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Genetic variability and molecular responses of root penetration in cotton

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Abstract

Compacted soils restrict root penetration hindering productivity. In this paper, genetic variability of cotton (*Gossipium* spp.) root capacity to penetrate hard soil layers and the patterns of gene expression during penetration event were investigated. To mimic hard soil layers, wax–petrolatum mixtures were used. Genetic variability among 27 cotton genotypes for the root capacity to penetrate wax–petrolatum disks of 500–700 g wax/kg of mixture was high indicating that breeding efforts targeted to improve this trait can be successful. In the root tips of a cotton strain with high root penetrating ability (*G. hirsutum* HS 200) which penetrated through wax–petrolatum disks (P), quantity of four polypeptides with molecular weights 35–66 kDa increased compared to those root tips which grew in the absence of mechanical impedance (NP). Differential display showed significant differences in the sets of mRNA expressed in P and NP roots. Out of a total of 917 cDNAs scored in the differential display experiment, 118 cDNAs, or 13%, were specific to P roots and hence could be associated with the root penetration event. Further detailed study of gene expression in penetrated roots will pinpoint molecular factors involved in root penetration ability in cotton.

Keywords: Cotton genetics; Root system; Mechanical impedance; Gene expression

1. Introduction

Successful cotton (*Gossypium* spp.) establishment and subsequent growth are frequently hindered by tillage or pedogenic pans characterized by high bulk densities that restrict root penetration, thus limiting the soil volume that can be explored for water and nutrients [1]. This mechanical impedance increases when soil density increases, as happens with soil compaction, and also when soil–water content decreases.

The root system plays a critical role in ensuring an adequate water flow and nutrient supply to the canopy. Root growth, however, is frequently restricted by mechanical impedance associated with soil compaction or decreasing soil water content under drought stress. Genetic variability in root characteristics, especially root morphology and root penetration through compacted layers of soil, has been observed by plant breeders and physiologists. Additional work is needed to characterize the extent of genetic variation of these root traits using a diverse collection of germplasm. This work should be followed by a detailed analysis of the genetic control and heritability of these root characteristics for the development of plant breeding strategies. However, genetic improvement of root traits such as root penetration ability is difficult

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using conventional phenotypic selection criteria because of the underground nature of the roots. Recent advances in plant molecular genetics hold great promise to overcome this difficulty in breeding for improved root characteristics in crop plants.

Knowledge of cotton root capacity to penetrate mechanically impeded layers is essential to develop better cotton genotypes. Root distribution of field-grown cotton has been documented [2]. Development of the cotton root system has been described [2–5]. Genotypic differences in cotton root penetration of compacted subsoil layers were reported [6]. Wax layers were evaluated as impedances for root growth [7,8]. Root growth decreased as impedance increased. Furthermore, Taylor and Gardner [9] reported that 'soil strength at the time the root penetration occurred — not soil bulk density — was the critical impedance factor…' Selection for root systems has been difficult for plant breeders due to the lack of reliable screening techniques [10].

In plant biology, there has been a lack of genetic and molecular studies of roots as compared with the shoots [11]. Moreover, studies of root ability to penetrate through hard soil layers are lacking. We are aware of a single report of gene expression induced by physical impedance in maize [12]. This experiment was carried out in the conditions of physical impedance induced by controlled gas pressure that increased the rigidity of the clay matrix containing roots of maize seedlings. While the molecular approach has been used to dissect gene expression specific to reproductive tissues of cotton [13], to date, the root penetration event in cotton has not been studied at the molecular level. Our objectives in this study were to investigate genetic diversity in root penetration ability of cotton and to characterize gene expression involved in the root penetration process.

We report the extent of cotton germplasm diversity for root penetration ability which is essential for future genetic investigations and tagging of root penetration trait using molecular markers. Moreover, we obtained basic information on the alteration in gene expression patterns resulting from the root penetration event in the cotton genotype HS200 which has a high root penetration ability.

2. Materials and methods

2.1. Plant material and experimental conditions for root penetration experiment

Wax–petrolatum disks of different mechanical strengths were used to simulate mechanical impedances encountered by roots in soil and hard pans [14]. For cotton genotype screening experiments, 27 cotton (*Gossypium* spp.) germplasms (Table 1, R. Cantrell's collection at New Mexico State University, Las Cruces, NM) were grown in polyvinyl chloride (PVC) tube sections of 5 cm in diameter by 5 cm in length. Four of these sections were secured together using duct tape. Between the third and fourth section, wax/petrolatum disks of 4.8 cm in diameter by 5 mm thickness were inserted. Disks were constructed from wax/petrolatum mixtures containing canning wax (Gulfwax™ Household Paraffin Wax) and purified petrolatum in differing proportions. Three mixtures containing 500, 600 and 700 g wax/kg of wax–petrolatum mixture (50, 60 and 70% wax, correspondingly) were used. Below the wax–petrolatum disk, a fifth 5-cm PVC segment was attached. A commercial grade of growing medium consisting of moss, perlite, and vermiculite (Ball Growing-on Mix) was used to fill the growing tubes.

Uniform seeds of each genotype were selected and placed into perforated plastic containers with lids which were immersed into a heated (31.5°C) aerated water bath for 24–30 h for germination. After radicle emergence, two seedlings were planted into each growth tube and covered with ~ 2 cm of growth medium. Five growth tubes representing independent replications for each density of wax/petrolatum layers were planted for each genotype and arranged in the bottom tray in the randomized complete block design. Seedlings were grown for 20 days at 22°C under 300 μmol/m^2 s PPFD with a 16-h photoperiod with regular watering. Tap roots of each seedling (one per seedling) were scored as 'penetrated', if they emerged through the wax/petrolatum layer and 'nonpenetrated', if they did not.

Due to the qualitative nature of the scoring of the penetration events, the data were analyzed with '*G*' statistics based on a log-linear model that was used to analyze a three-way contingency table (wherein 'rows' = penetrated or not penetrated, response variable; 'columns' = wax concentration;

'depths' = genotype). We tested hypotheses involving statements about independence, in particular, independence between root penetration and genotype and independence between root penetration and wax concentration [15].

2.2. Plant material and experimental conditions for molecular analysis

To obtain root material for RNA and protein analysis, seeds of cotton genotype HS200 were soaked in distilled water for an hour before sowing. Seeds were sown in pots of 20 cm in diameter containing 15 cm of growing medium on top of a 5-mm thick wax/petrolatum disk (650 g of wax/kg mix) used as a mechanical impedance. A plastic grid supported the wax disk in each bottomless pot. The pots were placed in a tray with sufficient tap water to reach up to the wax/petrolatum disk. This allowed us to visually examine roots penetrated through the wax/petrolatum layer and eliminated the danger of injuring them by removing soil. Seedlings were grown at 25°C with a 16-h photoperiod at ~ 300 μmol/m^2 per s PPFD and 30% relative humidity. After 5–6 days of seedling growth, root tips of 10 mm long which penetrated the wax/petrolatum layers were excised and immediately frozen in liquid nitrogen. These were called 'penetrated' (P) roots. Only healthy root tips from the primary tap root were used. The amount of tissue obtained was limited, as we used only the tap root tip. The secondary roots do not penetrate the wax layer but remain in the soil and grow laterally; hence they were not used. Each root weighed ~ 2 mg, and 100–200 mg of the root tip tissue was used in each experiment. 'Nonpenetrated' (NP) roots were collected from 5-day-old seedlings that were grown in similar pots without wax/petrolatum layers and hence were not subjected to a mechanical impedance.

Table 1
Gossypium germplasm evaluated for root penetrance of wax-petrolatum disks

Cultivar	Genus and species	Origin or comment	Root penetration capacity
Acala 1517-91	*G. hirsutum*	New Mexico	Good
Acala 15170SR3	*G. hirsutum*	New Mexico	Intermediate
Acala 1517-75	*G. hirsutum*	New Mexico	Intermediate
Acala B4222	*G. hirsutum*	N.M. experimental strain	Intermediate
Acala GC10	*G. hirsutum*	California	Intermediate
Acala Maxxa	*G. hirsutum*	California	Intermediate
Acala Prema	*G. hirsutum*	California	Poor
Acala Royale	*G. hirsutum*	California	Poor
CB1210	*G. hirsutum*	Texas–New Mexico F_2	Good
DPL50	*G. hirsutum*	Grown beltwide	Intermediate
DPL90	*G. hirsutum*	Grown beltwide	Poor
Georgia King	*G. hirsutum*	Georgia, Carolinas	Intermediate
McNair 220	*G. hirsutum*	Texas–New Mexico	Poor
Stoneville 132	*G. hirsutum*	Delta	Poor
Stoneville 324	*G. hirsutum*	Delta	Poor
Paymaster HS-26	*G. hirsutum*	Texas	Good
Paymaster HS-200	*G. hirsutum*	Texas	Good
Pima S-6	*G. barbadense*	Arizona	Intermediate
Pima S-7	*G. barbadense*	Arizona, California	Intermediate
MSI 1518	*G. barbadense*	Monserrat Sea Island	Good
MSI 1310	*G. barbadense*	Monserrat Sea Island	Intermediate
20812-1/20813-1	*G. barbadense*	Pima S2/MSI, F4 dwarf	Good
1468-23469X	*G.b* × *G.h.*	F_1 hybrid	Good
ASAF90040	*G.b.* × *G.h.*	Interspecific R-line	Good
ASAF92015R	*G.b.* × *G.h.*	Interspecific R-line	Good
IBMF89063	*G.b.* × *G.h.*	Interspecific R-line	Good
IV4F91048R	*G.b.* × *G.h.*	Interspecific R-line	Good

2.3. Two-dimensional gel electrophoresis of proteins

Total protein was isolated from ~100 mg of root tip tissue by standard methods described earlier by Hendershot et al. [16] from P and NP root tips and analyzed by two-dimensional denaturing polyacrylamide gel electrophoresis followed by silver staining of the gels according to [17].

2.4. RNA isolation

Total RNA was isolated from 100 to 200 mg of root tip tissue by the guanidinium hydrochloride method as described by Logemann et al. [18] with a modification. When RNA was isolated from root tip tissue according to the original procedure of Logemann et al. [18], the yield and quality of RNA were rather low, although leaf RNA yields were moderate. We modified the procedure to obtain better quality and higher yield of RNA. Polyvinylpolypyrrolidone (PVPP) at 0.01% was added to the RNA extraction buffer [19] which improved significantly the quality of RNA (absorbance ratio 260/280 = 1.6–1.8) and increased yields from 20–50 μg to 180–200 μg/g of root tip tissue.

2.5. Differential display

Differential display was carried out according to [20,21] using RNAmap kit (GenHunter, Massachusetts). In short, total RNA was treated by DNAse and used for RT-PCR. Four random primers [AP-1, AP-2, AP-11, AP-12] were used as 5′- end primers and T12MN [N = A/C/G/T] primer was used as the 3′-primer for the reactions. The reaction mixtures were run on a 6% denaturing polyacrylamide gel for 3–4 h. The gels were dried and exposed to X-ray films for 2–3 days.

3. Results and discussion

The wax/petrolatum mixtures containing 500, 600 and 700 g wax/kg of mixture were chosen to evaluate root ability to penetrate through hard layers in 27 cotton genotypes listed in Table 1. The test of the three-way interaction between penetration, wax, and genotype was not significant ($G = 65.3$, $P > 0.0713$). We concluded that the degree of association between penetration and wax was independent of genotype, or, equivalently, the degree of association between penetration and genotype was independent of wax concentration. Because the three-way interaction was not significant, we tested the two-way interactions [22]. The interaction between penetration and wax concentration was significant ($G = 76.8$, $P < 0.0142$) indicating that penetration differed between levels of wax concentration. The interaction between penetration and genotype also was significant ($G = 97.5$, $P < 0.0416$). Hence, root penetration capacity differed between genotypes. We observed that across all wax concentrations the *G. hirsutum* × *G. barbadense* crosses were better penetrators than the *G. hirsutum* and *G. barbadense* strains. Overall, *G. barbadense* strains were better penetrators than *G. hirsutum* strains. The group of genotypes rated 'good' in their root penetration ability included an F1 hybrid, all four interspacific lines, two *G. barbadense* strains (MSI 1518 and 20812-1/20813-1), and four *G. hirsutum* strains (Acala 1517-91, CB1210, Paymaster HS-26, and Paymaster HS-200). While improved root penetration ability of interspecific crosses may be a consequence of heterotic vigor, the above genotypes with high root penetration ability are good candidates for future genetic studies and for use in breeding programs as donors of high root penetration capacity.

Overall, root penetration in the set of 25 cotton genotypes was affected by wax concentration ($P < 0.0142$) and by genotype ($P < 0.0416$). However, the effect of wax concentration on penetration was independent of genotype ($P > 0.0713$). This points to significant inherent variability among cotton genotypes for the capacity to penetrate hard wax/petrolatum layers. This genetic variability is manifested at all densities of the wax/petrolatum layers. Hence, cotton breeding may result in improved root penetration through soils with high mechanical impedance. Our data also confirm the usefulness of the wax–petrolatum layer technique to quickly and reliably screen cotton genotypes for the differences in their root penetration ability in laboratory conditions.

We subjected a selected cotton genotype HS200 ('good penetrator') to molecular studies of alterations in gene expression during the root penetration event. We chose root tips, because they are the major site of activity during penetration

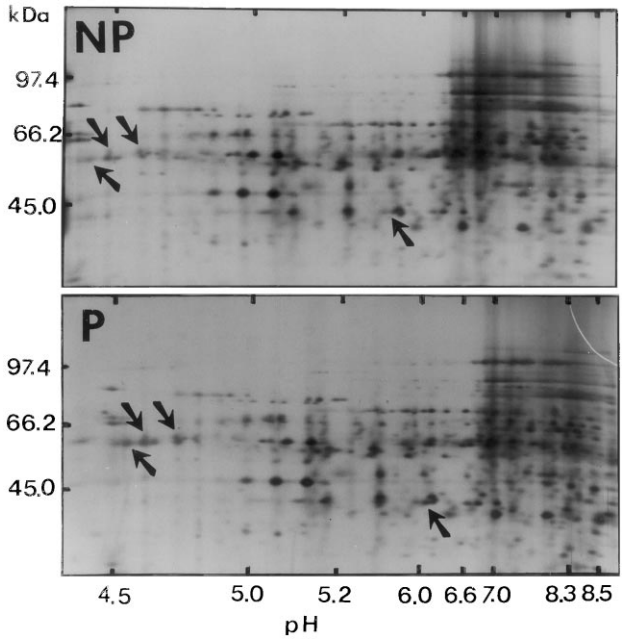

Fig. 1. Two-dimensional gel electrophoresis profiles of proteins from NP and P cotton root tips. Total protein was isolated from ~100 mg of cotton root tips that penetrated (P) and that did not penetrate (NP) through wax–petrolatum layers and analyzed by two-dimensional denaturing polyacrylamide gel electrophoresis followed by silver staining. Approximately 100 mg of total protein was loaded on each gel. Four polypeptides whose quantities were higher in P roots than in NP roots are marked with arrows.

through hard soil layers. Two approaches were used. One approach compared total protein patterns in the roots of the seedlings that were subjected to mechanical impedance and control roots not subjected to this treatment by two-dimensional gel electrophoresis followed by silver staining. In a second approach, we attempted to compare the pattern of expression of specific mRNAs in root tips subjected to mechanical impedance and without it by the differential display (DD) method.

Two-dimensional electrophoresis gels showed that a wide array of proteins is expressed in both NP and P root tips ranging in size from 14 to 98 kD. There are more proteins in the high molecular weight range between 30 and 98 kD compared with the low molecular weight range (below 30 kD). We did not observe any qualitative differences (as the appearance of a new polypeptide) between patterns of proteins from P and NP roots, but we observed four polypeptides that were present in higher quantity in P roots compared with NP roots. Three of these polypeptides had apparent molecular weights of 50–56 kD with p*I* values between 4.0 and 4.6. The fourth polypeptide of molecular weight 43 kD has a p*I* value of 5.6 (Fig. 1). Thus, relatively few differences in the protein expression and accumulation pattern could be observed between NP and P roots of cotton. This could be partly due to the level of sensitivity of the 2D-PAGE technique and silver staining. The underground nature of roots as well as the elaborate setup of the experiment precludes the use of radioactive isotope tracers for the detection of newly synthesized proteins in this system.

To circumvent this limitation, differential display (DD) was carried out. DD is a PCR-based technique for cloning 3′-ends of individual sample-specific mRNAs. The major advantages of DD include: (1) requirement of very small quantities of total RNA; (2) ability to visualize quantitative as well as qualitative differences in gene expression patterns in the side-by-side display; (3) fastness; and (4) possibility to clone and further characterize differentially expressed cDNAs fragments and clone corresponding full-length genes. After the first use for the identification of differentially expressed genes in normal and tumorogenic mammalian cell lines [20], this method is currently widely used for cloning the differences between complex mRNA mixtures in various species, including plants [21,23]. Special methods of RNA purification were required to obtain pure and intact RNA from cotton roots as described in Section 2. The total RNA was first treated with DNAse to remove genomic DNA contamination that is commonly observed in total RNA preparations. Four reverse transcription reactions each were set from penetrated (P) and nonpenetrated (NP) roots of cotton using primers T12VA, T12VC, T12VG, and T12VT (where V is A/C/G). These eight first-strand cDNA populations were then used as templates for radioactive PCRs using the same specific T12VN as 3′ primer and one of the random primers, AP-1, AP-2, or AP-12 as 5′ primers. Small aliquots from these radioactive reaction mixtures were electrophoresed on a denaturing polyacrylamide gel for 4 h. The DD patterns obtained showed distinct differences between P and NP RNA samples (Fig. 2). The number of bands that are clearly visible in each lane and their specificity to penetrated or nonpenetrated cotton roots are shown in Table 2. We considered only clearly scorable bands for our analysis. Out of a total of 917 bands, 530 bands

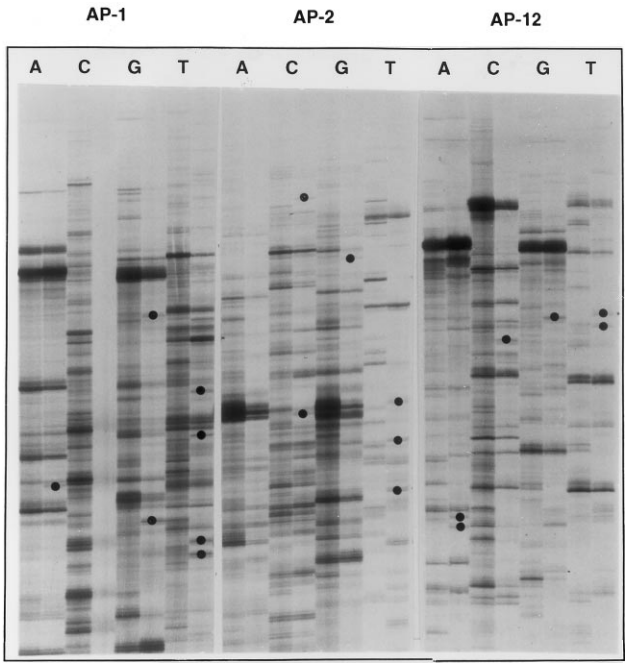

Fig. 2. Differential display patterns of the total RNA from NP and P cotton root tips using three 5′ random primers (AP-1, AP-2, AP-12) and four 3′ anchored oligo-dT primers (T_{12}VA, T_{12}VC, T_{12}VG, T_{12}VT). In each pair of samples, first corresponds to NP, and second correspond to P cotton root tips. Several prominent cDNA species specific to P roots are marked. Top portions of the sequencing gels are presented.

(58%) were common to both P and NP roots, 269 bands (29%) were specific to NP roots, and 118 bands (13%) were specific to P roots. It can be concluded, therefore, that during the penetration process, a number of mRNAs characteristic to control roots are turned off, while a set of novel mRNAs is induced. These novel mRNAs may be associated with the root penetration event.

In the low molecular weight range, several doublets or even sets of three or four bands of equal intensity were observed. They are typical of DD patterns and are the result of either visualization of both strands of one fragment or molecules with or without additional adenine base known to be added by Taq polymerase at the 3′ ends [24]. Larger cDNA fragments rarely show this anomalous pattern.

Our data indicate that DD technique allows the detection of more alterations in gene expression patterns than the two-dimensional gel electrophoresis of proteins. However, it remains unclear why observed changes in mRNA populations due to the root penetration event did not correspond to more significant changes in protein patterns. A possible explanation is a lack of sensitivity of the 2D-PAGE technique. Further work is required to determine whether any of the observed changes in gene expression in cotton root tips correlate with root penetration ability.

Recently, using differential cDNA screening strategy, two cDNA clones corresponding to mRNAs that accumulate in maize root tips subjected to a short physical impedance were isolated [12]. One of these cDNAs was highly similar to maize cDNA encoding cortical cell-delineating protein and shared some homology with other plant stress-induced cDNAs, while the second one did not have significant similarity to any other published sequence and the predicted protein possessed a bi-partite nuclear targeting signal. These findings indicate that physical impedance in plant roots may induce numerous proteins with variable functions, including transcription factors, signal

Table 2
Analysis of cDNA band patterns observed in differential display (DD) experiments between penetrated and nonpenetrated root tip tissue of cotton

	5′ Primer															
	AP-1				AP-2				AP-11				AP-12			
	3′ Primer ending nucleotide															
	A	C	G	T	A	C	G	T	A	C	G	T	A	C	G	T
Common	51	nd	44	39	40	60	44	27	30	26	33	21	31	nd[a]	60	24
NP-specific	5	nd	27	40	22	10	24	15	16	36	25	16	17	nd	6	10
P-specific	2	nd	4	15	13	5	4	20	7	6	9	17	1	nd	4	11
Total	58	nd	75	94	75	75	72	62	53	68	67	54	49	nd	70	45

[a] nd, not determined.

transduction components, cell wall components, and other proteins. Our survey of cDNA populations by differential display in this study confirms an induction of multiple new impedance-induced proteins in cotton roots and points to a need to study root penetration event in its full complexity.

Our future goals are to identify specific genes governing root penetration ability and molecular markers which are useful in the selection of cotton germplasm with a desirable root penetration ability.

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