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Research

The remarkable morphological diversity of leaf shape in sweet potato (*Ipomoea batatas*): the influence of genetics, environment, and G×E

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Summary

- Leaf shape, a spectacularly diverse plant trait, varies across taxonomic levels, geography and in response to environmental differences. However, comprehensive intraspecific analyses of leaf shape variation across variable environments is surprisingly absent. Here, we performed a multilevel analysis of leaf shape using diverse accessions of sweet potato (*Ipomoea batatas*), and uncovered the role of genetics, environment, and G×E on this important trait.
- We examined leaf shape using a variety of morphometric analyses, and complement this with a transcriptomic survey to identify gene expression changes associated with shape variation. Additionally, we examined the role of genetics and environment on leaf shape by performing field studies in two geographically separate common gardens.
- We showed that extensive leaf shape variation exists within *I. batatas*, and identified promising candidate genes associated with this variation. Interestingly, when considering traditional measures, we found that genetic factors are largely responsible for most of leaf shape variation, but that the environment is highly influential when using more quantitative measures *via* leaf outlines.
- This extensive and multilevel examination of leaf shape shows an important role of genetics underlying a potentially important agronomic trait, and highlights that the environment can be a strong influence when using more quantitative measures of leaf shape.

Introduction

Leaf shape varies spectacularly among plant species at multiple taxonomic levels (Klein *et al.*, 2017; Shi *et al.*, 2019), across geography (Wyatt & Antonovics, 1981; Gurevitch, 1988) and in response to environmental differences (Andersson, 1991; Jones, 1995; McDonald *et al.*, 2003). Leaves can vary with respect to their degree of dissection, length-to-width ratio, venation patterning, prominence of tips and petiolar sinus, or any combinations of the above, meaning that leaf shape variation across species is multifaceted and complex. Leaf shape diversity is also present within species (Hilu, 1983). For example, accessions of grapevine and cotton vary with respect to leaf complexity, whereas lineages within tomato and apple show ample variation in the length-to-width ratio of leaves (Chitwood *et al.*, 2013; Andres *et al.*, 2016; Klein *et al.*, 2017; Migicovsky *et al.*, 2017). Although a large number of species exhibit variation in leaf shape, examinations within species are often limited to only a few accessions, with a few notable exceptions (Conesa *et al.*, 2012; Chitwood *et al.*, 2014a,b). Moreover, these studies often focus on circularity and length-to-width ratio, which are the most common leaf shape descriptors. Therefore, for most species, truly quantitative analyses of the diversity of leaf shape variation within species remain largely unexamined.

Leaf shape variation is regulated by genetics, the environment and the interaction of genes and environment (G×E). Although the genetic and transcriptomic basis underlying leaf shape diversity has been uncovered in only a small number of species (i.e. tomato, Arabidopsis, cotton and a few others; Kim *et al.*, 2002; Kimura *et al.*, 2008; Ichihashi *et al.*, 2014; Vlad *et al.*, 2014; Andres *et al.*, 2016; Chitwood & Sinha, 2016), there are many examples showing the influence of different environments on leaf shape (McDonald *et al.*, 2003; Zwieniecki *et al.*, 2004; Hopkins *et al.*, 2008; Royer *et al.*, 2009; Nicotra *et al.*, 2011; Royer, 2012; Campitelli & Stinchcombe, 2013; Glennon & Cron, 2015). For example, submerged leaves of aquatic plants are often highly dissected as compared to their aerial counterparts (Arber, 2010) and leaves growing in colder environments tend to be more complex than similar ones growing in warmer environments (Huff *et al.*, 2003; Royer *et al.*, 2005). Moreover, the environment can interact with genes to further modulate leaf shape. For instance, Nakayama *et al.* (2014) found that changes in temperature lead to abrupt changes in *KNOTTED1-LIKE HOMOEOBOX1* (KNOX1) activity, a key regulator of circularity in multiple

species, thus altering leaf complexity. Although we are beginning to understand how genetics, environment, and G×E separately influence aspects of leaf shape, few studies have partitioned the effect of genetics vs the environment on leaf shape variation, and most examinations are limited to only one environment, such that the role of G×E on leaf shape is often not considered within species.

Leaf shape is most commonly quantified using the 'traditional' leaf shape traits – circularity (a measure of leaf dissection, or 'lobedness'), aspect ratio (the length-to-width ratio of a leaf) and solidity (the relation of the area and convex hull). These traditional morphometric parameters have previously been used to quantity leaf shape in diverse species, such as grapes (Chitwood *et al.*, 2014b), tomato (Chitwood *et al.*, 2015) and sweet potato (Rosero *et al.*, 2019), among others. Although these traits are linked to important yield traits in crops (Chitwood *et al.*, 2013; Vuolo *et al.*, 2016; Chitwood & Otoni, 2017; Klein *et al.*, 2017; Rowland *et al.*, 2019), and are important for understanding the broader aspects of plant adaptation to environment, they capture only a few components of leaf shape variation. A more comprehensive quantification of leaf shape can be captured with EFD analyses, which converts leaf outlines to harmonic coefficients allowing for Fourier analyses (Chitwood & Sinha, 2016). This approach captures extensive leaf shape variation due to both symmetry and asymmetry of the leaf; some examples include shape differences associated with the depth of the petiolar sinus, the prominence of the leaf tip, and the positioning of the lobes. This approach has been applied to a handful of species like tomatoes, passiflora, and grape (Chitwood *et al.*, 2013; Chitwood & Otoni, 2017; Klein *et al.*, 2017), where it was shown that leaf shape based on EFD analysis is highly heritable. Therefore, traditional measures along with consideration of leaf outlines holds greater power to comprehensively measure and characterise leaf shape, which may yield important insights about the genetic basis of leaf shape variation. Interestingly, while leaf shape based on EFD analysis is heritable, no studies have yet examined the genetic or transcriptomic basis of leaf shape based on leaf outlines.

Ipomoea batatas, the sweet potato, is an important staple root crop worldwide (Khoury *et al.*, 2015) as it produces the highest amount of edible energy per hectare (Khoury *et al.*, 2015) and also provides an important source of nutrients in the form of vitamin A, calcium, and iron (Kays & Kays, 1998). Sweet potato displays striking morphological variation in leaf shape across its *c.* 6000 documented varieties (Huaman, 1987), but very few studies have examined the extensive leaf shape diversity in this species (Huaman, 1987; Hue *et al.*, 2012; Rosero *et al.*, 2019). Studies that have examined leaf shape phenotypes in sweet potato are limited to a few cultivars and/or present traditional measures of leaf shape traits. Additionally, the genetic or transcriptomic basis of leaf shape variation in this species has yet to be considered. The vast unexamined diversity of leaf shape in this species, along with its role as a staple food crop worldwide makes *I. batatas* an ideal study system to investigate leaf shape diversity at the species level and how this diversity is influenced by the interplay between genetics and environment.

Here, we examine the extensive leaf shape variation within accessions of *I. batatas*, and uncover the role of genetics, environment, and G×E in influencing leaf shape traits. We specifically ask: (1) How diverse is leaf shape at a species-wide level? (2) What are the candidate genes associated with leaf shape (extending beyond the traditional shape descriptors)? and (3) To what degree does the environment and G×E influence leaf shape traits? We show that extensive natural variation exists in leaf shape within this species and that most of this variation is largely controlled by genetic factors, with a low proportion of variance in leaf shape attributable to environmental differences. We also identified promising candidate genes associated with the broad differences in multiple leaf shape traits. The results of our work fill critical gaps in current knowledge of leaf shape evolution by expanding analysis beyond that of the traditional measures of leaf shape and by using many distinct lineages of the species. We unite this with transcriptomics of these traits along with a multiple-environment assessment of leaf shape variation in the field. Therefore, this work allows us to comprehensively assess leaf shape in this agronomically important species and partition the role of genetics, environment, and G×E on leaf shape within this species.

Materials and Methods

Leaf shape variation within *I. batatas*

We ordered vegetative slips for 68 publicly available accessions of sweet potato from USDA and online resources. The location of the origin of 68 accessions is represented in Fig. 1 (Supporting Information Table S1). The accessions represent the majority of the genetic variation in the species; we identified three of the four population structure clusters among our chosen accessions as per a recent study (Wadl *et al.*, 2018). We grew slips at the UM Matthaei Botanical Garden under standardised growth conditions (16 h : 8 h, light : night cycle) for *c.* 6 months, at which time we sampled four to six mature leaves (third to sixth mature leaves from the beginning of the vine to control for age and exposure to light) of 57 randomly chosen accessions and scanned them for leaf shape analyses.

We used the scanned images to extract leaf shape trait values using custom macros in ImageJ (Abràmoff *et al.*, 2004). Briefly, we converted leaves into binary images and then used outlines from these binary images to measure circularity, aspect ratio and solidity, each capturing a distinct aspect of leaf shape (Li *et al.*, 2018). Circularity, measured as $4\pi(\text{area}/\text{perimeter}^2)$, is influenced by serrations and lobing. Aspect ratio, in comparison, is measured as the ratio of the major axis to the minor axis of the best-fitted ellipse and is influenced by leaf length and width. Lastly, solidity measured as area/(convex hull), is sensitive to leaves with deep lobes, or with a distinct petiole, and can be used to distinguish leaves lacking such structures. Solidity, unlike circularity, is not very sensitive to serrations and minor lobings, since the convex hull remains largely unaffected.

For a more global analysis of leaf shape via EFDs, we used the program Shape (Iwata & Ukai, 2002) as described in Chitwood *et al.* (2014b). EFDs capture variation in shape represented by the outline which is difficult to categorise via traditional shape

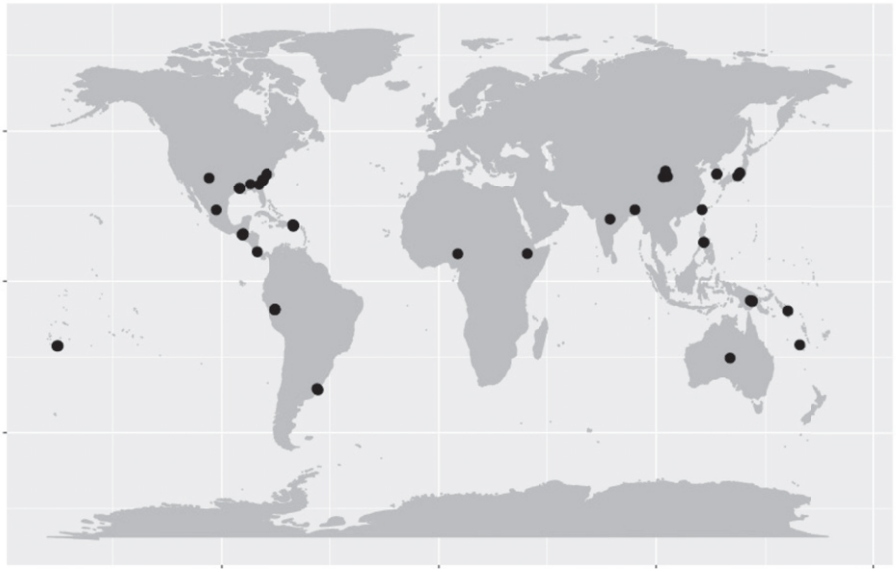

Fig. 1 Geographic diversity of the 68 chosen sweet potato, *Ipomoea batatas,* accessions. Black dots represent the origin of the chosen samples.

descriptors. From the EFD coefficients obtained, we used coefficients a and d only, thus analysing symmetric variation in leaf shape. Principal component analysis (PCA) was performed on the EFD coefficients to identify shape features contributing to leaf morphological variation (referred to as EFD symPCs below). We calculated the correlation matrices using the rcorr() function of the HMISC package v.4.0-3 (Harrell *et al.*, 2017) with multiple test adjustments using the p.adjust() function in R, which implements the Bonferroni correction.

RNA-seq library construction and sequencing

We sequenced and analysed transcriptomes of 19 individuals of *I. batatas* to examine gene expression differences associated with leaf shape variation associated with circularity, aspect ratio and EFD symPCs to obtain an initial set of candidate genes underlying these traits. We selected glasshouse-grown accessions with differing leaf shape trait values (Fig. S1). As high aspect ratio represents both longitudinally longer or latitudinally broader leaf shape phenotypes, we chose to only examine individuals that had a high aspect ratio due to latitudinal elongation. We chose multiple accessions to assess each leaf shape trait; 11 for circularity (six entire, five lobed), eight for aspect ratio (four high and low aspect ratio (AR), each), six individuals for EFD symPC1 (three high and three low) and four accessions each for EFD symPC2 and EFD symPC3 (two high and two low) (Fig. S1); EFD symPC4 was not considered for differential expression analysis.

We used three to five leaves that were in the P4–P6 stage of growth (fourth to sixth youngest primordium), from multiple branches of each individual accession for RNA extractions, and combined replicate leaves per individual to increase the depth of the transcriptome. We sampled all individuals on the same day within 1 h to reduce variation due to the developmental stage and/or time of collection. We froze samples in liquid nitrogen before preserving them at −80 for further processing. We performed RNA extraction using Qiagen RNeasy Plant mini kit with the optional DNase digestion step and constructed libraries using the TruSeq Stranded mRNA Sample Preparation protocol (LS protocol). After barcoding, we bulked all libraries and performed one lane of Illumina HiSeq2500 sequencing.

RNA-seq data processing and transcriptome analysis

An overview of our RNA-seq data processing and transcriptome analysis is given in Fig. 2, with detailed information presented in Methods S1.

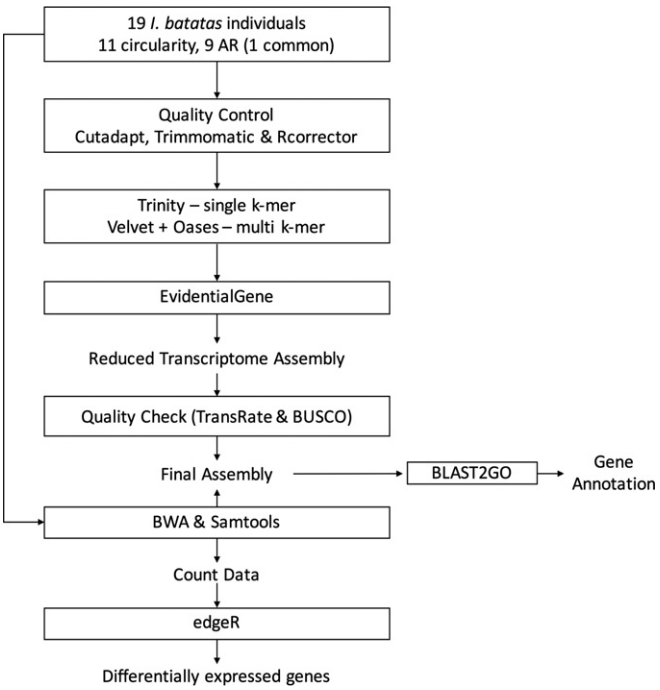

Fig. 2 Methodology for RNA-seq data processing for differential gene expression.

Differential gene expression We mapped reads from all 19 individuals to the *de novo* assembled transcriptome using BWA-MEM v.0.7.15 (Li & Durbin, 2009) and estimated read counts for uniquely mapped reads using SAMTOOLS v.1.9 (Li *et al.*, 2009). We then used read counts to filter out lowly expressed transcripts using the Bioconductor package EDGER v.3.18.1 (Robinson *et al.*, 2010) such that transcripts were retained only if they had > 0.5 counts per million in at least two samples. We then normalised libraries in EDGER (using the trimmed mean of *M*-values method) followed by differential gene expression analysis using the classic pairwise comparison of EDGER v.3.18.1. We extracted the significance of differentially expressed transcripts (DETs) with a FDR ≤ 0.05.

Field experiment

We performed a field experiment to determine the extent to which genetics, the environment, and G×E interactions influence leaf shape traits. We generated replicate individuals by planting 5 cm cuttings of the stem of each accession in 4-inch pots, randomly positioned on a mist bench at the Matthaei Botanical Gardens. During the first week of June, we planted three to seven replicates of each of the 68 accessions in two common gardens, one located at the Matthaei Botanical Gardens in Ann Arbor, MI (42.18°N, 83.39°W; from here on referred to as MI), and the other at the Ohio University Student Farm, West State Street Research Site in Athens, OH (40.46°N, 81.55°W; from here on referred to as OH). Replicates were planted in either three (MI) or seven (OH) blocks in a completely randomised block design with 14-inch spacing between individuals. Blocks were kept relatively weed free but were otherwise allowed to grow undisturbed. We randomly sampled two to five mature leaves from each individual in the first week of October, before the first frost and scanned them for leaf shape analyses as explained before.

Data analysis We first examined the potential for variation in leaf shape due to environmental differences (i.e. variation due to being grown in MI or OH) by performing an analysis of variance (ANOVA). To meet the assumptions of normality, we used the function TransformTukey from RCOMPANION v.2.0.0 (Mangiafico, 2018). TransformTukey is a power transformation based on Tukey's ladder of powers, which loops through multiple powers and selects the one that normalises the data most. These normalised leaf shape traits were then used as dependent variables and accession, garden, block effects and an interaction term of accession and garden as independent variables in the following fixed-effects model:

$$(\text{Trait} \sim \text{Accession} + \text{garden} + \text{block} + \text{Accession} : \text{garden}).$$

The term accession represents the genetic component, garden represents variation due to environment (plasticity), Accession: garden represents the G×E component and the block effect captures microenvironmental variation (and was nested within each garden). To quantify the relative effects of each of these variables on leaf shape, we calculated eta squared (η^2) as a measure of the magnitude of effect size using the Bioconductor package LSR v.0.5 (Navarro, 2013). Eta squared for an effect is measured as SS_{effect}/SS_{total}, where SS_{effect} is the sum of squares of the effect of interest and SS_{total} is the total sum of squares of all the effects, including interactions. In other words, it is a measure of the proportion of variance in the dependent variable associated with the independent variable and is one of the most commonly reported estimates of effect size for ANOVA (Levine & Hullett, 2002; Ialongo, 2016). Further, we calculated broad-sense heritabilities of leaf shape traits to determine the extent to which traits are genetically controlled within each environment. Broad-sense heritability was calculated using linear mixed modelling with the Bioconductor package SOMMER v.3.4 (Covarrubias-Pazaran, 2016) based on the phenotypic data collected from the two fields. The model used was:

$$\text{Trait} \sim 1,\ \text{random} = \sim \text{Accession} + \text{block} + \text{Accession} : \text{block},\ \text{rcov} = \sim \text{units}.$$

Variance components from the model were used to calculate the broad-sense heritability (H^2) using the formula:

$$H^2 = \frac{V_g + V_e + V_{g\times e} + V_r}{V_g},$$

where V_g is the genotype variance, V_e is the environmental variance due to the blocks, $V_{g\times e}$ is the variance associated with $V_{g\times e}$ (accession: block), and V_r is the residual variance.

Results

Leaf shape variation among accessions

We found a wide variation in leaf traits across 57 *I. batatas* accessions (Table 1). Among the three traditional traits examined, circularity is most variable with a phenotypic coefficient of variation (PCV; (standard deviation(x)/mean(x)) × 100; where x is the trait of interest) of 22.61% while AR is least variable with a narrow distribution and PCV of 4.76%. Fig. 3 shows the phenotypic diversity with respect to two leaf traits, circularity and AR. Of our 57 accessions, 10 exhibit low circularity (defined as circularity < 0.50). PI 599387, for example, exhibited leaves that are very deeply lobed and thus has a low circularity (0.09) value. By contrast, PI 566647 has no serrations or lobing (entire margins) and

Table 1 Leaf shape trait values across the 57 chosen sweet potato accessions.

Trait	Range	Mean	SD	PCV (%)
Circularity	0.09–0.71	0.50	0.12	22.61
Aspect ratio	1.03–1.26	1.10	0.05	4.76
Solidity	0.44–0.95	0.84	0.10	11.85

PCV, phenotypic coefficient of variation.

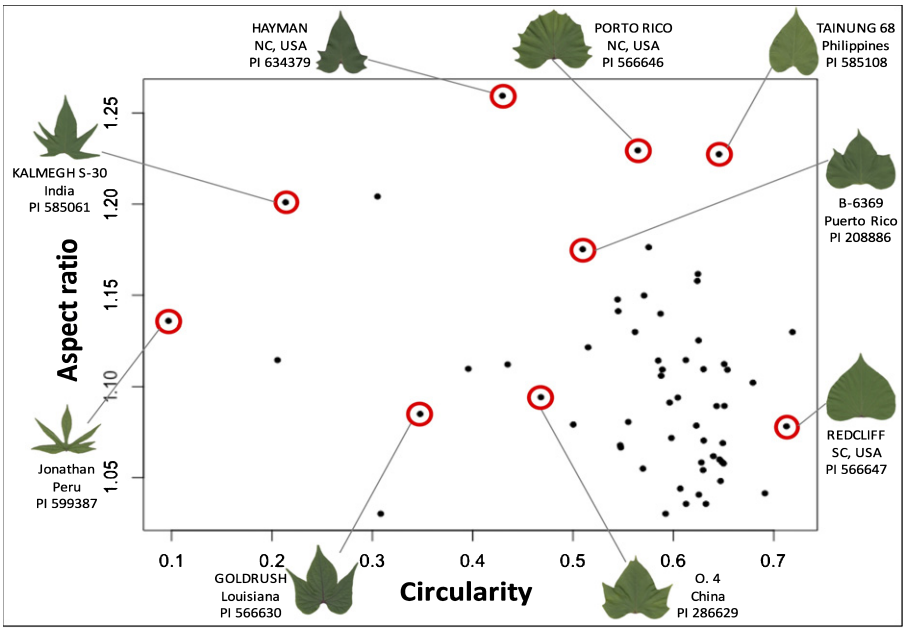

Fig. 3 Leaf shape variation in 57 diverse, glasshouse-grown, accessions of sweet potato, *Ipomoea batatas*, highlighting exceptionally high morphological variation.

thus exhibits high circularity (0.71; Fig. 3). Additionally, we found 22 of 57 accessions exhibited a high aspect ratio (AR > 1.11). For example, PI 531134 (AR = 1.03) has almost equal values of the major and minor axis and thus a low aspect ratio value. By contrast, the leaves of PI 208886 (AR = 1.268) are much wider, that is, a larger major to minor axis, and therefore has a high aspect ratio value. Most often this increase in AR in sweet potato manifests itself with increased leaf width (e.g. PI 566646, PI 208886) relative to length (e.g. PI 634379). Further, although solidity values range from 0.44 to 0.95, only five accessions had solidity values < 0.7 (PCV = 11.85%). The lack of low solidity values indicates that only a few accessions have deeply lobed leaves (e.g. PI 599387, solidity = 0.44), in contrast with accessions with slightly lobed leaved (e.g. PI 566630, solidity = 0.76).

We performed an EFD analysis on leaf outlines to get a more global estimation of leaf shape variation (Fig. 4). In total, we processed 292 leaves from 57 accessions to identify leaf shape traits that explain symmetrical shape variation in sweet potato. Low symPC1 values describe leaves with deep lobing, prominent tip and shallow petiolar sinus (PI 573318) whereas high symPC1 values explain nonlobed leaves with flattened leaf tips and enclosed petiolar sinus (PI 566646). symPC2 explains variation in leaf shape due to differences in breadth and lobing of the leaf (low symPC2 values describe broad leaves with two lobes whereas high symPC2 values depict narrow leaves with no lobes). symPC3 primarily captures leaf shape variation due to the depth of petiolar sinus (low symPC3 values describe leaves with highly enclosed petiolar sinus as compared with high symPC3 eigenleaves which have flattened sinus). Lastly, symPC4 represents variation in leaf shape attributed to the angle of lobe tips – low symPC4 eigenleaves have lobes with a high obtuse angle (almost 160°) whereas high symPC4 eigenleaves have lobes with a lower obtuse angle (almost 125°). The four symPC components together explain 87.79% of total variance relating to symmetrical leaf shape variance in sweet potato.

Further, we calculated correlation matrices for traditional shape descriptors and EFD symPCs to determine if they capture different aspects of leaf shape (Fig. S2). We found that symPC1 is correlated with circularity ($r = 0.20$; $P = 0.03$) and solidity ($r = 0.20$; $P = 0.02$), which is expected as symPC1 partially captures shape differences due to lobing. Additionally, circularity was highly correlated with solidity ($r = 0.96$; $P < 0.001$). This is not surprising as circularity is a measure of serrations and lobing whereas solidity is a measure of deep lobing; leaves having deep lobes (and lacking serrations) will therefore have similar values of circularity and solidity.

Sequencing and *de novo* assembly of *I. batatas* transcriptome

We performed a transcriptomic survey to identify gene expression changes associated with the leaf shape traits described above. For our analyses of the transcriptome, Illumina HiSeq2500 returned 266 million (125 bp) paired-end sequence reads; on average, each individual had 14 million (M) reads (GEO Submission ID-GSE128065) which was used to construct a *de novo* transcriptome assembly (sequence statistics are presented in Table 2). The results from BUSCO (Simão *et al.*, 2015) indicate that the *de novo* transcriptome assembly is of high quality with 91.32% (1315/1440) complete genes found (single copy genes *c.* 87%) of which only 4.51% were duplicates. Additionally, only 6.32% of genes were missing from the assembled transcriptome. Thus, our sequencing and assembly strategy produced a relatively complete transcriptome. Using blastx, 24 565 transcripts were annotated by the functional description of their top 20 hits. The transcriptome is available at Transcriptome Shotgun Assembly Database hosted by NCBI (TSA accession no. GHHM01000000).

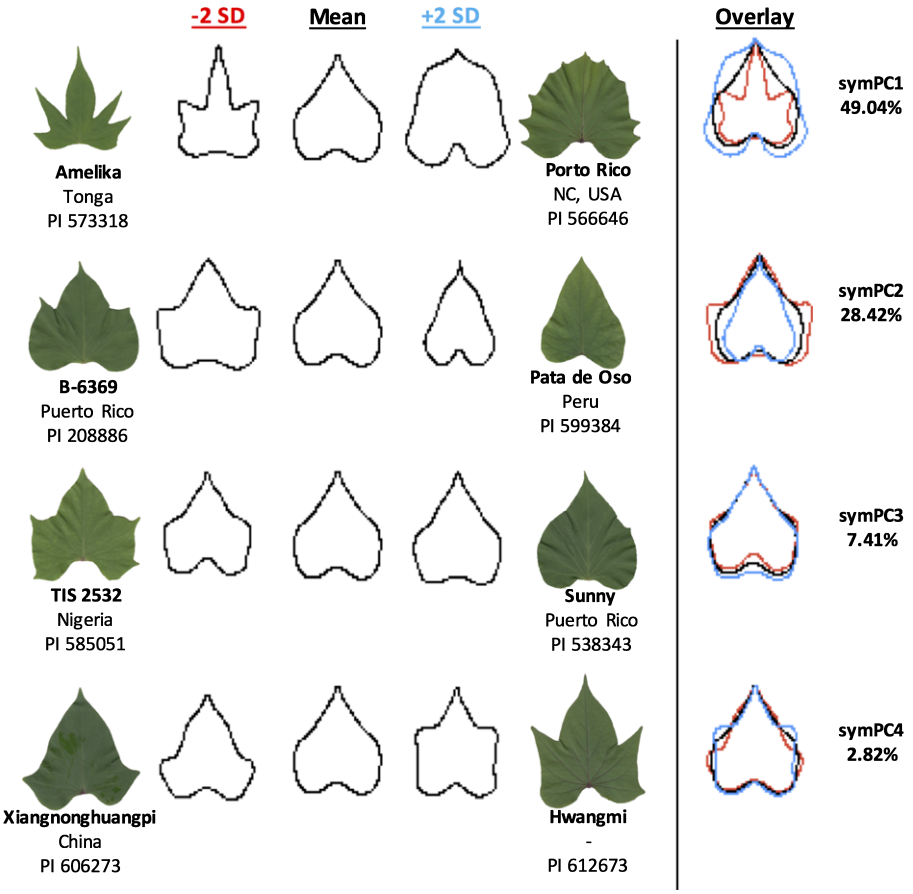

Fig. 4 Elliptical Fourier Descriptor (EFDs) of symmetrical shape variation in sweet potato, *Ipomoea batatas*. Contours represent eigenleaves resulting from principal component analysis (PCA) on symmetrical shape (symPC) on EFDs. Shown are the first four symPCs with the per cent variation explained by each; 87.79% of the total variation is explained. −2 SD (red) and +2 SD (blue) represent two units of SD from the mean along the symPC. Representative leaves of accessions with extreme symPC values are shown.

Table 2 Sequence statistics of the reference transcriptome obtained from the Evidential Gene pipeline.

Number of transcripts	Minimum length (nt)	Maximum length (nt)	Number of bases	Mean length (nt)	Open reading frame (%)	n50 (nt)	% reads mapped
33 684	200	16 428	35 769 411	1062	79.95	1608	77

Identification and functional annotation of DETs

As a first step towards understanding the genetic control of leaf shape, we identified gene expression changes associated with multiple leaf shape traits: circularity, AR (latitudinal expansion) and the symPCs obtained from the EFD analysis. We did not consider solidity and symPC4 due to their high correlation to circularity and low level of variation captured, respectively. On average, we found that 11 million unique paired-end reads per individual (range 7.66–14.23 M) mapped back to the reference transcriptome (net mapping efficiency of 89.65% with the paired-end high-quality reads). This indicates that we had sufficient read depth (> 10 M) to continue with our differential expression analysis (as shown by Wang *et al.*, 2011).

We uncovered 530 DETs associated with our leaf shape traits (Fig. 5; Table S2). Specifically, we found 47 DETs associated with circularity and 158 DETs associated with AR. For the symPCs examined, we found 121 DETs associated with symPC1, 148 DETs with symPC2 and 56 DETs with symPC3. Functional annotation of these DETs uncovered putative leaf shape genes (Table 3). As an example, for circularity, FAR1-related sequence 5 (or *FRS5*), a putative transcription factor involved in regulating light control of development, is differentially regulated with a log fold change of 5.77. Among other DETs for circularity, we found genes that are involved in regulating cell proliferation and organ morphogenesis (EXO70A1-like and extra-large guanine nucleotide-binding protein) and could be involved in regulating leaf dissection.

Among the 158 transcripts differentially expressed for AR (broad leaves vs rounder leaves), two genes have been shown in the literature to alter the longitudinal vs latitudinal expansion of the leaves. These are chalcone synthase (*CHS*), an enzyme involved in the production of chalcones involved in flavonoid biosynthesis, and feruloyl CoA 6′-hydroxylase which is involved in scopoletin biosynthesis and causes postharvest physiological deterioration in cassava (Liu *et al.*, 2017). Finally, we also found LIGHT-DEPENDENT SHORT HYPOCOTYL 10 (*LSH10*), to be significantly downregulated (log fold change of −1.85; *P*-value < 0.001).

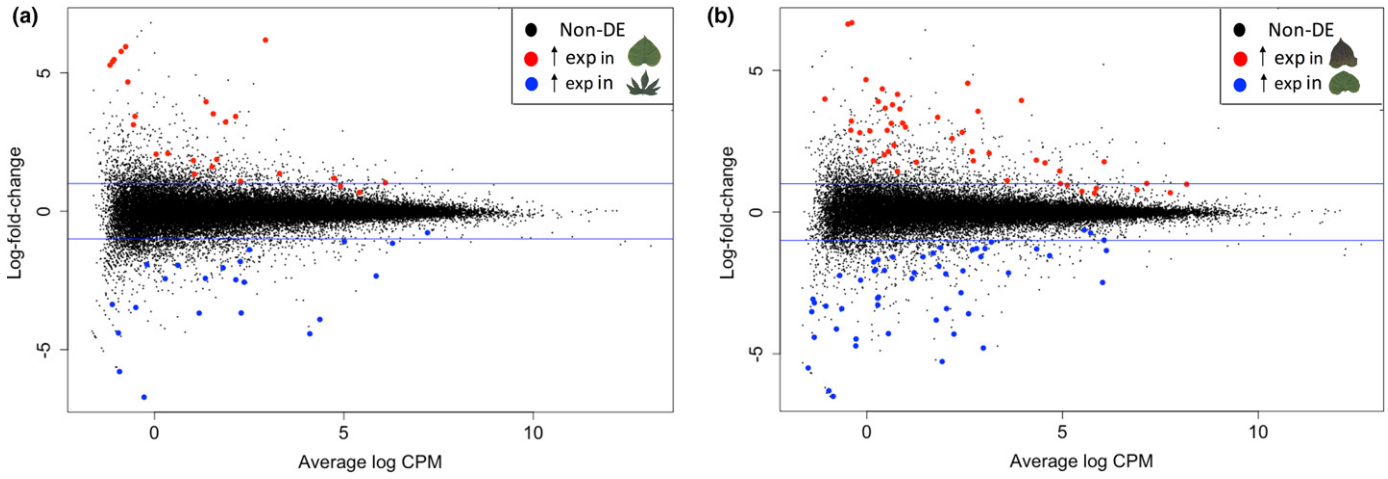

Fig. 5 Plot of log fold change against log CPM (counts per million) with differentially expressed transcripts highlighted (red and blue dots) for leaf shape in *Ipomoea batatas*. (a) Red and blue dots represent transcripts with higher expression in entire and lobed, respectively. (b) Red and blue dots represent higher expression in high aspect ratio and low aspect ratio individuals, respectively.

Table 3 Candidate genes maintaining variation in leaf traits (circularity, AR and symPCs) identified from the set of DETs in *Ipomoea batatas*.

Transcript ID	Log fold change (LogFC)	False discovery rate (FDR)	Gene description
Circularity			
trn22514	5.77	0.003	FAR1-RELATED SEQUENCE
trn27202	2.08	0.021	Exocyst complex component EXO70A1
trn24081	1.33	0.033	Extra-large guanine nucleotide-binding protein
Aspect ratio			
trn9778	−2.95	0.035	Chalcone synthase (CHS)
trn24267	2.55	0.00	Feruloyl CoA 6′-hydroxylase 2
trn25053	−1.85	0.021	Protein LIGHT-DEPENDENT SHORT HYPOCOTYLS 10
symPC1			
trn27227	1.56	0.018	Homeobox leucine zipper HAT22
trn23566	3.54	0.00	FAR1-RELATED SEQUENCE 7
symPC2			
trn27049	−3.09	0.009	Chalcone synthase
trn28352	−3.52	0.00	Chalcone synthase
trn9093	−2.21	0.00	Sporamin B

Individuals with extreme values of symPC1, a trait differentiating leaf shape based on lobing and prominence of tips and petiolar sinus, were also analysed for DETs. Of the 121 transcripts showing differential expression, two genes had interesting functional annotations. We found a homeobox gene (*HAT22*) to be upregulated in individuals with high symPC1 (leaves lacking lobes with flattened leaf tips and enclosed petiolar sinus), with a log fold change of 1.56. We also found another member of the *FRF1* family – FAR1-related sequence 7 (or *FRS7*) – to be upregulated in the high symPC1 individuals, as in the case of circularity.

We found 148 DETs for symPC2, which explains variation in leaf shape due to the differences in the broadness and lobing of the leaf. Again, we found that two copies of CHS were negatively regulated in high symPC2 individuals. We also found Sporamin B transcript, a tuberous root protein (Yeh *et al.*, 1997), to be significantly downregulated (log fold change of −2.76; *P*-value < 0.001). Finally, we identified 56 transcripts that were differentially expressed with respect to symPC3, however functional annotation revealed that most genes belonged to chloroplastic or mitochondrial genes.

Field experiment

We performed a field experiment to examine leaf shape in different environments, with the specific goal to determine the extent to which genotype, environment, and G×E altered leaf shape. We found significant variation among accessions (indicating genotypic or genetic variation) for circularity, AR and solidity ($F_{73} = 18.06$, $F_{73} = 4.22$, $F_{73} = 21.09$; $P < 0.001$), with accession explaining 73.23%, 38.40% and 77.18% of the total variation, respectively (Table 4). This high variance explained for circularity and solidity is reflected in high heritability values (Table 5; $H^2_{MI\_cir} = 0.79$, $H^2_{OH\_cir} = 0.73$; $H^2_{MI\_solidity} = 0.82$, $H^2_{OH\_solidity} = 0.76$). We also found evidence of significant block effect ($F_8 = 3.01$, $P = 0.002$; $\eta^2 = 1.33\%$) for circularity, whereas AR and solidity were not significantly influenced by block effects. Garden differences between OH and MI contributed 1.93% ($F_1 = 15.55$, $P < 0.001$) of the variability in AR while the accession by garden interaction contributed 12.95% (a significant G×E effect: $F_{69} = 5.01$, $P = 0.009$). AR also had lower heritability within each garden (Table 5; $H^2_{MI\_AR} = 0.39$, $H^2_{OH\_AR} = 0.26$). Circularity and solidity were not significantly altered by the environment and had no significant differences due to G×E.

Table 4 ANOVA table of the leaf shape traits model showing significant explanatory variables.

		Circularity			Aspect ratio			Solidity		
Variable	df	*F*	*P*	η^2 (%)	*F*	*P*	η^2 (%)	*F*	*P*	η^2 (%)
Accession	68	18.06	< 0.001***	73.23	4.22	< 0.001***	38.40	21.09	< 0.001***	77.18
Garden	1	3.64	0.056	0.20	15.5	< 0.001***	1.93	3.37	0.067	0.16
Block	8	3.01	0.002**	1.33	1.38	0.020	1.38	1.94	0.052	0.70
G×E	69	1.30	0.06	5.01	1.50	0.009**	12.95	1.30	0.065	0.40
Residuals	364	na	na	20.2	na	na	45.31	na	na	17.56

df, degrees of freedom; *F*, value of *F*-statistic; *P*, *P*-value; η^2, eta squared value.
na, not applicable; ** and *** represents a 0.01 and 0.001 significance, respectively.

Table 5 Broad-sense heritability (H^2) values for leaf shape traits in differing environments.

	H^2						
Env	Circularity	Aspect ratio	Solidity	symPC1	symPC2	symPC3	symPC4
MI	0.79	0.39	0.82	0.80	0.58	0.70	0.69
OH	0.73	0.26	0.76	0.59	0.67	0.63	0.47

We can not compare heritability values for Elliptical Fourier Descriptor (EFD) symPCs between MI and OH because the expression of traits varies between environments, and hence the information captured by symPCs differs between the two environments.

We also examined symmetrical leaf shape variation in both field sites by performing an EFD analysis (Fig. 6). EFDs from MI captured variation in leaf shape homologous to the symPCs estimated from glasshouse-grown individuals. There was general congruence in symPCs between glasshouse and field-grown leaves in MI (i.e. MIsymPC1 (field) ≈ symPC1 (glasshouse)), but leaf shape variation captured by EFDs from OH differed significantly in their order of variation explained (Fig. S3). OHsymPC1 explained leaf shape variation due to differences in the broadness and lobing of the leaf (similar to MIsymPC2), whereas OHsymPC2 explained variation due to lobing, tip and petiolar sinus differences (similar to MIsymPC1). This indicates that in OH the majority of leaf shape diversity is primarily due to the broadness of the leaf and secondly due to leaf lobing, while in MI, it is the opposite, the majority of leaf shape diversity is due to the leaf dissection rather than leaf width. Therefore, although traditional shape descriptors are only slightly influenced by the environment, leaf shape as a whole can be altered significantly by the environment.

We also calculated broad-sense heritability values for the symPCs in their respective environments and found that H^2 values ranged from 0.47 to 0.80 across the symPCs (Fig. 6). Heritability values in the OH garden were consistently lower than in the MI garden due to reduced genetic variance and increased environmental variance. Overall, the high heritability values indicate that leaf morphology is controlled to a great extent by genetic factors.

Discussion

In this study, we examined the extent of leaf shape variation within an agronomically important species, determined the role of genetics, the environment and G×E in altering leaf shape traits, and identified potential candidate genes associated with multiple leaf shape traits. We found evidence of extensive intraspecific morphological variation, with shape differences due to lobing, length-to-width ratio of leaves and the prominence of tip and petiolar sinuses explaining the majority of the variation. We also found that leaf shape has a strong genetic basis with most phenotypic variation attributed to accessional variation, with low or limited influence of G×E. Strikingly, we show that although traditional shape descriptors are only slightly influenced by the environment in this species, when measured comprehensively, leaf shape can be significantly altered by the environment (evident by the change in symPC1 across the MI and OH gardens). Below, we expand on each of our findings and place them in the context of current knowledge about leaf shape diversity at a species level as well as what is known about the environmental influence on leaf shape in other species.

High morphological diversity of leaf shape in *I. batatas*

A recurring question among plant morphologists is the extent to which leaf shape varies among genotypes in a species. This study quantified leaf shape variation among multiple replicated accessions of sweet potato and identified traits contributing most to leaf shape variation. We focused our morphometric study on three traditional shape descriptors (circularity, AR and solidity) and then expanded into the more comprehensive EFD measures.

In our analysis of traditional measures, circularity was found to be the most variable whereas AR was found to be the least variable. Further, the first two principal components of the EFD analysis together accounted for 77.46% of the total variation in leaf shape and described variation associated with petiolar sinus, tips and positioning of lobes. Additionally, the lack of correlation between symPCs and traditional leaf shape metrics suggests that

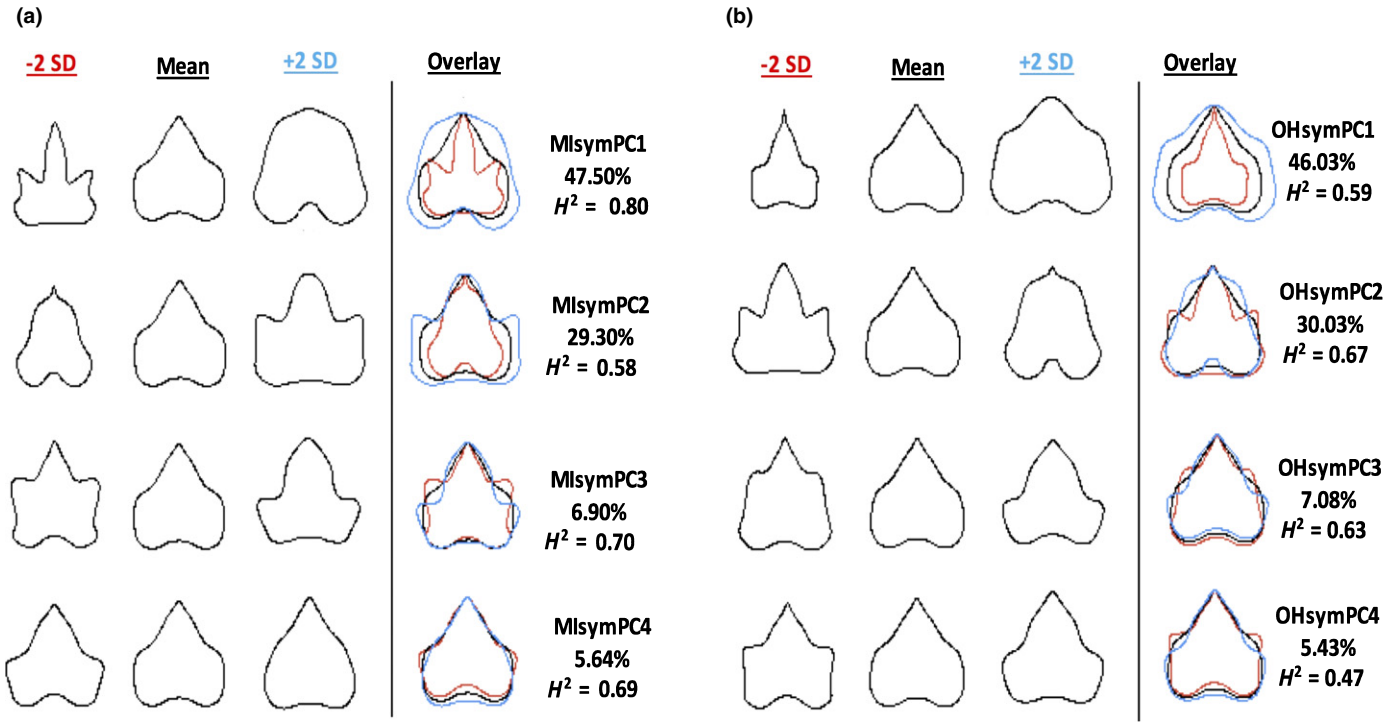

Fig. 6 Elliptical Fourier Descriptor (EFDs) of symmetrical leaf shape variation among 68 accessions of sweet potato, *Ipomoea batatas*, in the common gardens in Michigan (a) and Ohio (b), respectively. MIsymPC and OHsymPC represents the Michigan and Ohio symmetrical leaf shape variation, respectively; H^2 represents broad-sense heritability.

they capture different features of shape. Only symPC1 was slightly correlated with circularity and solidity. This is not surprising as symPC1 captures variation in leaf shape due to lobing, tip and sinus. No other traits were found to be correlated. Therefore, variation captured by the EFD symPCs would have been missed by simply quantifying traditional shape descriptors, suggesting that the use of comprehensive morphometric techniques can help quantify the full extent of shape variation across species. Further, combining the results from traditional morphometric approaches with EFDs revealed that variation in leaf dissection (circularity and symPC1) contributes most to the morphological variation in leaf shape in sweet potato (Figs 3, 4), similar to that seen in grape (Chitwood *et al.*, 2014b). In addition, AR explains a significant proportion of the remaining variation, unlike in tomato and apple where AR is the primary trait of variation in leaf shape (Chitwood *et al.*, 2013; Migicovsky *et al.*, 2017). This indicates that leaf shape variation does not follow a trend across species which is likely due to multiple independent evolution of leaf shape across phylogenetic taxa (Nicotra *et al.*, 2011).

Gene transcripts underlying leaf shape variation

To further our understanding of gene expression changes associated with leaf shape diversity, we sequenced transcriptomes of 19 accessions and assembled a high-quality gene expression database for performing a differential expression analysis in *I. batatas*. We found 47 genes that were differentially expressed for circularity and 121 DETs for symPC1, a trait that accounts for leaf shape differences due to leaf dissection, prominence of the tip and petiolar sinus. Functional annotations of these genes identified potential candidates that could contribute to leaf shape dissection in *I. batatas* (Table 3). The most promising candidate is the *FRS* gene; we found *FRS5* and *FRS7* to be upregulated in nondissected individuals in the differential analysis for circularity and symPC1, respectively. *FRS* is a putative transcription factor and contains the DNA binding domain needed to bind the RB-box promoter region of *SHOOT MERISTEMLESS* (*STM*; Aguilar-Martínez *et al.*, 2015), a protein required for leaf serrations (Kawamura *et al.*, 2010). *FRS* might bind to *STM* thus regulating its expression. However, we did not find *STM* to be differentially expressed in our datasets. This might be due to no real expression differences or it might indicate that the expression differences are really small and thus the gene is not detected to be differentially expressed.

Furthermore, genes containing homeobox domains have been shown to be associated with leaf dissection in multiple species, for example *PTS* in tomato (Kimura *et al.*, 2008), *STM* in *Arabidopsis* (Piazza *et al.*, 2010), *RCO* in *C. hirsuta* and other Brassicaceae (Vlad *et al.*, 2014; Sicard *et al.*, 2014) and *LMI1* in cotton (Andres *et al.*, 2016). Most of these genes are differentially regulated in the shoot apical meristem (SAM) and P0 (the youngest primordium) to determine the extent of leaf dissection and complexity for the genotype. However, we did not find any homeobox domain containing genes to be differentially expressed in sweet potato accessions that varied for circularity (i.e. lobed vs entire) (Table S3) but found a homeobox leucine zipper protein (*HAT22*) to be upregulated for high symPC1 individuals. This mismatch could represent a caveat to our transcriptomic

sampling stage (P4–P6), which is past the leaf dissection morphogenic stage of development. Thus, although preliminary, our data indicate that the degree of lobing in *I. batatas* might be maintained in later stages of leaf development (P4–P6) by the action of a gene containing a homeobox domain and that the difference in expression required might be very small.

Further, we found 158 differentially expressed genes associated with AR and 148 DETs associated with symPC2 (leaf shape due to the differences in the broadness and lobing). Based on the function of the homologues of these genes, we identified promising putative candidate genes associated with broad leaved phenotypes (Table 3). In apples, a transgenic *CHS* silenced individual developed longer leaves when supplied with naringenin, thus altering leaf AR. This indicates that higher expression of *CHS* (and thus naringenin) is responsible for the longitudinal expansion of the leaves and thus downregulation of *CHS* could lead to broader leaves due to the lack of longitudinal expansion. Another gene of interest that we found differentially expressed for AR, feruloyl CoA 6′-hydroxylase, produces broader leaved phenotypes of cassava when silenced (Liu *et al.*, 2017). Interestingly, however, we found a *higher* expression of feruloyl CoA 6′-hydroxylase2 in broader leaved, compared with the rounder-leaved individuals. Finally, the differentially expressed *LSH10* belongs to the family of *LSH* genes, which have been shown to interact with BLADE-ON-PETIOLE (BOP) and regulate PETROSELINUM (*PTS*) expression, a gene that regulates *KNOX* genes, and thus leaf complexity (Ichihashi *et al.,* 2014). This indicates the potential role of the *LSH* gene in regulating both leaf broadness and complexity in this species.

Factors influencing leaf shape traits in multiple environments

While studies often examine the potential for plasticity in leaf shape traits (McLellan, 2000; Royer *et al.*, 2009; Viscosi, 2015), the relative influence of genetic background, environment and gene by environment interactions are less commonly examined. We show that leaf shape traits (circularity, AR and solidity) in sweet potato are influenced by multiple effects. Variation in circularity and solidity were mostly attributed to accession (or genotype) and showed little to no effect due to environment or gene by environment interaction. Circularity and solidity have exceptionally high broad-sense heritability values in *I. batatas* (0.76 and 0.79, respectively, averaged between gardens). These traits have similarly been shown to be highly heritable in tomato with heritability values being 0.65 and 0.67, respectively (Chitwood *et al.*, 2013). The high PCV for circularity and solidity in *I. batatas* (22.61% and 11.85%) along with high broad-sense heritability indicates that there is a lot of standing variation for these traits that can be actively selected for (or against) by breeders. Furthermore, the lack of plasticity and G×E demonstrate the stability of these simple leaf shape descriptor traits, at least in the environments tested.

Contrary to our results, multiple studies have found that leaf dissection, captured here by our measure of circularity is a plastic trait that responds to changes in temperature. For example, Royer and colleagues (2009, 2012) found that leaves of *Acer rubrum* were more dissected when grown in cooler environments as compared to warmer environments. A similar trend was observed in grapevine (*Vitis* spp.) (Chitwood *et al.*, 2016). However, we found that leaf dissection in sweet potato is not influenced by the environment. This could reflect that our gardens were not different enough to lead to plastic responses in these two measures of leaf shape. The Ohio garden was consistently warmer (by 2°C on average) and experienced less precipitation than the Michigan garden, the difference between the two gardens was 662.43 mm month^{-1} on average throughout the growing season. Although there were environmental differences between gardens, before we conclude that circularity in *I. batatas* is not strongly environmentally responsive, multiple studies in environments that range more widely for temperature will need to be performed.

Comparatively, we found significant variation in AR due to environment and G×E, explaining 1.93% and 12.95% of the total observed variation in this measure of leaf shape, respectively. This is reflected in the significant alteration of trait values between environments. There were small yet significant differences observed ($P < 0.001$; 95%CI, 0.009–0.03) between gardens, with clones grown in Michigan consistently showing less round, more elliptical leaves than clones grown in the Ohio garden. However, we still found that 38.40% of the variation in the trait was due to accessional variation which was also indicated in the estimated heritability value of the trait ($h^2 = 0.24$). AR has been found to be a major source of leaf shape variation in apples and tomatoes with high heritabilities of 0.75 and 0.63, respectively (Chitwood *et al.*, 2013; Migicovsky *et al.*, 2017). By contrast, we found that this important leaf shape trait is globally not as variable in sweet potato (4.76% PCV), but it still presents a selection potential. The considerable effect of G×E on AR indicates that this trait has a genetic component that interacts with the environment leading to varied values between environments.

Furthermore, comparing leaf outlines between two environments, we found that although the traits explaining leaf shape variation are homologous between the two environments, these traits vary in the percent of variation they explain. The heritability of EFD symPCs measured in MI and OH was found to be very high, yet the changes in the amount of variation they explain in their respective environments indicate a strong environmental (and/or G×E) influence on EFD symPCs measured. Although traditional shape descriptors were only slightly controlled by the environment (AR), we found that the more comprehensive measure of leaf shape can be altered significantly by the environment. This further signifies the importance of measuring leaf shape using methods apart from traditional shape descriptors in multi-environment conditions.

Overall, this work highlights the extensive natural variation in leaf shape within the globally important domesticate *I. batatas*. More broadly, and considering leaf shape analyses from other, mostly domesticated species, leaf shape variation appears to be species specific; there is no evidence of a shared trait between species that explains the majority of within-species variation. Additionally, we found that most of the variation in the

traditional measures of leaf shape appears to be largely controlled by genetic factors in sweet potato, with a low proportion of variance in leaf shape attributable to environmental differences between gardens. However, when leaf shape was considered more comprehensively and by the use of leaf outlines, we identified a significant influence of the environment, suggesting that studies relying solely on circularity or AR to describe leaf shape may not capture the extent to which environmental factors can impact leaf development. This multilevel examination highlights the importance of examining morphological variation at the species level in multiple environments, and using a range of leaf shape phenotypes to comprehensively understand the mechanistic basis (morphological, molecular and environmental) of leaf shape.

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Author contributions

RSB and DMR conceived the research idea; SG, RSB and DMR performed the experiments and SG performed data analyses with RSB's supervision. SG wrote the manuscript in consultation with RSB, DMR, JRS.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Glasshouse-grown accessions selected for transcriptomic analysis.

Fig. S2 Correlation plot between leaf shape traits (traditional and EFD PCs).

Fig. S3 Leaf shape variation captured by EFDs from MI (Michigan garden) and OH (Ohio garden) differing significantly in their order of variation explained.

Methods S1 RNA-seq data processing and transcriptome analysis.

Table S1 Accession IDs with their source and location of origin used in this study.

Table S2 Differentially expressed transcripts associated with leaf shape traits found in this study.

Table S3 Raw read counts of orthologues of homeobox domain genes within the assembled transcriptomes, for accessions chosen for circularity RNA-seq analysis.

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