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A Molecular Diagnostic for Phosphorus Deficiency in Potatoes

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Introduction

Phosphorus (P) is an essential element for plant growth and fecundity (White & Hammond 2008). Crop plants require P in large amounts, and acquire it from the rhizosphere solution as phosphate (Pi). Because of the low solubility product of Pi salts, Pi concentrations in soil solutions are extremely low (2-10 μM), which restricts the diffusion and mass flow of P to the rhizosphere (White & Hammond 2008). For this reason, Pi-fertilisers are applied to young crops to support their P demands. The use of Pi-fertilisers can incur both financial and environmental costs. For example, in the UK the volatile price of inorganic Pi-fertilisers fluctuated over four-fold between 2007 and early 2009 (Farmers Weekly, UK), and it is estimated that 11,800 tonnes (19.5%) of the P reaching the surface waters of Great Britain (GB) arises from agriculture (White & Hammond 2009). In addition, commercially viable reserves of phosphate rock for the production of Pi-fertilisers could be exhausted within the next 100 to 400 years at current rates of consumption (Johnston 2008).

Potatoes have a high demand for P fertilisers. For example, this crop occupied about 3.1% of the cultivated arable land in Great Britain in 2004, but consumed about 9.4% of the inorganic Pi-fertiliser applied to all arable crops (White et al. 2007). Several agronomic strategies have been suggested to reduce this Pi-fertiliser burden. These include Pi-fertiliser placement and the optimization of the amounts and timing of Pi-fertiliser applications through informed recommendations (Heathwaite et al. 2003, Gregory & George 2005, White et al. 2005, 2007, Bryson 2005, Zhang et al. 2007, Hammond & White 2008a). Recommendations for Pi-fertiliser applications are often based on analyses of soil solution and/or plant P concentrations, and/or the occurrence of visible P-deficiency symptoms in crops (Hammond & White 2008a). However, these methods are subject to methodological vagaries and cannot rectify production losses due to P-deficiency during the growing season. This paper describes a novel method to monitor the physiological status of a potato crop in the field, which could allow remedial Pi-fertilization to prevent losses of yield due to P-deficiency. The method is based on global gene expression (transcriptome) patterns in diagnostic leaves.

Materials and Methods

Glasshouse Experiment

Potato (*Solanum tuberosum* L. var. Kennebec) micro-plants obtained from Higgins Agriculture (Doncaster, UK) were grown hydroponically in a glasshouse at Wellesbourne, UK, using the NFT system described by Broadley et al. (2004). The experiment was performed between May 2003 and June 2004. The glasshouse was set to maintain temperatures of 20°C by day and 15°C at night using automatic vents and supplementary heating. The NFT system comprised 12 individual gullies (Fig. 1B). Each gully was connected to one of two tanks each holding 200 L of a nutrient solution containing 2 mM $\text{Ca}(\text{NO}_3)_2$, 2 mM NH_4NO_3 , 0.75 mM MgSO_4 , 0.5 mM KOH, 0.25 mM KH_2PO_4 , 0.1 mM FeNaEDTA, 30 μM H_3BO_3 , 25 μM CaCl_2 , 10 μM MnSO_4 , 3 μM CuSO_4 , 1 μM ZnSO_4 , 0.5 μM Na_2MoO_4 . Nutrient solutions were adjusted daily to pH 6 using H_2SO_4 , and solutions were replaced completely twice a week. Once plants were established, P was removed from the nutrient solution supplying half the plants by replacing the KH_2PO_4 with 0.125 mM K_2SO_4 (P-starved plants), whilst the other plants continued to receive complete nutrient solution (P-replete plants). This timepoint was defined as day 0. Three plants from each treatment were harvested periodically over the following 28 days and for 7 days following the resupply of P to P-starved plants on day 28. Shoot biomass and leaf P-concentrations were determined on all plants harvested. Diagnostic leaves, defined as the second

fully expanded leaf below the crown of the plant (Ulrich 1993), were taken from plants harvested on days 0, 1, 3, 7, 17, 29 and 31 and snap frozen in liquid nitrogen for subsequent analyses.

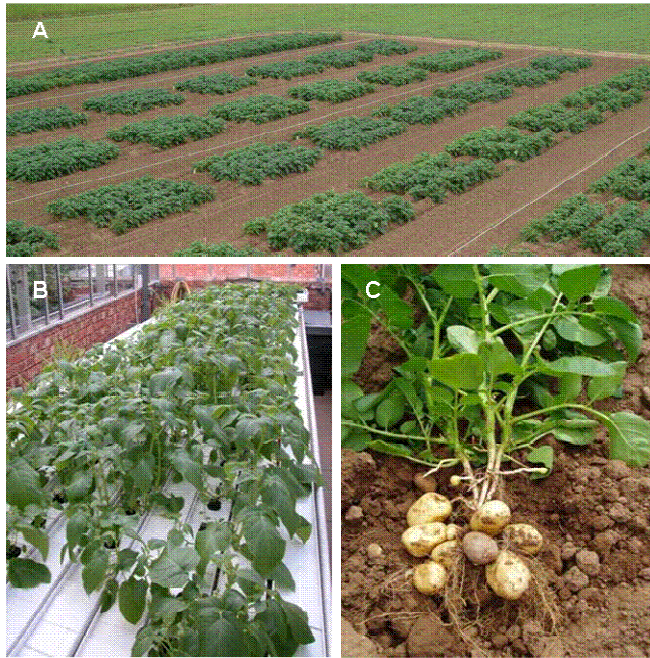


Figure 1. (A) Potato plants growing in the field at Wellesbourne, UK. (B) Potato plants growing hydroponically in a glasshouse at HRI-Wellesbourne in the NFT system described by Broadley et al. (2004). (C) Young potato plant excavated from a field plot.

Field Experiment

To test the diagnostic transcriptome, samples were obtained from diagnostic leaves of Kennebec potatoes grown on a field site of low P-status at Wharf Ground, Wellesbourne (Fig. 1A). Seed potatoes were planted on the 13th April 2005. Field plots had received 185 kg ha⁻¹ N, supplied as NH₄NO₃, 325 kg ha⁻¹ K₂O, supplied as K₂SO₄, and either 0 or 180 kg ha⁻¹ P₂O₅, supplied as triple super-phosphate (21% P, TSP), prior to planting. Potatoes were cultivated according to best agronomic practice and treated with irrigation and pesticides when appropriate. Diagnostic leaves were sampled at midday on 7th July 2005. Three biological replicates, each constituting diagnostic leaves from three plants sampled at random, were snap frozen in liquid nitrogen in the field.

Determination of Leaf P Concentrations

The fresh weight (FW) of diagnostic leaves was recorded immediately after harvest. Leaf dry matter (DM) was determined after oven-drying at 80°C for 72 h. Leaf-P was determined by inductively coupled plasma emission spectrometry (JY Ultima 2, Jobin Yvon Ltd, Middlesex, UK) following digestion of a 100 mg DM subsample as described by Bradstreet (1965).

Assay for Gene Expression in Diagnostic Leaves

To identify genes that respond specifically and rapidly to P deficiency, total RNA was extracted from diagnostic leaves of potato plants of contrasting P-status grown hydroponically in the glasshouse. Predictive genes identified using these samples were then tested using total RNA obtained from diagnostic leaves of plants growing in the field. Total RNA was extracted according to Hammond et al. (2006). Total RNA from all samples was labelled and hybridised to the Potato Oligo Chip Initiative (POCI) oligonucleotide array, representing 42,034 potato sequences (Kloosterman et al. 2008). Labelled cRNA was generated from RNA using the Low

RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Palo Alto, CA, USA). Dye incorporation for labelled cRNA was 17.02 ± 0.43 pmol dye μg^{-1} cRNA (mean \pm SE). Hybridisation cocktails were prepared using the *In situ* Hybridisation Kit (Agilent Technologies) and cocktails contained between 0.5 and 3 μg labelled cRNA per sample. Cocktails were hybridised to microarrays rotating at 10 rpm in a hybridisation oven (Agilent Technologies) at 65°C for 17 hours. Following hybridisation, microarrays were washed according to manufacturer's instructions. Microarrays were scanned on an Agilent DNA Microarray Scanner BA using the Extended Dynamic Range function and data were extracted from the scanned images using the Feature Extraction software package. For further analysis 'processed' signal values were used. Microarray scans were checked for quality using data from the Feature Extraction software and distribution of data in GeneSpring GX analysis software.

Identification of Discriminatory Genes

The processed signal values were imported into GeneSpring GX (Agilent Technologies). Data from individual microarrays were subjected to a Lowess normalisation and the signal value for each gene was divided by the median of its measurements in all samples for an experiment. Data were pre-filtered by (1) removing genes whose raw signal value was less than 50 in five of the seven time points; (2) removing genes flagged as absent; and (3) removing genes whose normalised signal value remained between 0.8 and 1.2 at all time points to leave 28,946 genes for further analysis. To identify genes that were significantly differentially expressed between treatments an ANOVA with a Benjamini & Hochberg FDR multiple testing correction was used. Gene Ontology terms assigned to genes were analysed using the Gene Ontology Browser in GeneSpring GX. For class prediction, the support vector machine implemented in the Class Prediction tool of GeneSpring GX was used to classify the data. Sets of diagnostic genes were selected using the Golub method with the Polynomial Dot Product (Order 1) kernel function. Different kernel functions and sets of diagnostic genes were changed systematically to optimise the classification of samples.

Results

The shoot dry weight of P-replete plants growing hydroponically in the glasshouse increased regularly over the experimental period (Fig. 2 legend). A significant reduction in shoot dry weight ($P < 0.05$) was observed in P-starved plants 15 days after removing P from the nutrient solution, and this was not recovered by subsequently resupplying P (Fig. 2A). The P concentration in diagnostic leaves of P-replete plants was 8.5 ± 0.14 mg g^{-1} DM (mean \pm SE, $n = 16$ timepoints), which is above that considered sufficient for maximal growth of potato plants (1.5-2.5 mg P g^{-1} DM; White et al. 2007, 2009). A significant ($P < 0.05$) reduction in the P concentration of diagnostic leaves of P-starved plants was observed within one day of removing P from the nutrient solution and was restored to concentrations significantly ($P < 0.05$) greater than those found in leaves of P-replete plants within 7 days of resupplying P (Fig. 2B).

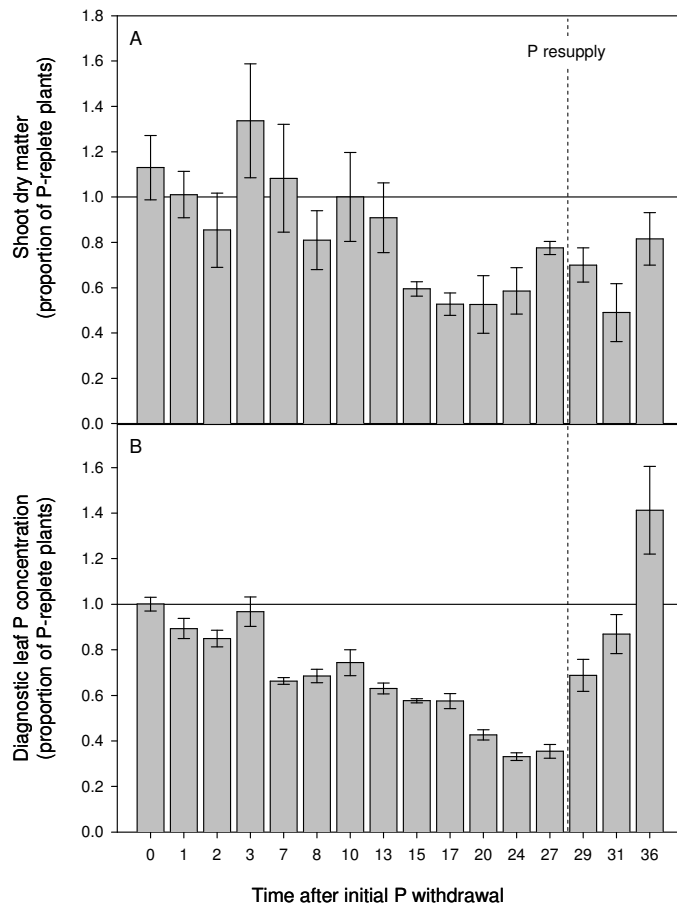


Figure 2. Response of (A) shoot biomass and (B) shoot P concentration of young potato plants subject to P-starvation and P-resupply in hydroponics. Values are expressed as the quotient of P-starved/P-replete plants. Shoot biomass (y) accumulated with time (t) in P-replete plants according to the equation $y = -6.75 + 7.58e^{0.04t}$. The shoot P concentration for P-replete plants was $8.5 \pm 0.14 \text{ mg g}^{-1} \text{ DM}$ (mean \pm SE, $n = 16$ timepoints).

A total of 1,659 genes were significantly ($P < 0.01$) differentially expressed in diagnostic leaves of P-replete and P-starved plants in at least two of the seven time points assayed during the experimental period. Many of these were characteristic of the acclimation of leaf metabolism to P-starvation and included genes involved in re-routing carbon metabolism to reduce the demand for phosphorylated metabolites, genes encoding enzymes involved in alternative lipid metabolism to reduce the P-demand of cellular membranes, and genes encoding ribonucleases and cellular phosphatases that release P from RNA and vacuolar sources during P starvation (Hammond et al. 2003, 2005, Amtmann et al. 2006, Hammond & White 2008b, White & Hammond 2008).

A support vector machine algorithm was used to define 200 genes whose expression in diagnostic leaves allowed the most accurate discrimination between P-replete and P-starved plants grown hydroponically in the glasshouse. Table 1 lists the twenty genes whose expression in diagnostic leaves discriminates with the greatest power between P-replete and P-deficient potato plants growing hydroponically in the glasshouse.

Table 1. Identity of the twenty genes whose expression in diagnostic leaves discriminates with the greatest power between P-replete and P-deficient potato plants growing hydroponically in the glasshouse. na = not available

Microarray Probe ID	Predictive power	Genbank ID	Description	StGI ID
STMJA76TV	0.555	ABE82661	Tyrosine protein kinase	BQ516953
STMEU15TV	0.724	NP_565730	poly(ADP-ribose) glycohydrolase	BQ120909
PotatoF0996.scf	0.579	na	-	TC152755
POAC664TV	0.549	NP_191747	Cytochrome P450	TC154689
MICRO.7275.C1	0.518	P21568	Peptidyl-prolyl cis-trans isomerase	TC138212
MICRO.712.C31	0.567	AAN76811	PEP carboxylase kinase	TC134483
MICRO.6284.C1	0.545	NP_195300	unknown protein	TC136718
MICRO.5327.C4	0.530	NP_849715	unknown protein	TC139374
MICRO.4091.C1	0.498	AAF61863	DNA-binding protein 3	TC151567
MICRO.3180.C2	0.575	EAY76147	hypothetical protein	TC158148
MICRO.14933.C4	0.478	na	-	CV499811
MICRO.14151.C1	0.540	ABL61268	putative zinc transporter	TC133838
MICRO.13119.C1	0.543	ABE89190	Protein kinase	TC154081
MICRO.12238.C1	0.803	NP_177557	protein binding	TC160980
MICRO.11800.C1	0.589	NP_191964	MGDG2	TC142634
BPLI5I11TH	0.495	AAX83943	Sgt1b	BG590437
bf_mxlfxxx_0065f11	0.479	na	-	TC152755
bf_mxflxxx_0027g02	0.495	AAT38751	RNA-directed RNA polymerase	TC150928
bf_ivrootxx_0061c03	0.547	NP_566159	glycerophosphodiester phosphodiesterase	CO502533
bf_arrayxxx_0037e12	0.552	ABO81782	Protein kinase	DR034267

As an independent test of the diagnostic transcriptome, diagnostic leaves were sampled from plants growing in the field at HRI-Wellesbourne. An analysis of gene expression in these leaves correctly identified that this potato crop was P-deficient. Thus, the diagnostic transcriptome can identify P-deficiency in field-grown potato crops and, therefore, has potential as a viable management tool for precision agriculture. It will allow farmers to monitor the immediate physiological P status of their crops and, thereby, optimise Pi-fertiliser applications and reduce P losses to the environment and commercial losses due to P-deficiency.

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