona Common	-5.6 a
Mirage	-6.1 ab
Jackpot	-6.3 abc
Guymon	-7.4 bc
OKS 91-11	-7.6 c

Although data from the fairway study should be interpreted with caution, it appears that GN-1 and Baby were the least hardy with Tmids around -6 C. Freeze tolerance increased from Tifway (-6.6 C), Tifton 94 (-7.4 C), Quickstand (-8.0 C) to Midlawn (-8.4 C). If these values represent true winter survival capacity, genotypes such as GN-1 and Baby will be at greater risk of freeze damage than Quickstand and Midlawn.

Among the bermudagrasses propagated from seed, Arizona Common (-5.6 C) was significantly less cold hardy than Guymon (-7.4 C) and OKS 91-11 (-7.6 C). Mirage (-6.1 C) and Jackpot (-6.3 C) were not significantly hardier than Arizona Common. Although we have not previously examined this combination of genotypes, Tmids of several genotypes were substantially lower (greater hardiness) in previous reports (Anderson and Taliaferro, 1999) where plants were propagated clonally. Although we did not compare seed vs clonal propagation, it is possible that the Tmids of our recently seeded materials reflect the frequent field observation of increased susceptibility to winter injury the first season after establishment.

Seven bermudagrass genotypes are being evaluated for relative freeze tolerance. The genotypes include recent selections from Oklahoma State University's breeding program (OKC 18-4, OKS 95-1) and representative standards or emerging grasses with similar intended use (Tifway, Tifsport, Midlawn, U-3, and Princess). Plants were clonally propagated and established in cone-tainers in a greenhouse. Bermudagrasses were transferred to a controlled environment chamber at 8/2°C (day/night) temperatures for acclimation beginning in early September 2000. Plants were divided into four groups to allow replication in time. Bermudagrasses are being subjected to low temperatures in a freeze chamber and responses visually evaluated as regrowth in a growth chamber.

Freezing evaluations began in early October and will be completed in November 2000. Survival versus temperature data will be fit to a nonlinear model to estimate the midpoint (T_{mid}) of the sigmoidal response curve. Significant differences in freeze tolerance between the genotypes will be determined by mean separation following analysis of variance. Relative freeze tolerance estimates will provide information useful to turfgrass managers selecting genotypes adapted to the transition zone.

Isolation and characterization of genes induced during cold acclimation in *Cynodon* sp.

The recent success of recombinant DNA technology in many aspects of crop improvement demonstrates its potential as a tool to further enhance or complement plant breeding efforts towards the development of more cold hardy turf bermudagrass cultivars. One way by which this goal can be accomplished is through the discovery of genes whose expressions contribute either directly or indirectly to increased survival of turfgrasses following periods of freezing stress. Some bermudagrass cultivars are capable of surviving under conditions of freezing temperatures by their ability to cold acclimate at temperatures slightly above 0°C before the occurrence of freezing conditions, a process known as cold acclimation or hardening. The main goal of this research project is to dissect the molecular basis of this biological phenomenon in *Cynodon* through the use of recombinant DNA techniques. Efforts in this area will lead to the discovery of novel genes that may have potential use for genetic improvement of the freezing tolerance not only of bermudagrasses but of other turfgrass species as well.

For the last seven years we have probed the molecular basis of cold acclimation and freezing tolerance in *Cynodon*. One of our earlier findings was the possible involvement of pathogenesis-related (PR) chitinase proteins that may also confer freezing tolerance in bermudagrass crown tissues. We have evidence from two-dimensional protein electrophoretic studies showing that some chitinases are synthesized in larger amounts in response to cold acclimation in the freezing tolerant cultivar Midiron than in the moderately freeze tolerant Tifgreen bermudagrass (Gatschet et al., 1996). A similar situation was recently documented by the Hon et al. (1995) and Antikainen et al. (1997) on winter rye, and by Hincha et al. (1997) in spinach. The results of these studies pointed to the possible secondary roles of PR proteins as antifreeze factors. It is now hypothesized that the biochemical basis for the involvement of PR proteins in freezing tolerance is by virtue of their structural ability to bind to growing extracellular ice crystals, thereby preventing further crystallization, a situation analogous to the mode of action of the AFPs originally isolated from polar fishes (Davies and Hew, 1990). Although still speculative, this possibility is very attractive due to the widespread occurrence of this phenomenon not only in winter rye but also in other overwintering cereal species. This leads to a further hypothesis that this may be an adaptive response specific to monocots (Antikainen et al., 1997). This also points to a possible pleiotropic nature of some PR-protein genes that occur as members of multigene families.

In line with the findings discussed above, we initiated a project with the aim of isolating chitinase genes from Midiron. Our major goal is to characterize members of the chitinase

gene families in *Cynodon*. Expected outputs from this project include cloning and sequencing of chitinase genes, analysis of their temporal and spatial expression patterns in relation to cold acclimation and freezing, and functional analysis on the products of the low temperature inducible bermudagrass chitinase gene/s.

Major accomplishments that have been reported in the previous progress report include: (1) construction of a *Cynodon* genomic library from 'Midiron' (*Cynodon dactylon* X *Cynodon transvaalensis*); (2) isolation and sequencing of CynCht1 and CynCht2; and (3) induction of CynCht genes in response to low temperature, drought and exogenous ABA. Much of the earlier work is detailed in the attached manuscript 'Induced expression of class II chitinase during cold acclimation and dehydration of bermudagrass (*Cynodon* sp.)'. Additional results and current studies follow:

Amino Acid Sequence Homology

The amino acid sequence of the proteins encoded by CynCht1 and CynCht2 genes were determined. The primary structure of the preprotein encoded by the bermudagrass genes are divided into two regions, the signal and the catalytic domains. CynCht1 and CynCht2 have highly homologous N-termini with only a single amino acid substitution at the signal peptide (Figure 1).

Computer analysis using ExPASy Proteonomics Tools predicted that both proteins are cleaved at the glycine-phenylalanine junction, removing a signal peptide consisting of 22 amino acid residues. The signal peptides of CynCht1 and CynCht2 are 95% identical and are both hydrophobic as indicated by the calculated Grand Average Hydropathicity (GRAVY) of 1.286 and 1.450, respectively (Kyte and Doolittle, 1982).

The CynCht mature polypeptide is composed of 227 amino acid residues with a molecular weight of 25 kDa, and a calculated pI of 8.10. The CynCht2 mature polypeptide consists of 229 amino acid residues and about 25.5 kDa. Computer calculations indicates that CynCht2 is basic with a pI of 8.82. Both proteins are hydrophilic with GRAVY values of –0.493 and –0.465, respectively.

Alignment of the amino acid sequences of the mature polypeptides encoded by the two genes revealed significant homology with a number of known chitinases from higher plants (Table 1, Figure 1). The homologous regions are located at the catalytic domain of the two proteins defined by amino acid 43-243 and aligned quite well with the catalytic regions of both Class I and Class II chitinases (Table 1). Apparently, these highly conserved regions of the two bermudagrass proteins correspond to the functional domain for catalytic activity of these chitinases (Flach et al., 1992; Beintema, 1994). The results of the sequence alignments also revealed that both bermudagrass chitinase genes are most closely related to Class II chitinases from peanut (Kellmann et al, 1996) and tomato (Harikrishna et al., 1996). No sequence homology was detected with known Class III chitinases.

Computer analysis failed to detect any intracellular targeting signals in both genes. Most notable was the absence of the hydrophobic C-terminal extension that characterize chitinases which are targeted to the vacuole (Bednarek and Raikhel, 1991; Chrispeels and Raikhel, 1992). Thus, the mature proteins encoded by both bermudagrass genes are all predicted to be extracellular. This result is consistent with the extracellular location of known class II chitinases of higher plant species.

Selection of Suitable *Arabidopsis* Ecotype for Transformation

Seeds of the following *Arabidopsis* ecotypes (Ms-0, Wil-3, Pi-0, St-0, Lm-2, Tu-0, Pa-1, Ct-1,Col-0 and RLD) collected from a variety of cold and warm habitats were requested from *Arabidopsis* Biological Resource Center (ABRC). The 30 seeds obtained from ABRC were sowed in pots containing Metromix 350 and were subjected to cold treatment (4 °C) for 5 days. After the cold treatment, the seeds were transferred to a controlled environment chamber set at 22 °C to 24°C with light and dark periods of 14 hours and 10 hours, respectively. The plants were allowed to self-pollinate and were maintained until maturity. After 28-30 days, the seeds from each ecotype were harvested and then dried for a period of 2-3 weeks.

At least 120 seeds of each ecotype were planted for cold tolerance evaluation. Cold hardiness of non-acclimated plants (24°C/20°C) and 24-hour cold acclimated (8°C/2°C) plants were determined using the electrolyte leakage test method (Gilmour et al., 1988, Sukuraman and Weiser, 1972, Jaglo-Ottosen et al, 1998). Non-acclimated and cold acclimated plants were placed in test tubes with distilled water and then submerged for 1 hour in a -2°C bath containing ethylene glycol. Ice crystals were added to nucleate freezing and the sample were allowed to equilibrate for additional 1 hour.

After equilibration, samples (three replicates for each temperature point) were cooled in decrements of - 1°C each hour until the temperature -15 °C was reached. The samples were kept in ice water bath for 1 hour and were thawed for 15 hours inside a refrigerator set at 4°C. The initial and final conductivities of the resulting solutions were measured using a conductance meter. The conductivity data and temperature were fittted to a nonlinear model to estimate the midpoint (T_{mid}) of the sigmoidal response curve (Table 2).

Results suggested that the Tmid values (using electrolyte leakage test) slightly differ between the non-acclimated and cold acclimated plants and there is no significance difference among the cold hardiness of the 10 ecotypes evaluated. S.J. Gilmour (unpublished) compared freezing tolerance of eight ecotypes and found that the LT_{50} values were small (the maximum difference is only about -3°C) . The above results taken together suggest that there is no much genetic variation in the ability of *Arabidopsis* ecotypes to cold acclimate. Hence, any of the ecotypes so far evaluated can be used for the transfomation experiment.

Preparation of Construct

A chimeric gene construct containing the CaMV 35 S promoter and the CynCht1 gene is being made. Binary T- DNA vectors (pBECKS19, pBECKSgen and PBECKS400 series) that are being used in making the construct were given by Dr. Alex McCormac from the University of Southampton, UK. The coding region (1.2 kb) of the CynCht1 from the main clone Stul 456-1 was PCR amplified using forward (7847) and reverse (7848) primers. All the PCR products were gel purified and were sequenced before cloning. Procedures for inserting the CynCht1 gene into the binary T- DNA vector are being optimized.

Research in Progress

It is now well established that some plant species synthesize proteins with antifreeze function during cold acclimation. Many of these proteins have been identified as pathogenesis-related (PR) proteins (Hon et al, 1995;Griffith et al, 1997; Yu and Griffith, 1999). The salient features of the two PR protein genes encoding chitinases (*CynCht1*, *CynCht2*) from freeze-tolerant cultivar 'Midiron' are consistent with the possibility that they may be involved in freeze-tolerance mechanisms. The data on the temporal and spatial expression patterns strongly indicated low temperature-induced expression. These results have significant implications especially with regard to the hypothesized function of PR proteins as antifreeze molecules. Despite this information, the direct involvement of the products of these genes in bermudagrass freeze-tolerance mechanisms needs to be further confirmed experimentally. A major question that needs to be answered concerns the magnitude of increase in cold hardiness and drought tolerance if a chitinase gene is overexpressed without cold acclimation. To address this question, we will overexpress the bermudagrass chitinase gene (CynCht-1) in a suitable *Arabidopsis* strain. Chitinase overproducing transgenic plants will be evaluated at the phenotypic, biochemical and genetic levels in order to determine the role of chitinases in freeze-tolerance mechanism/s.

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Table 1. Homology of the catalytic region of bermudagrass chitinases with some class I and class II chitinases in higher plants.

Chitinase	% Identity	% Similarity
Cht1/Peanut (Class II)	73	87
Cht1/Tobacco (Class II)	62	89
Cht1/Tomato (Class II)	60	76
Cht1/Class II (Average)	**65**	**84**
Cht1/Alfalfa (Class I)	59	74
Cht1/Pea (Class I)	59	74
Cht1/Potato (Class I)	57	73
Cht1/Class I (Average)	**58**	**73.6**
Cht2/Peanut (Class II)	70	84
Cht2/Tobacco (Class II)	60	77
Cht2/Tomato (Class II)	57	74
Cht2/Class II (Average)	**62**	**78**
Cht2/Alfalfa (Class I)	55	69
Cht2/Pea (Class I)	56	69
Cht2/Potato (Class I)	53	69
Cht2/Class I (Average)	**54.6**	**69**

Table 2 . Tmids of *Arabidopsis* ecotypes that were evaluated for cold hardiness using the electrolyte leakage test.

Temp (°C) of the site of collection Spring/Autumn	Accession Number	Name	Non-acclimated T mid (°C)	24-hr Cold Acclimated (8/2°C) Tmid (°C)
< 0-2 / < 5-6	CS905	Ms-0	-2.7359	-2.8015
< 0-2 / < 9-10	CS1598	Wil-3	-2.6665	-3.6241
3-4 / 5-6	CS1454	Pi-0	-1.2931	-2.2131
3-4 / 7-8	CS1534	St-0	-2.2453	-2.5176
9-10 / 13 -14	CS1344	Lm-2	-2.1328	-1.8068
9-10 / 15 -16	CS1566	Tu-0	-2.0758	-2.1596
13-14 / 21-22	CS1438	Pa-1	-2.3090	-3.5536
13-14 / 21-22	CS1094	Ct-1	-1.6449	-3.8135
No record	CS1092	Col-0	-1.3567	-3.8397
No record	CS913	RLD	0.3713	-2.0693

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CHT1      maysdallfavtavaslvtsggFFAEARWYGPGGKCSSVEAL--------------------------------
CHT2      maysdallfavtavaflvtsggFFAEARWYGPGGKCSSVEAL--------------------------------
Pn(CII)   malfsfsfssfcltifviyssslslsAESRVSPIAPISSLISKTLFDSIFLHKDDNACPARNFYTYESFVE----
Tm(CII)   mrllvlglfsvlclkcvlsQNISSLISKNLFERILVHRNDAACGAKGFYTYEAFIT------------------
Af(CI)    mlmkmrlalvttvvlliigcsfaEQCGKQAGGALCPGGLCCSKFGWCGSTGEYCGDGCQSQCGGSSGGGGDLGS
          Signal Peptide          Cysteine-rich domain                        Linker

CHT1      ---------------------------------AARAFPKFAGTGDLATRKRELAAFFAQISHETTGGWATAPDGP
CHT2      ---------------------------------AARAFPKFAGTGDLATRKRELAAFFAQISHETTGGWATAPDGP
Pn(CII)   ---------------------------------ATSSFPAFGSTGCSATRKREVAAFLAQISHETTGGWATAPDGP
Tm(CII)   ---------------------------------ATKTFAAFGTTGDTNTRNKEIAAFLAQTSHETTGGWATAPDGP
Af(CI)    LISRDTFNNMLKHRDDSGCQGKGLYTYDAFISAAKAFPNFANNGDTATKKREIAAFLGQTSHETTGGWATAPDGP
          Hypervariable Region             Catalytic region

CHT1      YSWGLCYKEEISPASNYCDATDKQWPCYPGKSYHGRGPIQLSWNFNYGPAGQALGFDGLRNPEIVANCSDTAFRT
CHT2      YSWGLCYKEEIRPASNYCDATDKKWPCYPAKSYHGRGPIQLSWNFNYGPAGQALGFDGLRNPEIVANCSDTAFRT
Pn(CII)   YAWGLCFKEEVSPQSDYCDSSNKEWPCYPGKSYKGRGPIQLSWNYNYGPAGKALGFDGLKNPDIVSNNSVIAFKT
Tm(CII)   YSWGYCYKQEQGSPGDYC-ASSQQWPCAPGKKYFGRGPIQISYNYNYGAAGSAIGVNLLNNPDLVANDAVVSFKT
Af(CI)    YAWGYCFVREQNP-STYCQPSS-EFPCASGKQYYGRGPIQISWNYNYGQCGRAIGVDLLNNPDLVATDPVISFKT

CHT1      ALWFWMTPRRPKPSCHEVMVGEYRPTADVAGNRMPGFGLVTNIVNGGLECNRTDDARVNNRIGFYRRYCQIFNV
CHT2      ALWFWMTPRRPKPSCHEVMVGEYRPTAVDVAGNRMPGFGLVTNIVNGGLECNRTDDARVNNRIGFYRRYCQIFNV
Pn(CII)   ALWFWMTEQKPKPSCHNVMVGNYVPTASDRAANRTLGFGLVTNIINGGLECGVPDDARVNDRIGYFQRYAKLFNV
Tm(CII)   ALWFWMTAQQPKPSAHDVITGRWSPSVADSAAGRVPGFGVITNIINGGMECNSGSNALMDNRIGFYRRYCQILGV
Af(CI)    ALWFWMTPQSPKPSCHDVITGRWSPSSADRAAGRLSGYGTVTNIINGGLECGRGQDGRVQDRIGFYKRYCDILGV

CHT1      DTGPNLDCAHQQPY
CHT2      DTGPNLDAHTINRISR
Pn(II)    DTGPNLDCAYQKSF
Tm(II)    DPGNNLDCANQRPFG
Af(I)     GYGANLDCFSQRPFGSSLSLSSLFLNSIDT
                          Hydrophobic Extension
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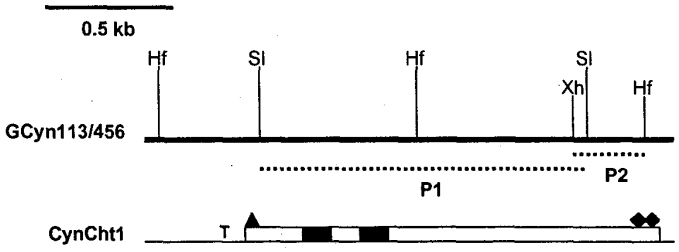
Figure 1. Amino acid sequence alignment of CHT1 and CHT2 with known class I and class II chitinases from other plant species. Pn(CII): Peanut, class II=*S65069*; Tm(CII): Tomato, class II=*S69184*; Af(CI): Alfalfa, class I= *U83591*. The putative signal peptides are in lower case letters. The highlighted sequences indicate the mismatched amino acids between *CynCht1* and *CynCht2*. The structural domains of class I chitinase not found in the class II chitinases are also indicated.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Xh
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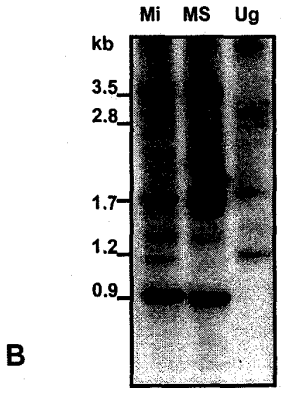
P1
P2
CynCht1
T

A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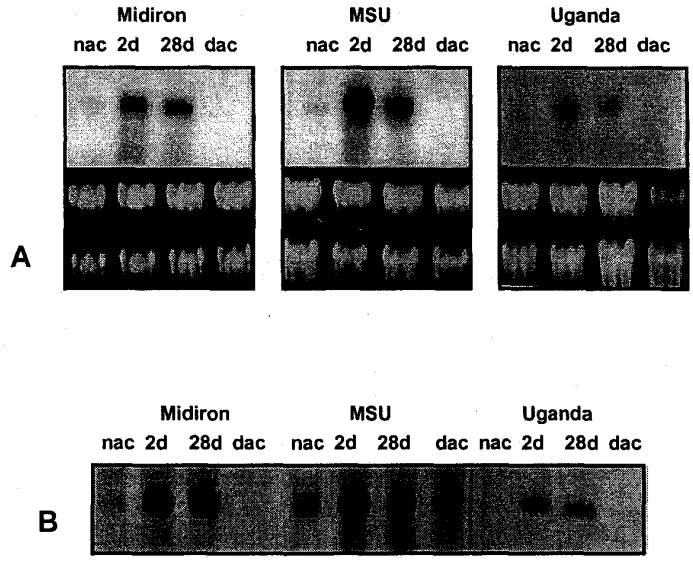
0.9

B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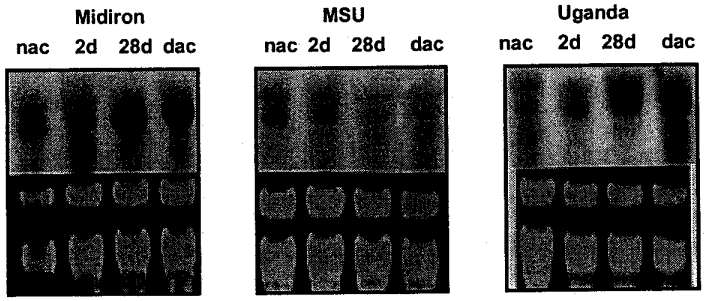
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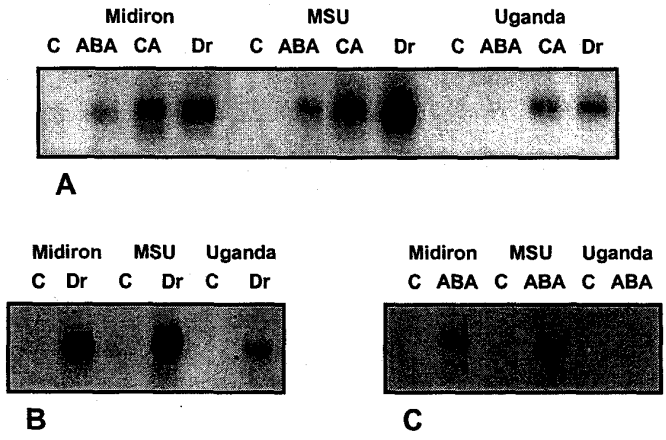
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C