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Direct Detection of Transcription Factors in Cotyledons during Seedling Development Using Sensitive Silicon-Substrate Photonic Crystal Protein Arrays

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Transcription factors in seedling cotyledons are directly measured using novel silicon photonic crystal protein arrays and their levels are correlated with transcript abundances
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ABSTRACT

Transcription factors control important gene networks, altering the expression of a wide variety of genes including those of agronomic importance, despite often being expressed at low levels. Detecting transcription factor proteins is difficult, as current high-throughput methods may not be sensitive enough. One-dimensional, silicon-substrate photonic crystal (PC) arrays provide an alternative substrate for printing multiplexed protein microarrays which have greater sensitivity through an increased signal-to-noise ratio of the fluorescent signal as compared to performing the same assay upon a traditional aminosilanized glass surface. As a model system to test proof of concept of the silicon-substrate PC arrays to directly detect rare proteins in crude plant extracts, we selected representatives of four different transcription factor families (zinc finger GATA, bHLH, NAC, and YABBY) which have increasing transcript levels during the stages of seedling cotyledon development. Antibodies to synthetic peptides representing the transcription factors were printed on both glass slides and silicon-substrate PC slides along with antibodies to abundant cotyledon proteins, seed lectin and Kunitz trypsin inhibitor. The silicon-substrate PC arrays proved more sensitive than those performed upon glass slides, detecting rare proteins that were below background on the glass slides. The zinc finger transcription factor was detected on the PC arrays in crude extracts of all stages of the seedling cotyledons, while YABBY appeared to be at the lower limit of their sensitivity. Interestingly, the bHLH and NAC proteins showed developmental profiles consistent with their transcript patterns indicating proof of concept for detecting these low abundance proteins in crude extracts.
INTRODUCTION

Transcription factors act as master regulators controlling the expression of suites of multiple genes. Despite this important function they are often expressed at very low levels, both as RNA and as protein, because only a small amount may be necessary to activate a cascade of other genes. This makes transcription factors difficult to study, especially at the protein level where fewer sensitive, high-throughput tools are currently available.

One-dimensional photonic crystals (PC) have been developed as an alternative surface to the aminosilanized glass slides which have been successfully used for high-throughput study of gene expression via cDNA microarrays (Jones et al., 2010). As shown in Figure 1, a precise, nanoscale grating of silicon dioxide topped with layers of highly refractive materials such as titanium dioxide allows the structure to be tuned to provide two resonance wavelengths—one at the excitation wavelength of a desired fluorescent reporter molecule, and the other at the fluorophore’s emission wavelength (reviewed in Cunningham and Zangar, 2012 and Chaudhery et al., 2013). Referred to as photonic crystal enhanced fluorescence (PCEF), this dual resonance property increases the signal-to-noise ratio (SNR) for fluorescent tags that are captured on the PC surface, allowing fluorescent molecules from the sample that attach to capture spots on the surface to be more readily distinguished from the background than they are on a regular aminosilanized glass slide. The sensitivity of the photonic crystal structure was further enhanced by the use of low-autofluorescent silicon as the bottommost layer. In this report, we refer to arrays printed on these PC devices as silicon-substrate photonic crystal arrays (PCs) while those printed on aminosilanized GAPSII glass slides are referred to for simplicity as glass slides.

Work with a cDNA array printed on a photonic crystal surface (Mathias et al., 2010) showed that the PC surface doubled or tripled the number of genes that could be detected above background compared to traditional aminosilanized glass slides. Furthermore, studies in which photonic crystal surfaces (Huang et al., 2011; George et al., 2013) were printed with antibodies related to a set of cancer biomarkers demonstrated detection of proteins at concentrations in the range of 0.3 pg/ml to 10 ng/ml.

Other types of PC structures that do not use fluorescence include biosensors in which detection is performed by measuring shifts in the PC resonant wavelength for various analytes that bind to the surface (Pal et al., 2011; Scullion et al., 2011; Chakravarty et al., 2013; Zou et al.,
2014). One recent report illustrates use of the microcavity biosensors to detect a lung cancer antigen in lysates of a recombinant lung cancer cell line in which expression of the antigen is induced (Chakravarty et al., 2013). However, neither PCEF arrays nor PC biosensors have been tested for performance to detect proteins in plant systems. Using antibodies to well-known seed proteins as controls, alongside antibodies generated to synthetic peptides representing transcription factors, we tested the silicon-substrate PCs for biological performance with plant crude extracts to detect changes in these low abundance transcription factors in cotyledons during early seedling growth.

The antibodies were printed as arrays on silicon-substrate PCs in parallel during the same printing run with traditional aminosilanized glass substrate slides in order to examine the sensitivity of each to detect changes in abundances of six proteins over seven stages of cotyledons dissected from germinating seedlings. During development of the immature seed, the cotyledons are the storage organs of the seed, filling with a large number of transcripts and proteins, some highly abundant. The seeds dehydrate as they mature and may lie quiescent for months or years before imbibition of water causes germination to begin (Bewley, 1997). The previously stored products in the cotyledons are used as food for the seedling in its early growth; but around stage 3 (approximately 3 days after germination), the cotyledons begin to turn from yellow to green and become photosynthesizing organs that produce nutrients for the seedlings, in essence serving as the first leaves. This is a complete transformation in function which occurs in just a few days.

After approximately a week, the first true leaves become functional and the cotyledons eventually senesce. This complex developmental process is certainly accompanied by many changes in gene regulation as the cellular machinery is repurposed. Global views of the gene expression changes over these seven stages of seedling cotyledon development in soybeans have been performed using both microarrays (Gonzalez and Vodkin, 2007) and RNA-Seq (Shamimuzzaman and Vodkin, 2014). Here we examine six proteins—including four transcription factors—at these same stages, pairing the protein abundance data from silicon photonic crystal slides with gene expression data starting from the very early stages of immature seed development. We demonstrate proof of concept with this sensitive technology by using it to gain a more complete understanding of the changes in low abundance transcription factors occurring during this dynamic sequence of seedling cotyledon development.
RESULTS

Selection of Four Transcription Factor Genes That Increase in Expression in the Cotyledons during Early Seedling Growth

Cotyledons were taken from seven stages of early seedling development of soybean, beginning with dry mature seeds that had been soaked in water for approximately 24 hours (Stage 1) and ending with seedlings approximately 6 to 7 cm tall above ground, fully green, with the first true leaves prominent (Stage 7) as shown in Figure 2. Unless otherwise stated, all biological samples and data were from the cultivar ‘Williams’. Seedling cotyledons from the same seven stages of this cultivar had been used to produce high-throughput next generation transcriptome sequencing data using Illumina technology to yield 46 to 75 million RNA-Seq reads per stage, which is sufficient to detect low abundance transcripts representing transcription factors (Shamimuzzaman and Vodkin, 2014). The soybean genome contains approximately 5600 gene models that are annotated as transcription factors (Jones and Vodkin, 2013). In each of the 7 stages of seedling cotyledons, approximately 1000 to 1700 transcription factors were expressed with normalized RPKM levels (reads per kilobase per million mapped reads) of ≥5, and 479 showed notable expression of at least 30 RPKM in at least one of the 7 seedling cotyledon stages (Shamimuzzaman and Vodkin, 2014). From that data set, we selected four transcription factors that increased in the cotyledons during early seedling growth, as shown in Figure 2 and Supplemental Table S1. The four transcription factors included members of the zinc finger, bHLH, NAC, and YABBY families that have been investigated in other plant systems (Bailey et al., 2003; Manfield et al., 2007; Bartholmes et al., 2012; Kang et al., 2013) using genetic and other approaches. However, their detection at the protein level has rarely been described without the use of reporter fusions or overexpression in transgenic plants. Our goal here was to directly detect these low-abundance proteins from crude plant extracts using a potentially high-throughput, highly sensitive microarray format.

Polyclonal Antibodies Generated to Synthetic Peptides Representing Epitopes of the Four Selected Transcription Factor Proteins

The four selected transcription factor proteins (bHLH037, BTF3/NAC, YABBY, zinc finger GATA) are presumably of very low abundance based on RNA-Seq data and therefore,
they are not amenable to direct purification. Thus, we chose to synthesize peptides of 15 amino acids to predicted antigenic regions for each transcription factor as shown in Table I. (See more information in Supplemental Table S2). The rabbit polyclonal antibodies were purified by affinity chromatography with the cognate peptide. For two of these transcription factors, BTF3/NAC and YABBY, the same antibodies have been shown to bind the transcription factors in chromatin immunoprecipitation experiments with the same tissue of seedling cotyledons, coupled with high-throughput sequencing to reveal the suites of genes that they regulate (Shamimuzzaman and Vodkin, 2013).

In addition, we used two polyclonal antibodies that had previously been produced against the native purified seed proteins, soybean lectin and soybean trypsin inhibitor, to serve as controls. The lectin and trypsin inhibitor antibodies were also affinity purified and have been used many times in conventional immunoprecipitation experiments and Western blots (Vodkin, 1981; Jofuku and Goldberg, 1989; Lindstrom et al., 1990) demonstrating their specificity.

**Comparison of Conventional Glass Arrays to Silicon-Substrate Photonic Crystal Arrays**

Primary antibodies for the six soybean proteins were printed on either glass slides or silicon-substrate PC devices attached to glass slides during the same printer run. Crude protein was extracted from the seedling cotyledon tissue at each stage and diluted to 25ug/ml total protein. A sandwich assay approach was used, with an equal number of glass and silicon-substrate PC slides. Figure 3 illustrates the assay in which slides were incubated first in the crude protein mixture, then in biotinylated secondary antibodies, and finally in streptavidin-Cy5, providing a reporter molecule visible when the slides were scanned. The slide images were then quantitated, yielding intensity values on a per-spot basis. A total of four slides across two biological replicates were used for each stage. Background and negative controls were subtracted from the average of all replicate spots for each protein.

Spots printed on silicon-substrate PC slides consistently generated higher fluorescence intensity values than spots on glass slides, under the same incubation and scanning conditions. Figure 4 shows fluorescence intensity of pixels on images of identical rows on both a silicon-substrate PC slide and a glass slide for the same stage. As can be seen both by eye and in the plot of values, the silicon-substrate PC slide produced a higher intensity for most protein spots, whether high abundance or low abundance. The optical properties of the silicon-substrate
photonic crystal slide allowed it to have a better signal-to-noise ratio (SNR) than the ordinary glass slide, meaning that the intensity of Cy5-labeled pixels stood out better from the background of the slide. Thus, fainter spots were distinguished on the silicon-substrate PC that were not differentiated from background on the glass slide.

Profiles of Transcription Factor Proteins Detected in Early Seedling Cotyledons on Silicon-Substrate PC versus Glass Arrays

The protein expression patterns of four transcription factors are shown in Figure 5 and direct values are detailed in Table II for each of the seven stages of seedling cotyledon development. Each value represents 16 spots (32 for the control lectin protein) across 4 slides (including two biological replicates). The four transcription factor proteins showed differing patterns across seedling cotyledon development as well as differing results from the two types of slides. Two of the transcription factors, zinc finger GATA and bHLH037, had high values at most stages on the silicon-substrate PCs though bHLH037 had lower numbers than zinc finger GATA at every stage. However, on the glass slides, the two proteins showed different profiles. Zinc finger GATA was found at every stage, but bHLH037 was not found above background at four of seven stages on the glass slides.

The other two transcription factors in this study, BTF3/NAC and YABBY, showed much lower protein abundance throughout the seedling cotyledon stages. BTF3/NAC displayed a dramatic rise in protein abundance on the silicon-substrate PCs, increasing over 500 times between Stages 3 and 7. On the glass slides, BTF3/NAC was not detected above background until the final two stages of highest abundance. In contrast, the YABBY protein showed consistently low abundance on the silicon-substrate PCs, and at two stages it was not detected above background; however, on the glass slides, it was not detected above background at any stage.

The protein abundance data for two seed storage proteins in the same tissues are shown in Table II and Figure 6. These proteins displayed very high abundance on both types of slides. On the silicon-substrate PCs, they both had values over a range of 8 to 10 fold. Thus, the abundance of these proteins was relatively stable compared to some of the transcription factors, such as BTF3/NAC, with its increase of 500 fold over time. On the glass slides, the seed proteins had similar abundance profiles, though always with smaller numbers indicative of the higher
sensitivity of the silicon-substrate PC slides. The most variation in values was introduced by the biological replicates as shown in Supplemental Figure S1; the standard error calculations for final values are shown in Supplemental Table S3.

In summary, the transcription factors and seed proteins display a variety of expression profiles, including dramatic changes in abundance across the seven stages of development. The silicon-substrate PC slides also detect low-abundance transcription factors whose values fall below background on the glass slides.

**Comparative Transcript Profiles of the Six Proteins throughout Immature Cotyledon and Seedling Cotyledon Development**

As shown in Figures 5 and 6 and Supplemental Table S1, we charted the expression of the genes representing these six proteins from RNA-Seq data derived from immature seed cotyledons (Jones and Vodkin, 2013) as well as seedling cotyledons (Shamimuzzaman and Vodkin, 2014). The RPKM values for the transcription factors tended to be fairly low, with the highest value being around 60 for BTF3/NAC. Both BTF3/NAC and YABBY had similar gene expression profiles, which peaked at the 5-6mg whole seed stage, BTF3/NAC being about twice as high as YABBY. In the seedling cotyledon stages, both genes peaked sharply at Stage 4, YABBY having slightly lower expression overall.

In the immature seed stages, bHLH037 had extremely low gene expression, with RPKMs less than 1 at all stages until a slight increase at the dry seed stage, which appeared to be part of the build-up to the peak at seedling cotyledon Stage 3. In contrast, zinc finger GATA peaked sharply at 22-24 DAF whole seed, though it also started to increase again at the dry whole seed stage, leading to a relatively high value at Stage 1 in the seedling cotyledons. After dropping to a low at Stage 5, its gene expression began to increase again.

The two storage protein genes had very similar expression profiles to each other, as expected. Both had tremendously high expression in the 100-200mg cotyledons with RPKMs around 10,000, but then declined rapidly. During the seedling cotyledon stages, gene expression for these gene models was quite low with RPKMs less than 40 at all stages.
Use of a Lectin Negative Isoline Demonstrates Lack of Non-Specific Cross Reaction in Cotyledon Extracts using the Silicon-Substrate PC Arrays

A naturally-occurring transposon insertion that abolishes lectin mRNA and protein production has previously been thoroughly described at the molecular level (Pull et al., 1978; Vodkin, 1981; Vodkin et al., 1983; Goldberg et al., 1983). This mutation has been backcrossed into the genetic background of the cultivar ‘Williams’ to create genetic isolines that are either lectin positive or lectin negative. Crude extracts from either normal or lectin-negative soybean cotyledons were incubated with the silicon-substrate PC arrays. As shown in Figure 7, lectin is detected comparably to trypsin inhibitor in the normal lectin-positive line. However, in the lectin-negative soybean line, trypsin inhibitor levels are unchanged but lectin is undetectable, indicating that there was no spurious attachment of other proteins to the lectin antibody and also demonstrating the low autofluorescence of the silicon-substrate PC arrays.

DISCUSSION

Abundant Proteins in Crude Extracts Saturate the Sensitive Silicon-Substrate PC Arrays

The carbohydrate-binding lectin and the protease inhibitor Kunitz trypsin inhibitor are both well-characterized proteins in soybean which may have roles in nutrient storage and defense (Vodkin, 1981; Jofuku and Goldberg, 1989; Van Damme et al., 2008; De Hoff et al., 2009). They are both highly abundant proteins, especially in seedling cotyledons, where previous studies have found lectin and trypsin inhibitor at approximately 2mg and 3mg, respectively, per gram of fresh weight (Pueppke and Bauer, 1978; Tan-Wilson et al., 1982) during the early stages of germination. This amount does not markedly decrease until approximately 8 days after germination (Pueppke and Bauer, 1978; Wilson et al., 1988), beyond the timeframe of our study. This pattern is consistent with the results we found of high, relatively steady levels of protein throughout all stages examined. These high protein abundances in the seedling cotyledons result from high levels of gene expression in the immature cotyledons and storage of the proteins in specialized storage vacuoles, the protein bodies.

The consistent, relatively narrow range of protein levels displayed by lectin and trypsin inhibitor may be due to a saturation effect on both types of slides, both glass and silicon-
substrate PC, whereby the proteins were so abundant neither technique could accurately quantify them past a certain point. A previous report with PCEF slides has suggested their range is from 0.3 pg/ml to 10 ng/ml (Huang et al., 2011; Cunningham and Zangar, 2012). With total seed protein at 25ug/ml in our assays, and lectin representing approximately 1% of the total extractable seed protein (Pull et al., 1978), the estimated amount of lectin in the solution is about 250 ng/ml, well over the amount needed to saturate both the glass and the silicon-substrate PC arrays. Since our goal was to detect low abundance transcription factors as shown below, the higher abundance proteins in the same crude extracts will necessarily be out of range.

**Silicon-Substrate PC Arrays Outperform Glass to Directly Detect Scarce Proteins Such as Transcription Factors in Crude Plant Extracts**

Silicon-substrate PC slides distinctly outperformed glass slides by detecting in measurable amounts the proteins that otherwise fell below background on the glass slides. For example, bHLH037 had a protein abundance pattern on the silicon-substrate PC slides similar to that of zinc finger GATA, being relatively high; however, on the glass slides, it was undetectable above background at four stages. Likewise, BTF3/NAC, which increased in abundance in the seedling cotyledons during development as determined by the silicon-substrate PC arrays, was not detected at all during the first five stages on the glass slides. YABBY was not detected on glass at all; it had such low abundance that even the silicon-substrate PCs did not detect it at two stages.

These results highlight the strength of the silicon-substrate PC arrays in detecting low but critical proteins such as transcription factors, which act as master regulators controlling the expression of suites of other genes. Such proteins are found in far too low abundance to be purified from tissue by conventional techniques, yet the timing of their appearance can have major downstream effects. A technique that allows a large number of such low abundance proteins to be measured in crude extracts of many stages and tissues would be very useful for elucidating large-scale protein changes and effects. Our results also demonstrate the practicality of using antibodies generated to synthetic peptides representing regions of the proteins.
Biological Context of the Developmental Changes in Levels of the Four Transcription Factor Proteins

Because we had both protein abundance data and gene expression data from the same seedling cotyledon stages for these proteins, as well as gene expression data from the immature seed stages immediately preceding the seedling cotyledon stages, we could examine how changes in gene expression levels might affect changes in protein levels. For many transcription factors, there are few data measuring their levels directly over developmental time. It is likely that they have high turnover since their protein causes changes in the expression of other genes, which is a tightly regulated part of the developmental program. For the transcription factors shown in Figure 5, three had single, sharp peaks in gene expression during the seedling cotyledon stages, which could be driving a sudden increase in protein abundance a stage or two later. Notably, these peaks occurred in Stages 3 or 4, when the seedling cotyledon undergoes a functional transition from yellow storage organ to green photosynthesizing tissue. The zinc finger GATA protein had a more sustained presence at both the transcript and protein levels.

Following its Transcript Pattern, bHLH037 Protein Levels Increase in Seedling Cotyledons

In the case of bHLH037, its gene expression peaked at Stage 3. This stage is the point of minimum protein abundance according to the silicon-substrate PCs; however, protein abundance peaked at the next stage, Stage 4, before decreasing. It is possible this delay indicates the time needed for the transcripts to be translated and accumulate. The timing of gene expression appeared to be sharply defined, while the protein abundance persisted at measurable levels on the silicon-substrate PCs throughout the rest of the stages studied.

The bHLH037 factor is part of the basic-helix-loop-helix family of transcription factors, which is found in a wide variety of eukaryotes, including humans, plants, and yeast (Heim et al., 2003). The bHLH037 protein examined in this study corresponds to the soybean homologue of the HECATE family, first identified in Arabidopsis (Gremski et al., 2007). This family has mainly been studied in the context of floral development in Arabidopsis (Gremski et al., 2007), as well as in shoot apical meristem tissue under short-day treatment in soybean (Wong et al., 2013). However, little additional function beyond their important role in female reproductive tissue development has been found for the HECATE genes (Feller et al., 2011). With the peak in
gene expression in the seedling cotyledons and notable protein abundance throughout seedling cotyledon development, there may be additional unknown functions for this gene family.

**The Zinc Finger GATA Protein is Found at Appreciable Levels in All Seven Seedling Cotyledon Stages**

In contrast to bHLH037, zinc finger GATA had a peak of gene expression during the immature cotyledon stage. Its expression increased again during very late seed development, carrying over into relatively high gene expression during Stage 1 of the seedling cotyledons (which are separated from the dry mature seeds only by twenty-four hours’ imbibition in water). Following this, the zinc finger GATA protein was detectable at appreciable levels in all seven stages, although there was about 10-fold variation in the levels (Table II). It is possible these results indicate that the zinc finger GATA protein is not turned over rapidly during seedling development; and that it initially accumulates to a higher level, perhaps enough to cause a saturation effect with the sensitive silicon-substrate PC slides. Some of the highest average values recorded from the silicon-substrate PC slides were found with this zinc finger GATA protein during the seedling cotyledon stages.

The zinc finger GATA protein used in this study corresponds to the *GATA transcription factor 5-like* protein from soybean. *GATA5* has been specifically noted to have low but constant gene expression in many parts of the adult *Arabidopsis* plant such as roots, stems, and leaves (Manfield et al., 2007). Another GATA gene in the same subfamily, *GATA8* or *BME3* (*Blue Micropylar End 3*), has been implicated in the initial process of seed germination (Liu et al., 2005), which would be consistent with the high values for gene expression and protein abundance we see in the early stages of the seedling cotyledons. However, the GATA family, like bHLH, is found in a number of eukaryotes, and the plant members are associated with a wide variety of processes such as light- and circadian-responsiveness, sugar and nitrogen metabolism, and flower and shoot apical meristem development (Manfield et al., 2007). The relatively high abundance of *GATA5* protein in the seedling cotyledons suggests it may have an important role to play in early seedling development.
The BTF3/NAC Protein Accumulates Significantly in Stages 6 and 7 Following Peak Transcript Abundance

Another transcription factor, BTF3/NAC, showed the dynamic range of the silicon-substrate PC slides, its protein abundance increasing dramatically from values around 1 at Stage 3 to about 650 at the final Stage 7. The RNA-Seq data peaked sharply at Stage 4, while the protein abundance began to rise noticeably in Stage 6. Similar to bHLH037, the delay may indicate the time needed to translate the gene transcripts and accumulate the protein, which likely peaks later in development than Stage 7. It is notable that the glass slides only detected this protein during the last two stages, when its abundance was highest according to the silicon-substrate PCs, demonstrating the threshold below which the glass slides are not as sensitive.

*BTF3* is part of the NAC transcription factor family, with members found in plants, animals, and yeast (Kang et al., 2013). It may be involved in directing newly-synthesized peptides to organelles such as chloroplasts and mitochondria—silencing it has been found to result in yellowed leaves, as chloroplasts become smaller with less chlorophyll and reduced gene expression (Yang et al., 2007). This finding meshes well with the results from our study in that this gene’s expression peaked at Stage 4, in the middle of the seedling cotyledon’s functional transition from yellow storage organ to green photosynthesizing organ, with the protein increasing dramatically in abundance coincident with the greening of the seedling cotyledon. Other studies have suggested *BTF3* has roles in biotic and abiotic stress response, and pollen development (Huh et al., 2012; Wang et al., 2012; Kang et al., 2013).

Recently, we elucidated the binding sites for the same members of the NAC and YABBY transcription factor families, as well as identifying some of the genes they may regulate in seedling cotyledon development, using chromatin immunoprecipitation coupled with high throughput sequencing (ChIP-Seq) (Shamimuzzaman and Vodkin, 2013). BTF3/NAC was found to have up to 72 targets it may regulate, including lipoxygenase and pectin methyl esterase inhibitor.

The YABBY Protein Appears to be Turned Over Rapidly in the Seedling Cotyledons

The YABBY protein is an interesting case, being of such low abundance that even the silicon-substrate PCs could not detect its protein above background at two stages. However, it was not detected at all on the glass slides, and thus the silicon-substrate PCs provided
information that would otherwise have been unattainable. As with BTF3/NAC, the gene expression of the two YABBY models peaked at Stage 4, before the peak in protein abundance at Stage 6. It would appear that the YABBY protein is turned over quickly by the seedling cotyledons, as its abundance dropped to undetectable at the next stage, Stage 7.

In contrast to other transcription factors studied here, the YABBY family is found only in flowering plants (Bartholmes et al., 2012). YABBY5, the protein used here, is part of the ‘vegetative YABBY’ branch which is mainly involved in various aspects of leaf development (Juarez et al., 2004; Liang et al., 2009; Bartholmes et al., 2012). It is possible that the YABBY or some of the other transcription factors discussed here are found only in sub-layers of the seedling cotyledons and not throughout the whole organ, which would dilute their concentrations in the crude extracts. Another hypothesis for the low protein abundance of YABBY is that its transcripts are not as efficiently translated as, for example, bHLH037, given that their gene expression levels are comparable but bHLH037 has much higher protein abundance. It is also possible that YABBY protein is turned over at a much higher rate than bHLH037.

Alternatively, the antibodies to the YABBY proteins may not be as effective in recognizing the protein as the antibodies to bHLH037, zinc finger GATA, and BTF3/NAC. However, this seems unlikely, as the same antibodies to the YABBY protein have been used in ChIP-Seq experiments with Stages 4 and 5 seedling cotyledons, identifying 96 genes potentially regulated by the YABBY transcription factor, including an AP2 transcription factor, fatty acid desaturase, and a WRKY transcription factor (Shamimuzzaman and Vodkin, 2013). Thus, the antibodies appear to be functional by two different experimental techniques, ChIP-Seq and direct detection of the YABBY protein on the silicon-substrate PC arrays, albeit at a level near the lower limit of the arrays.

CONCLUSIONS

Our results demonstrate the power of the silicon-substrate PC arrays to directly detect low abundance, critical proteins such as transcription factors in crude plant extracts. Direct detection of transcription factors has been very limited; generally the presence of the protein is inferred indirectly, from transcript data or detection in transgenic plants after the protein is fused
to fluorescent reporter molecules. Alternatively, the binding sites of the transcription factor may be explored by ChIP-Seq. As a model system to test proof of concept of the silicon-substrate PC arrays to directly detect rare proteins, we selected representatives of four different transcription factor families which showed increasing transcript levels during seedling cotyledon development. Our results also demonstrate the practicality of using antibodies generated to synthetic peptides representing regions of the low abundance proteins. For antibodies to both NAC and YABBY proteins, the antibodies were confirmed as functional by a second experimental technique, ChIP-Seq (Shamimuzzaman and Vodkin, 2013). Here, the zinc finger GATA transcription factor was detected at high and possibly saturating levels on the silicon-substrate PC arrays. Most interestingly, the bHLH037 and BTF3/NAC proteins showed developmental profiles consistent with their transcript patterns, indicating proof of concept for detecting these low abundance proteins in crude extracts.

METHODS

Silicon-Substrate PC Fabrication

The silicon-substrate PC is composed of a periodic surface structure fabricated in a low refractive index (RI) silicon dioxide (SiO₂) layer on a silicon substrate (see Figure 1). The grating structure is coated with a high RI titanium dioxide (TiO₂) thin film with a thickness of 130nm. The PC has a period of 360nm, a duty cycle of 36%, and a grating depth of 40nm. A commercial vendor (Novati Technologies Inc., Austin TX) was contracted for performing photolithography and reactive ion etching (RIE) of the SiO₂ grating structure over 8-inch diameter wafers, while TiO₂ thin films were deposited upon whole wafers at a second vendor (Intlvac Inc., Niagara Falls NY). Following lithography, etching, and TiO₂ deposition, the wafers were diced into 1.0x0.5 inch² pieces. These pieces were affixed to standard microscope slides with optically transparent double sided adhesives (3M).

Plant Material and Protein Extraction

Soybean seeds (Glycine max cv. Williams, maturity group III) were used. Separate lots of seed, which had been grown and harvested in different years, were used for the two biological
replicates to account for natural variation in protein abundance. Dry mature seeds were soaked in water for approximately 24 hours to obtain Stage 1 cotyledons from which the seed coats and radicles were removed. For Stages 2 to 7, seed were grown in a standard soil mix in the greenhouse for approximately 2 to 7 days and cotyledons harvested at the appropriate seedling developmental stage as described in Shamimuzzaman and Vodkin, 2013. Tissue was fresh-frozen in liquid nitrogen for 10 min then stored at -80 degrees. Total protein was extracted from ground tissue with 0.01M phosphate buffered saline, pH 7.0 (PBS, Sigma-Aldrich, St. Louis MO). Extractions generally represented 2 to 3 cotyledons from different plants. Seed of Williams (PI 548631) or the Williams lectin-negative isolate L90-8047 (PI 591534) can be requested from the USDA at GRIN (Germplasm Resources Information Network) at http://www.ars-grin.gov.

**Origin of Primary and Secondary Antibodies**

Primary antibodies for the four transcription factor proteins (bHLH037, BTF3/NAC, YABBY, and zinc finger GATA) were produced by the GenScript Corporation (Piscataway NJ). Using the sequences indicated by GenBank accession numbers in Supplemental Table S2, GenScript designed synthetic peptides for the production of polyclonal antibodies. These antibodies were purified using affinity chromatography to the synthetic peptides. For the two seed proteins, lectin and trypsin inhibitor, affinity purified primary antibodies were produced to the native whole protein as described in Vodkin, 1981.

To create secondary antibodies, primary antibodies were biotinylated using the EasyLink Biotin (type A) Conjugation Kit (Abcam, Cambridge MA).

**Printing of Arrays**

Arrays were printed using a QArray2 robot (Genetix/Molecular Devices, Sunnyvale CA) on either aminosilanized GAPSII glass slides (Corning, Corning NY) or epoxysilanized silicon-substrate photonic crystal devices mounted on glass slides. Printing was performed at 50-55% humidity and ambient temperature, and in subdued light.

The array consisted of 4 identical rows, each containing 20 spots. Of the 20 spots per row, 10 represented antibodies (0.24mg/ml to 0.973mg/ml; includes some proteins not discussed here). Each type of antibody was printed once per row, except for lectin, which was printed twice per row. Thus each antibody had 4 replicate spots per slide, except for lectin, which had 8
replicate spots per slide (see Table I). Other spots in the array included an orientation spot (0.1mg/ml; Alexa-633-labeled donkey anti-sheep IgG antibody, Molecular Probes/Life Technologies, Grand Island NY) and phosphate buffered saline spots (0.01M), which functioned as negative controls.

**Incubations**

The incubation protocol was adapted from Huang et al., 2011. Arrays were generally incubated within a week of being printed. First, slides were blocked for 1 hr in a solution of 1% casein in PBS (Bio-Rad, Hercules CA). Then slides were incubated overnight at room temperature in the dark with gentle shaking (50 rpm) in the protein extract, which was diluted to 25ug/ml total protein with 0.1% casein in PBS. Next slides were incubated overnight under the same conditions in the secondary antibody mixture, which was diluted to about 0.025 ug/ml for each antibody, using PBS with 0.05% Tween (PBS-T, Sigma-Aldrich, St. Louis MO). Finally slides were incubated for 30 min under the same conditions with streptavidin-Cy5 (SA-Cy5, GE Healthcare/Amersham, Pittsburgh PA), 1ug/ml in PBS-T. Washes in between steps were performed in PBS-T.

**Slide Scanning**

Both silicon-substrate PC and glass slides were scanned from the top in the same manner with a commercially available confocal laser microarray scanner (Tecan LS Reloaded). See Figure 1. This scanner was fitted with a 632.8 nm 5 mW laser for Cy5 excitation and a Cy5 emission filter (bandpass, 670-715nm). The incident light was TM polarized and made incident on the substrates at an angle of 5° so that maximum laser coupling efficiency could be achieved. Scans were obtained at a resolution of 10μm and the photomultiplier tube (PMT) gain was adjusted to 80 so that the largest fluorescence intensities did not saturate the PMT.

**Data Analysis**

GenePix Pro 6.1 software (Molecular Devices, Sunnyvale CA) was used to find the spots and quantitate their fluorescence intensity levels. The final value for each protein represents 16 spots across 4 slides (32 spots for lectin), with slide background and negative control values removed, and includes 2 biological replicates. More specifically, the F635 median values of
replicate spots on one slide were averaged, then the average background (B635) of all 80 spots on the slide was subtracted from this number. The average F635 median value of all 36 PBS spots on a slide (negative controls, minus background) was also subtracted from the protein spot average. The values for each protein from all replicate slides (4 glass or 4 silicon-substrate PC) were then averaged together to give the final values. Negative values (less than background and negative controls) were transformed to 0.001. The largest variation in values was introduced by the biological replicates (see Supplemental Figure S1). Standard error calculations for final values are shown in Supplemental Table S3.

**RNA-Seq Data and Gene Models**

For further details about the seedling cotyledon or immature seed RNA-Seq data, see Shamimuzzaman and Vodkin, 2014 and Jones and Vodkin, 2013, respectively. Briefly, total RNA was sequenced using the Illumina method by the Keck Center (University of Illinois, Urbana IL) and the sequence data were processed through the standard Illumina pipeline. The reads were then aligned to 78,773 Glyma1 cDNA soybean gene models (a1.v1.0) determined by the Soybean Genome Project, Department of Energy, Joint Genome Institute (Schmutz et al., 2010), using the alignment program Bowtie (Langmead et al., 2009). Data are given in RPKMs (reads per kilobase of gene model per million mapped reads; Mortazavi et al., 2008).

Seedling cotyledon RNA-Seq data represent the average of two biological replicates across the same seven stages as were used for protein extractions, described above. Immature seed RNA-Seq data represent the average of two biological replicates across seven stages of either whole seeds (4, 12-14, or 22-24 days after flowering; 5-6 mg fresh weight; dry mature seeds) or just cotyledons (fresh weight of whole green seeds either 100-200 mg or 400-500 mg). RNA-Seq data derived from Jones and Vodkin, 2013 and Shamimuzzaman and Vodkin, 2014 is shown in Supplemental Table S1.

To connect protein sequences to soybean gene models, the GenBank protein sequences listed in Supplemental Table S2 were used to search the soybean gene models at Phytozone (www.phytozone.net) using BLASTP with default parameters. The gene models with the best match (e-value and percent identity) to the proteins were selected, with a minimum of 90% identity. See Supplemental Table S2 for more information. For the BTF3/NAC, three other gene
models have high matches at the antigenic region (Shamimuzzaman and Vodkin, 2013) but the entire protein is only an 85% match so these gene models did not meet the criteria used.

SUPPLEMENTAL DATA

Supplemental Figure S1. Intensity values for six proteins from four silicon-substrate PC slides using Stage 1 or 5 seedling cotyledon tissue.

Supplemental Table S1. RNA-Seq data for gene models linked to six proteins.

Supplemental Table S2. Six soybean proteins linked to gene models.

Supplemental Table S3. Standard error of intensity values for six proteins.

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FIGURE LEGENDS

Figure 1. Schematics of the photonic crystal structure and detection instrument. The photonic crystal device (A) is comprised of a periodic surface structure fabricated in a low refractive index silicon dioxide (SiO₂) layer on a silicon substrate and then overcoated with a thin film of high refractive index TiO₂. The device is further coated with a layer of epoxysilane and the protein array is printed on top. The detection instrument (B) is a Tecan LS Reloaded confocal laser microarray scanner fitted with a 632.8nm 5mW laser for Cy5 excitation and a Cy5 emission filter (bandpass, 670-715nm). See Methods for more details.

Figure 2. Seven stages of soybean cotyledon development and transcription factor expression. Stage 1 is after 24 hours’ imbibition of dry mature seed. Stage 7 is approximately 7 days after planting. During Stages 3, 4, and 5 the cotyledons begin to turn green and undergo a functional transition from a storage organ to a photosynthesizing organ. Stage numbers are indicated above each image. RNA-Seq data for transcription factor gene models at each stage is included at bottom (YABBY is represented by 2 gene models). See Supplemental Table S1 for the full RNA-Seq data and gene model numbers.

Figure 3. Illustration of the sandwich assay as described in the text. Primary antibodies were printed onto silicon-substrate PC or glass slides. Slides were then sequentially incubated with crude protein extract, biotinylated secondary antibodies, and streptavidin-Cy5. Cy5 is the reporter molecule visible to laser scanners.

Figure 4. Fluorescence intensity of spots on silicon-substrate PC and glass slides. Top, identical rows from a silicon-substrate PC (SiPC) slide and a glass slide. Red dotted line indicates spots with intensity plotted in the graph below. Spots to the left of red dotted line are orientation spot and negative control spot, respectively. Below, blue: silicon-substrate PC slide intensity values. Red: Glass slide intensity values. Slides were incubated with the same tissue extract and scanned under identical conditions.

Figure 5. Developmental gene expression and protein abundance data for four transcription factors. RNA-Seq data (left) in RPKMs on the Y axis show gene expression at seven stages of
immature seeds (A-G) and seven stages of seedling cotyledons (1-7). Multiple lines indicate data from multiple gene models. Protein array data (right) show protein abundance at seven stages of seedling cotyledons for both silicon-substrate PC slides (blue) and glass slides (red). A data point of 0.001 indicates the protein was undetected at that stage. All protein data is shown on a log scale. DAF, days after flowering. Stages on the X axis are (A) 4 DAF; (B) 12-14 DAF; (C) 22-24 DAF; (D) 5-6mg whole seeds; (E) 100-200mg cotyledon; (F) 400-500mg cotyledon; (G) dry mature seeds. Numbers 1-7, seedling cotyledon stages as described in Fig. 2 and the text.

Figure 6. Developmental gene expression and protein abundance data for two storage proteins. All graphs are log scale. RNA-Seq data (left) in RPKMs on the Y axis show gene expression at seven stages of immature seeds (A-G) and seven stages of seedling cotyledons (1-7). Multiple lines indicate data from multiple gene models. Protein array data (right) show protein abundance at seven stages of seedling cotyledons for both silicon-substrate PC slides (SiPC, blue) and glass slides (red). A data point of 0.001 indicates the protein was undetected at that stage. All protein data is shown on a log scale. DAF, days after flowering. Stages on the X axis are (A) 4 DAF; (B) 12-14 DAF; (C) 22-24 DAF; (D) 5-6mg whole seeds; (E) 100-200mg cotyledon; (F) 400-500mg cotyledon; (G) dry mature seeds. Numbers 1-7, seedling cotyledon stages as described in Fig. 2 and the text.

Figure 7. Differential detection on silicon-substrate PC slides of trypsin inhibitor and lectin proteins in lectin positive and lectin negative isolines. Histogram showing relative fluorescence levels representing either lectin (red) or trypsin inhibitor (blue) in dry seed from soybean lines with normal lectin levels (Lec POS) or no lectin (Lec NEG). Two slides (technical replicates) are shown for each soybean line. Lectin is the average of 8 spots per slide, trypsin inhibitor 4 spots; average slide background and negative controls were subtracted out and zero and negative values transformed to 0.001.
Table I. Antibodies printed on arrays used to detect transcription factors and control seed proteins

Antigen sequences, concentrations, number of spots printed per slide, and other information shown. Soybean gene model names here are identical between genome versions Gm109 a1.v1.0 and Gm189 a1.v1.1 unless otherwise noted. The sequences from Gm189 a1.v1.1 were used to determine the amino acid position numbers. All matches between peptide sequence and gene model were 100%. All antibodies are polyclonal rabbit and affinity purified.

| Antibody     | Peptide Sequence (Antigen) | Soybean Gene Model | Amino Acid Position | Conc (mg/ml) | Spots/Slide | Reference                     |
|--------------|----------------------------|--------------------|---------------------|--------------|-------------|-------------------------------|
| Zinc finger GATA\(^a\) | SQSQDRGEDDSN SNC          | Glyma06g01110.1    | 73-86               | 0.404        | 4           | This report                   |
| bHLH037\(^a\) | DPESIKPPKRRNV KC          | Glyma17g19830.1    | 140-153             | 0.810        | 4           | This report                   |
| BTF3/NAC\(^a\) | VRTGGKGMRR KKKC          | Glyma01g18780.1    | 13-26               | 0.376        | 4           | Shamimuzzaman and Vodkin 2013 |
| YABBY\(^a\)  | CTEERVVNRPPE KRQ          | Glyma13g22620.1    | 99-112              | 0.622        | 4           | Shamimuzzaman and Vodkin 2013 |
| Lectin       | Whole protein             | Glyma02g01590.1    | N/A                 | 0.880        | 8           | Vodkin 1981                   |
| Trypsin inhibitor | Whole protein          | Glyma01g10900.1 Glyma08g45530.1\(^b\) | N/A | 0.340 | 4 | Vodkin 1981 |

\(^a\)From synthetic peptides produced by GenScript Corporation. A C residue in the first or last position (C-terminus or N-terminus) of the antigen sequence was added to facilitate conjugation. \(^b\)Glyma08g45531.1 in a1.v1.1 of soybean genome.
**Table II.** Protein array data over seven stages of seedling cotyledon development on glass and silicon-substrate PC slides

Average intensity of 16 measurements (32 for lectin) per protein from four slides, minus background and negative controls. See Methods for details of calculation. Negative values transformed to 0.001.

| Antibody       | Slide Type | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 | Stage 6 | Stage 7 |
|----------------|------------|---------|---------|---------|---------|---------|---------|---------|
| Zinc finger GATA | PC         | 583.3   | 2555.1  | 250.4   | 2878.5  | 891.9   | 2821.5  | 396.2   |
| bHLH037        | PC         | 146.8   | 747.7   | 37.1    | 1589.5  | 568.6   | 791.2   | 337.1   |
| BTF3/NAC       | PC         | 5.3     | 19.9    | 1.3     | 34.3    | 20.5    | 79.1    | 647.5   |
| YABBY          | PC         | 15.2    | 13.6    | 0.4     | 0.001   | 7.6     | 25.6    | 0.001   |
| Lectin         | PC         | 232.2   | 762.4   | 151.1   | 782.5   | 931.6   | 1580.3  | 643.8   |
| Trypsin inhibitor | PC         | 288.6   | 2345.6  | 346.0   | 1598.4  | 665.4   | 267.0   | 866.7   |
| Zinc finger GATA | Glass     | 32.9    | 237.8   | 97.4    | 138.9   | 103.1   | 104.2   | 214.9   |
| bHLH037        | Glass     | 0.001   | 36.6    | 6.1     | 6.7     | 0.001   | 0.001   | 0.001   |
| BTF3/NAC       | Glass     | 0.001   | 0.001   | 0.001   | 0.001   | 0.001   | 5.2     | 53.9    |
| YABBY          | Glass     | 0.001   | 0.001   | 0.001   | 0.001   | 0.001   | 0.001   | 0.001   |
| Lectin         | Glass     | 87.7    | 99.9    | 33.7    | 203.1   | 418.1   | 294.1   | 144.5   |
| Trypsin inhibitor | Glass   | 139.1   | 181.4   | 18.5    | 300.8   | 234.7   | 339.6   | 1622.8  |
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