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GENOMICS OF HYBRIDIZATION

Molecular mechanisms of postmating prezygotic reproductive isolation uncovered by transcriptome analysis

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Abstract

Little is known about the physiological responses and genetic mutations associated with reproductive isolation between species, especially for postmating prezygotic isolating barriers. Here, we examine changes in gene expression that accompany the expression of 'unilateral incompatibility' (UI)—a postmating prezygotic barrier in which fertilization is prevented by gamete rejection in the reproductive tract [in this case of pollen tubes (male gametophytes)] in one direction of a species cross, but is successful in the reciprocal crossing direction. We use whole-transcriptome sequencing of multiple developmental stages of male and female tissues in two *Solanum* species that exhibit UI to: (i) identify transcript differences between UI-competent and UI noncompetent tissues; (ii) characterize transcriptional changes specifically associated with the phenotypic expression of UI; and (iii) using these comparisons, evaluate the behaviour of a priori candidate loci for UI and identify new candidates for future manipulative work. In addition to describing transcriptome-wide changes in gene expression that accompany this isolating barrier, we identify at least five strong candidates for involvement in postmating prezygotic incompatibility between species. These include three novel candidates and two candidates that are strongly supported by prior developmental, functional, and quantitative trait locus mapping studies. These latter genes are known molecular players in the intraspecific expression of mate choice via genetic self-incompatibility, and our study supports prior evidence that these inter- and intraspecific postmating prezygotic reproductive behaviours share specific genetic and molecular mechanisms.

Keywords: hybridization, reproductive isolation, speciation, transcriptomics

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Introduction

A critical step in the formation of new species is the evolution of reproductive isolating barriers. Identifying the loci contributing to reproductive isolation can reveal both the specific classes of genes and mutations underlying isolating traits, as well as the evolutionary and genomic factors that drive change at these loci. Although the genetics of reproductive isolation has been examined in numerous systems (Rieseberg & Blackman 2010; Seehausen *et al.* 2014), the specific loci underlying reproductive isolation phenotypes have been described in relatively few instances, most notably for male sterility and hybrid inviability in model organisms (Presgraves 2010). Therefore, dissecting the genetic basis of reproductive isolation to identify the specific causal loci remains a goal in many systems.

Of the stages that can contribute to reproductive isolation, comparative analyses have suggested that prezygotic isolating mechanisms—those that act before hybrids are formed—could play a disproportionately

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large role in preventing gene exchange between closely related species (e.g. Lowry *et al.* 2008). Genetics of these isolating mechanisms has been investigated for some premating traits, including mate signal traits such as male courtship (e.g. Yeh & True 2014) and chemical (including pheromone) signals (e.g. Chung *et al.* 2014) in animal species. Among flowering plants, the genetics of prezygotic reproductive isolation is arguably best understood—including at the molecular level—for traits that influence pollinator preference, including flower colour, scent and shape (Hoballah *et al.* 2007; Hopkins & Rausher 2011; Xu *et al.* 2012). Species that differ in these traits are likely to experience reduced pollinator-mediated gene flow, therefore preventing the formation of hybrids (Kay & Sargent 2009). In addition to premating reproductive isolation phenotypes, however, prezygotic isolation can also act after mating but before fertilization takes place. These postmating, prezygotic mechanisms include dysfunctional interactions between male gametes (sperm) and female reproductive tracts, between gametes themselves (sperm-egg interactions) and via reduced competitive ability of heterospecific gametes (conspecific sperm or pollen precedence). Because these barriers involve traits that are less conspicuous and experimentally accessible than those mediating premating isolation, they have received less attention from both a phenotypic and genetic perspective. Still, the genetic loci contributing to postmating prezygotic interactions, such as sperm–egg interactions (Lessios 2011) and sperm competition (e.g. Castillo & Moyle 2014), have been described in several cases.

Just as in animals, flowering plant postmating prezygotic reproductive isolation operates after mating (in this case, after transfer of pollen between species) but before fertilization; these barriers are due to the reduced effectiveness of heterospecific male–female interactions or, in some cases, because of active heterospecific rejection mechanisms (reviewed in Moyle *et al.* 2014). In this study, we focus on postmating isolating barriers among plant species that manifest after the male pollen grain germinates to produce a pollen tube (the haploid male gametophyte), while it is growing down through the female pistil (the diploid maternal floral reproductive tract composed of the stigma and style) and into the ovary (Fig. 1). These pollen–pistil interactions are roughly analogous to interactions between sperm and the female reproductive tract in animals; however, unlike animal sperm, growing pollen tubes are known to actively express a large proportion of their own haploid genome (Rutley & Twell 2015).

The genetic mechanisms underlying heterospecific pollen–pistil barriers have been investigated in several systems. Arguably, the clearest genetic work that connects pollen–pistil interactions to the expression of

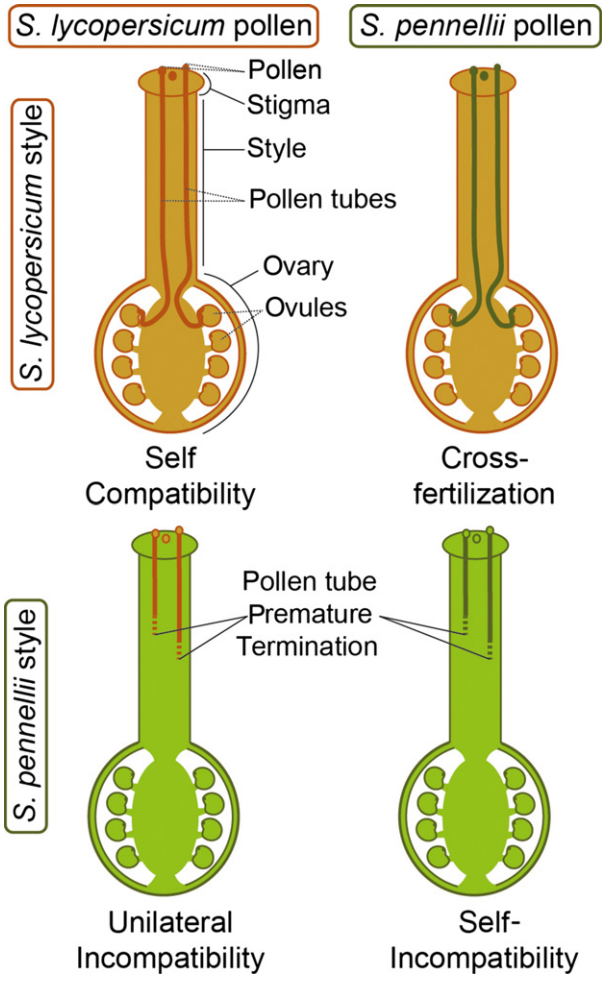

Fig. 1 Pollination of *Solanum pennellii* pistils by *Solanum lycopersicum* pollen (upper left) does not result in fertilization, but the reverse pollination (lower right) is successful, resulting in unilateral incompatibility (UI) in this species cross. Pollen rejection is accomplished by premature arrest of pollen tubes via pistil-side molecular mechanisms.

species barriers focuses on the phenomenon of 'unilateral incompatibility' (UI). UI is the observation of a strong pistil block of pollen tube growth in one direction of an interspecific cross, such that pollen tubes germinate and grow but are subsequently arrested at some distance down the style (Covey *et al.* 2010); in contrast, the reciprocal crossing direction produces successful pollen tube growth down the full length of the style (Fig. 1). Based on classical crossing studies (e.g. Grun & Aubertin 1966; Hardon 1967), it has long been recognized that UI often accompanies lineage differences in genetically determined self-incompatibility (SI), such that SI species can reject pollen from self-compatible (SC) species but not vice versa (Bedinger *et al.* 2011; Baek *et al.* 2015). This observation (the 'SI × SC' rule; Lewis & Crowe 1958) suggests that some molecular

mechanisms contributing to genetic SI within species could also be directly involved in the expression of unilateral crossing barriers between species (Pandey 1981). Early transformation studies in *Nicotiana*, for example, showed that restoring functional S-RNase (the pistil-side factor involved in normal SI rejection) is sufficient to confer UI (Murfett *et al.* 1996), implying a direct role for intraspecific SI molecular mechanisms in the control of interspecific UI. Nonetheless, S-RNase clearly varies in its contribution to UI. In other *Nicotiana* species pairs, for example, S-RNase either contributes to this response but is not individually sufficient or is not involved in UI (Murfett *et al.* 1996; McClure *et al.* 2011). Among *Solanum* (tomato) species, studies similarly indicate that UI can have both S-RNase-dependent and S-RNase-independent mechanisms (Bedinger *et al.* 2011), including cases where UI expression does not require the presence of a functional S-RNase (Covey *et al.* 2010). UI can also involve additional molecular players known to be involved in SI responses (Covey *et al.* 2010), including pollen-expressed genes (Li & Chetelat 2010). Together, these observations suggest that multiple loci can contribute to UI, including molecular mechanisms both shared with and independent of SI (Bedinger *et al.* 2011).

As a complementary approach to these detailed genetic analyses, short-read sequencing and microarray approaches have also been used to identify and evaluate new candidate loci for pollen–pistil interactions (Beale & Johnson 2013; Dresselhaus & Franklin-Tong 2013). While these genomic approaches have not specifically focused on understanding the genetic basis of interspecific barriers, and have yet to pinpoint specific molecular mechanisms of interspecific pollen–pistil isolation, they could uncover loci that are promising candidates for reproductive isolation at this postmating stage. These analyses could be particularly powerful when coupled with data on the stage- and tissue-specific expression of these barriers, and on potential candidate loci, drawn from more traditional approaches.

Here, we examine whole-transcriptome differences between reciprocal crosses of a *Solanum* species pair that is known to exhibit UI. Overall, our goals were to: (i) identify transcript differences between UI-competent and UI noncompetent tissues; (ii) characterize transcriptional changes specifically associated with the phenotypic expression of UI; and, (iii) using these comparisons, evaluate the behaviour of a priori candidate loci for UI as well as identify additional new candidates for future manipulative work to confirm the underlying genetic mechanisms. We also test the hypothesis that styles from the nonrejecting species fail to express UI because of a paedomorphic transition that arrests non-UI styles at an immature stage of development, making them unable to mount a UI response. Finally, because some of our samples are a mix of pollen and pistil tissues, we present a methodology for differentiating the expression of the two tissues of origin in interspecific crosses, based on SNP differences between species.

Materials and methods

Study system and material

The wild tomato clade (*Solanum* sect. *Lycopersicon*) includes species isolated by a range of pre- and postzygotic reproductive isolating barriers (Moyle 2008). Known isolating barriers include UI which in *Solanum* often, but not always, follows the SI × SC rule (Bedinger *et al.* 2011; Baek *et al.* 2015). In this study, we examined the transcriptional basis of UI between two well-analysed *Solanum* species: the wild species *Solanum pennellii* and the domesticated tomato *Solanum lycopersicum*. Pollen from *S. pennellii* (an historically SI species) will successfully germinate and grow within the pistil of *S. lycopersicum* (an SC species); however, in the reciprocal cross, *S. lycopersicum* pollen is arrested approximately one-third down the length of the style (Hardon 1967; Covey *et al.* 2010; Bedinger *et al.* 2011). We analysed tissues from *S. pennellii* genotype LA0716 and *S. lycopersicum* genotype LA3475; these specific genotypes have been analysed for proteomic profiles in pollen and pistil tissue previously (Lopez-Casado *et al.* 2012; Chalivendra *et al.* 2013). *Solanum pennellii* LA0716 has recently lost SI, but still exhibits UI against *S. lycopersicum* genotype LA3475; therefore, our analysis here focuses on S-RNase-independent mechanisms of UI response.

Plant cultivation and sample collection

Seeds for LA0716 and LA3475 were obtained from the C. M. Rick Tomato Genetics Resource Center, U.C. Davis. For all experimental individuals, seeds and plants were handled as in previous experiments (Moyle & Graham 2005; Moyle & Nakazato 2008). Our goal was to estimate quantitative gene expression in several developmental stages of male (pollen) and female (pistil) reproductive tissues from *S. pennellii* (*pen*) and *S. lycopersicum* (*lyc*; Table 1). For each of three biological replicates per species, we analysed pistil (the entire stigma and style) tissue from: flowers 5 days prior to opening (*S. pennellii* only: 'pen^{S-5}', where 'S' stands for 'style'); flowers 1 day prior to opening (both species: 'lyc^{S-}' and 'pen^{S-}'); flowers on the day of opening (both species: 'lyc^{S+}' and 'pen^{S+}'); flowers on the day of opening, 6 h after pollination with *S. lycopersicum* pollen:

Table 1 Tissues sampled from *Solanum lycopersicum* (*lyc*) and *Solanum pennellii* (*pen*)

Abbreviation	Description
pen^{S-5}	Unpollinated pistils 5 days before flowering time (*pen* only)
lyc^{S-}, pen^{S-}	Unpollinated pistils 1 days before flowering time
lyc^{S+}, pen^{S+}	Unpollinated pistils just after flowering time
lyc^{dry}, pen^{dry}	Ungerminated (dry) pollen
lyc^{germ}, pen^{germ}	Germinated pollen
lyc^{self}, pen^{self}	Self-pollinated pistils just after flowering time
lyc^{pen}	*lyc* pistils pollinated by *pen* pollen (interspecific compatibility)
pen^{lyc}	*pen* pistils pollinated by *lyc* pollen (unilateral incompatibility)

'pen^{lyc}' and 'lyc^{self}'; and flowers on the day of opening, 6 h after pollination with *S. pennellii* pollen: 'pen^{self}' and 'lyc^{pen}'. Unpollinated styles at 5 days prior to flower opening were collected from *S. pennellii* only (i.e. pen^{S-5}), because *S. lycopersicum* flowers have negligible style development at this early time point. For pollination treatments within species, pistils were always pollinated with pollen from the same biological individual (i.e. they were self-pollinated). In addition to pistil tissue, we analysed transcriptomes from dry (ungerminated) pollen (both species: 'pen^{dry}' and 'lyc^{dry}') and pollen germinated in liquid in vitro media (both species: 'pen^{germ}' and 'lyc^{germ}'). For each individual, pollen was manually collected from flowers on the day of opening (see Supporting information for additional details of sample handling and preparation).

RNA extraction and library preparation

In total, with three biological replicates, we generated 39 unique tissue pools (and therefore libraries) for sequencing and expression comparisons. Extraction of total RNA from ground tissue was performed using RNeasy® Plant Mini Kits from Qiagen (catalog number 74904). Tissue-specific total RNA was equi-molar pooled using the RiboGreen® RNA quantitation assay (Life Technologies: R11491) and then quality checked using an Agilent 2200 TapeStation System prior to library construction. Stranded, paired-end libraries of total RNA were generated from these pools for each accession using Illumina TruSeq Stranded total RNA HT Sample Preparation Kits (Illumina: RS-122-2203), and these libraries were pooled and distributed evenly (<6-fold difference among libraries, Appendix S1, Supporting information) across three lanes of Illumina HiSeq™ 2000 (Illumina Inc., San Diego, CA, USA). RNA QC, library preparation and pooling were performed by the Indiana University Center for Genomics and Bioinformatics.

Reference mapping, de novo assembly and mapping of unmapped reads

Prior to mapping and assembly, reads were trimmed using the SHEAR program (http://www.github.com/jbpease/shear) which incorporates the Scythe algorithm (https://github.com/vsbuffalo/scythe). The full command and parameters used for SHEAR can be found in Dryad (http://dx.doi.org/10.5061/dryad.j3s5r). Reads from all 39 samples were mapped to the *S. lycopersicum* reference chromosomes (v.SL2.50) using STAR RNA-seq spliced aligner (Dobin *et al.* 2013) with the default parameters and outputting unmapped reads in FASTQ format. The average proportion of uniquely mapped read pairs was 92.1% (min 83.8%, max 96.6%, Appendix S1, Supporting information).

Unmapped reads from each species (excluding cross-pollinated samples) were assembled de novo using TRINITY v. 2014-07-17 with default parameters (Grabherr *et al.* 2011). For *S. lycopersicum*, $n = 47\,559$ transcripts were assembled totalling 27.6 Mb ($N_{50} = 777$). For *S. pennellii*, $n = 77\,988$ transcripts were assembled totalling 45.2 Mb ($N_{50} = 772$). Unmapped reads from each sample were then individually mapped back to the de novo gene sets for each species using BWA v.0.7.10 (Li & Durbin 2009). Reads from the cross-pollinated samples that did not map to the transcripts from the stylar species were also mapped to the pollen species.

Quantification

Reads mapped to the reference were counted using FEATURECOUNTS v1.4.5-p1 (Liao *et al.* 2014) with the enhanced reference annotation. Reads mapped to de novo transcripts were counted using a PYTHON script (http://www.github.com/jbpease/mixtape/sam_read_count.py) that generates raw read pair counts from the SAM file. The counts from the reference-mapped and de novo-mapped reads were combined into the final table of counts utilizing both the combined mapping annotation and orthologous de novo gene table. Read counts were combined for loci with both directly mapped and de novo transcript-mapped reads. Loci in the final table were categorized as mapped/annotated reference loci (*Solyc##g######*), mapped/unannotated genes (*Solyc##x######*), unmapped de novo genes for which orthologs exist in both species (*Unmap######*) and unmapped de novo genes that do not have an ortholog in the other species (*LycoD#####* and *PennD#####*, respectively). A total of 660 581 594 read pairs were counted (83.0% of raw reads) in 273 253

exons in 53 939 genes from the extended reference (annotated loci, predicted coding region and de novo assembled transcripts).

Quantitative separation of pollen- and style-specific reads in interspecific mixed samples

Pollinated pistils contain tissues from two parental origins (paternal pollen and maternal style) that can be differentiated when the parents have different alleles at an aligned site and/or when specific transcripts appear to be exclusively expressed in only stylar or pollen tissue when these are assayed independently. The counts of alleles at all sites that were heterozygous in the self- and cross-pollinated samples were quantified using SAMTOOLS *mpileup* (Li *et al.* 2009) and custom PYTHON scripts (Dryad: http://dx.doi.org/10.5061/dryad.j3s5r). A site was identified as 'differentiable' in the mixed samples if it matched these criteria: (i) the mixed sample had two alleles mapped to that site (i.e. site was inferred to be heterozygous), (ii) at least two reads containing the given allele variant were observed in each orientation (forward/reverse) with a minimum base quality of 30 at that site and minimum read quality of 30, (iii) all unmixed (single-tissue pollen or style) samples from the pollen donor species exhibited only one of the two alleles in the mixed sample, (iv) all unmixed samples from the style donor species exhibited the other allele in the mixed sample, (v) each site was observed at least twice in each tissue, and (vi) pollen and style tissues expressed transcripts with nonoverlapping variants. For each locus that had variants meeting the above criteria, we then calculated a gene-specific pollen and style read proportion by separately summing pollen- and style-specific allele depths at all differentiable sites in the gene and dividing by the total pollen- and style-specific alleles counted in the gene. These ratios were then applied to the original counts table to split the mixed tissue columns into pollen- and style-specific columns for the *S. pennellii* pistils with *S. lycopersicum* pollen ('pen^{lyc}:pollen') and ('pen^{lyc}:pistil') and *S. lycopersicum* pistils with *S. pennellii* pollen ('lyc^{pen}:pollen', 'lyc^{pen}:pistil').

Differential expression testing

For pairwise comparisons between tissues, differential expression was tested using a linear model implemented by the LIMMA package (Ritchie *et al.* 2015; Law *et al.* 2014) and modules from the EDGER package (Robinson *et al.* 2010). Read counts were TMM normalized and transformed using *voom* (a weighted transformation based on the expected relationship between expression mean and variance; Ritchie *et al.* 2015). We computed *t*-statistics on the transformed expression values for each gene using an empirical Bayes adjustment of standard errors (with the *eBayes* function; Law *et al.* 2014). The normalized, untransformed read counts—in units of counts per million reads in library (cpm)—were used in downstream filtering steps (see Supporting information).

We identified genes that are differentially expressed in UI tissues by grouping samples in three categories: UI-competent styles (pen^{lyc}, pen^{self}, pen^{S-}, pen^{S+}), non-UI styles (lyc^{pen}, lyc^{self}, lyc^{S-}, lyc^{S+}) and immature styles (pen^{S-5}; see further, Results). We fit a linear model with these three categories as fixed effects and individual samples (i.e. biological replicates) as a random effect, to identify genes differentially expressed in UI-competent vs. non-UI styles, that also differ in expression (in the same direction) between mature style stages (pen^{lyc}, pen^{self}, pen^{S-}, pen^{S+}) and immature noncompetent (pen^{S-5}) styles in *S. pennellii* (Tables S2 and S3; see Supporting information for details of specific criteria cut-offs used for calling differential expression). Because these comparisons involve samples that are pollinated and unpollinated, they might be subject to background effects that are hard to evaluate. Therefore, we carried out an analysis identical to the one described above but including only unpollinated tissues (pen^{S-5}, pen^{S-}, pen^{S+}, lyc^{S-}, lyc^{S+}) and obtained fewer significant genes, all of which were identified in our primary analyses (see Supporting information).

To evaluate gene expression changes that specifically accompany activation of the UI response, we compared pistil tissues that are UI competent but not expressing UI (pen^{S-}, pen^{S+}) to UI-expressing pistil tissue (pen^{lyc}) (Table S4, Supporting information). Again, because this comparison involves both pollinated and unpollinated tissues, we also included a complementary analysis of just pollinated tissues that are and are not expressing UI (i.e. pen^{lyc} *vs.* pen^{self} and lyc^{pen}; Table S5, Supporting information). Finally, although not a primary focus of our study, we also tested allele-specific expression response during UI, in genes for which we were able to separate pollen- and pistil-specific expression (see above). Through these comparisons, we identified several genes with strong differential expression in just pollen (Table S6, Supporting information), just styles (Table S7, Supporting information) and both pollen and style tissues (Table S8, Supporting information) during the expression of UI.

A priori candidate gene, cysteine-rich peptide, gene ontology, and molecular evolution analyses

We examined gene expression patterns of several classes of loci or individual genes that have previously

been implicated in either SI or UI phenotypic responses in these or closely related Solanaceous species, focusing on 45 of these a priori candidates (Table S9, Supporting information). In addition, we also examined patterns of differential expression in cysteine-rich peptides (CRPs) as these are known to play an important role in mediating postmating reproductive interactions in flowering plants (Tang *et al.* 2002; Okuda *et al.* 2009; Marshall *et al.* 2011). We defined CRPs broadly as peptides that met the following criteria: (i) total length between 50 and 100 amino acids; (ii) contain at least 5% cysteine in the amino acid sequence; and (iii) contain a signal peptide domain, as identified by SIGNALP 4.1 (Petersen *et al.* 2011). We identified 136 ITAG2.4 reference proteins that met these criteria (Appendix S1, Supporting information).

To examine whether transcript differences between tissues and UI/non-UI conditions were significantly enriched for particular functional categories of genes, we examined Gene Ontology terms in these sets of loci. GO term reference (go.obo) v. 2015-09-25 was obtained from the Gene Ontology project (www.geneontology.org, Ashburner *et al.* 2000). GO terms for each gene in the SL2.50 (tomato genome) reference were obtain from SolGenomics (ftp://www.solgenomics.net). GO term enrichment analysis was performed using ONTOLOGIZER v2.0 (Bauer *et al.* 2008) using the parent–child analysis. We analysed three sets of genes for GO term enrichment: (i) 607 genes with significant increase in expression, (ii) 980 genes with significant decrease in expression and (iii) the combined set of 1587 genes with either significant increase or decrease in expression in UI-competent vs. noncompetent tissues (Appendix S1, Supporting information). Finally, to examine patterns of molecular evolution at the most highly differentially expressed loci uncovered by our expression analyses, we examined nonsynonymous changes per nonsynonymous site, compared to synonymous changes per synonymous site (d_N/d_S comparisons) in these loci. The merged alignments were translated to codons, and pairwise d_N/d_S values were calculated using PAML v4.8 (Yang 2007) via the 'PairwiseDNDS' analysis module in MVFTOOLS (Pease & Rosenzweig, In Press) (see Supporting information for details). Values of $d_N/d_S > 1$ are indicative of recent recurrent positive selection at a locus.

Statistical and visualization software

The Scipy PYTHON (http://scipy.org/) statistical library was used to compute Pearson (*pearsonr*) and Spearman (*spearmanr*) correlations. Graphs were prepared with VEUSZ (http://home.gna.org/veusz/), MATPLOTLIB (http://www.matplotlib.org) and R (https://cran.r-project.org/).

Results

Our experiment was designed to examine the gene expression differences that accompany unilateral pollen–pistil incompatibility, to evaluate expression in a priori candidate loci, and to identify new candidate loci associated with this postmating prezygotic reproductive isolating barrier. To do so, we collected RNA-seq data from seven unique tissue types in two species, including two pollen developmental stages and multiple developmental stages of pistil (female reproductive tract) tissue, each with three biological replicates, resulting in 39 unique libraries for expression analysis. In total, we obtained 56.6 Gb of sequence data, mapped 93% of total reads and assigned 90% of mapped reads uniquely to the reference genome and de novo assembled genes. Reads were counted in at least one tissue for 32 242 (93%) of reference genes, 15 316 unannotated genomic loci and 2203 de novo assembled transcripts. These data were used to identify loci that had gene expression differences associated with UI competence, the activation of the UI response, and with tissue and species specificity.

Differential gene expression in tissues competent for UI

To understand the pistil-side (female reproductive tract) loci that could be active specifically in the heterospecific pollen rejection response, we compared RNA expression in stylar tissues that are and are not competent to mount a UI rejection response. Prior work has demonstrated that this competence depends upon both species and the developmental stage of pistil tissue (Covey *et al.* 2010; Bedinger *et al.* 2011). Of our sampled tissues, only *Solanum pennellii* pistils from the day before flower opening onwards are developmentally competent to express UI; *S. pennellii* pistils from 5 days before opening are not yet UI competent, nor are any *Solanum lycopersicum* pistils. Therefore, pistil-specific loci that could be involved specifically in the UI response are those that differ between these two groups of tissues: (pen^{lyc}, pen^{self}, pen^{S-}, pen^{S+}) vs. (lyc^{pen}, lyc^{self}, lyc^{S-}, lyc^{S+}, pen^{S-5}) (see Materials and methods, and Table 1 for descriptions of each).

We identified 1587 loci that differed significantly in expression between UI- and non-UI-competent stylar tissues (Supporting information, Dryad: http://dx.doi.org/10.5061/dryad.j3s5r), according to our criteria for inclusion (see Materials and methods). Of these, 607 were expressed more highly in UI-competent tissues, while 980 were expressed more highly in non-UI-competent tissues, indicating that overall UI

competence was associated with a slight bias towards downregulated gene expression. The gene with highest differential expression in the UI conditions (expression is 7.0 cpm in immature *S. pennellii* styles and ~5500 cpm in mature and pollinated *S. pennellii* styles) appears in the *S. pennellii* reference genome as predicted protein *Sopen04g027820*, but there is no homologous gene in the *S. lycopersicum* reference genome. Searches of GenBank and the INTERPRO database (accessed on 8 August 2015) both indicate that this protein contains a 'plant invertase/pectin methylesterase inhibitor' (PMEI) domain. Orthologous sequences are also found in the genomes of *Solanum tuberosum*, *Nicotiana alata*, and *Nicotiana sylvestris*. While this class of genes has been previously highlighted in *S. pennellii* for its role in fruit maturation (Reca *et al.* 2012; Bolger *et al.* 2014), other studies have indicated a variety of other potential roles including biotic defence and pollen tube growth (recently reviewed in Levesque-Tremblay *et al.* 2015b).

Two other highly suggestive new candidates appear in the top 20 genes with largest expression increase in UI-competent tissues. The first is annotated as a PVR3-like protein (*Solyc01g109390*, whose ortholog is *Sopen01g051580*) from the class of lipid transfer proteins (*LTPs*). *LTPs* are known to be involved in cell wall-loosening activity specifically in mature *Nicotiana* styles (Nieuwland *et al.* 2005), although as a class of proteins they also have diverse functions including in pollen tube adhesion and in plant defence responses (Yeats & Rose 2008). The second new candidate encodes a pistil extension-like (*PELPIII*-like) protein (*Solyc02g078060*, whose ortholog is *Sopen02g0022900*). This locus is especially notable because in *Nicotiana*, this *PELPIII* protein is known to: (i) be produced in mature styles, (ii) localize to the transmitting tract, (iii) bind *S-RNases* and (iv) interact with growing pollen tubes (Bosch *et al.* 2001; Cruz-Garcia *et al.* 2005). Functionally, then, this locus is strongly implicated in pollen–pistil interactions relevant to UI (Eberle *et al.* 2013). Interestingly, in the SL2.50 genome annotation, this locus is annotated as two separate gene fragments (*Solyc02g078060* and *Solyc02g078070*), likely because the *S. lycopersicum* reference and LA3475 have a premature stop codon at the end of the *Solyc02g078070* annotated fragment, whereas *S. pennellii* LA0716 does not. An analysis of transcriptomic data from all wild tomato species (Pease *et al.* 2016) shows that only domesticated *S. lycopersicum* (and not other red-fruited species) have the premature stop codon (Pease *et al.* 2016). We confirmed that the expression levels of the two *lyc* gene fragments were indistinguishable, supporting the inference that they are fragments of the same locus. Moreover, this mutation is expected to disrupt expression in *S. lycopersicum*, and we confirmed that *lyc* had virtually no expression compared to *pen*.

We conducted Gene Ontology (GO) analyses on genes showing significant differential expression between UI- and non-UI-competent stylar tissues and found several significantly overrepresented GO terms. For the 607 genes with significantly higher expression in UI tissues, 143 genes (24%) were annotated as 'catalytic activity' (GO:0003824, $P = 0.0052$); other enriched categories ($P < 0.0025$) included 'oxygen binding' (GO:0019825), 'electron carrier activity' (GO:0009055) and 'protein methylesterase activity' (GO:0051723) (see full table in Appendix S2, Supporting information). In addition, the functional annotations of genes with the most substantial expression differences between UI and non-UI stylar tissues (Table 2) suggest that UI competence is accompanied by increased expression of multiple genes with catalytic activity that likely act on components of cell walls (e.g. pectin, xylan). Several of these cell wall-related classes of loci (including polygalacturonases) are also expressed during (nonrejecting, intraspecific) pollen–pistil interactions in *Arabidopsis thaliana* (Boavida *et al.* 2011).

For the 980 genes with significantly decreased expression during UI, 259 genes (26%) were also annotated as 'catalytic activity' (GO:0003824, $P = 1.07 \times 10^{-5}$) (Appendix S2, Supporting information). Other enriched categories ($P < 0.001$) include 'oxygen binding' (GO:0019825), 'regulation of proteolysis' (GO:0030162) and several categories involving peptidase regulation including 'peptidase inhibitor activity' (GO:0030414). Additionally, the top two genes with reduced expression in UI-competent tissues (Table 3) are both proteinase inhibitors (with two more in the top 20). This is intriguing as Kunitz proteinase inhibitors have been previously shown to be directly involved in SI in *Nicotiana* (Busot *et al.* 2008; Jiménez-Durán *et al.* 2013).

Among the top 20 genes with highest increase or decrease in expression, we had sufficient sequence from both species to test eight genes for positive selection (see Appendix S2, Supporting information for d_N/d_S results for all testable expressed loci). (We are unable to evaluate loci in cases where one species did not have any detectable expression.) Among these, only *Solyc02g078070* (pistil-specific extensin-like protein) and *Solyc01g110050* (Endo-1 4-beta-xylanase) had a value of $d_N/d_S > 1$ (see Appendix S2, Supporting information). Note also that when *Solyc02g078070* and *Solyc02g078060* are treated as a single gene (see above), the combined d_N/d_S at this locus is still >1 (in this case, $d_N/d_S = 1.763$; Appendix S2, Supporting information).

Table 2 Top 20 genes with largest increase in expression in unilateral incompatibility-competent tissues

Gene	Fold change	*P*-value	Annotation
*Sopen04g027820**	7082.3	4.30×10^{-24}	Predicted pectin methylesterase inhibitor
Solyc02g071400.2	2336.3	3.30×10^{-21}	1-aminocyclopropane-1-carboxylate oxidase
Solyc08g006360.1	1541.4	4.00×10^{-24}	UDP-glucose glucosyltransferase
Solyc05g051670.1	1176.3	9.20×10^{-17}	Gibberellin receptor GID1L2 (carboxylesterase)
Solyc01g105500.2	982.3	4.20×10^{-22}	Endo-1 4-beta-xylanase
Solyc08g006370.1	962.1	1.30×10^{-24}	UDP-glucosyltransferase family 1 protein
Solyc12g096620.1	903.9	6.30×10^{-10}	GDSL esterase/lipase (AT1G28590-like)
Solyc09g082660.2	843.4	1.10×10^{-26}	Caffeoyl-CoA O-methyltransferase
Solyc01g060120.1	719.1	3.60×10^{-21}	Ulp1 protease family C-terminal catalytic domain-containing protein
Solyc09x055675	694.6	1.10×10^{-30}	Unknown
Solyc10x061595	680.3	4.00×10^{-18}	Unknown
Solyc10g080210.1	617.4	2.10×10^{-19}	Polygalacturonase A
Solyc01g109390.2	556.4	2.60×10^{-30}	PVR3-like protein
Solyc09g056230.1	541.2	1.50×10^{-15}	O-methyltransferase
Solyc07g065110.1	526.4	8.20×10^{-13}	Protease inhibitor/seed storage/lipid transfer protein family protein
Solyc01g080680.2	515.6	1.20×10^{-29}	Glucose transporter 8
Solyc12g019120.1	512.0	7.80×10^{-21}	Polygalacturonase
Solyc02g078060.1†	491.1	8.40×10^{-26}	Pistil extensin-like protein
Solyc00x000758	471.1	2.50×10^{-24}	Unknown (similar to *Sopen02g001890*)
Solyc00g142170.2	467.9	4.90×10^{-21}	RNA-dependent RNA polymerase

*Note that this gene appears as 'Unmap000018' in some data sets.
†Note that this locus corresponds to *Solanum pennellii* gene *Sopen02g022900*.

RNA expression patterns that accompany the UI phenotype

To understand the transcriptome-wide changes that specifically occur when UI is activated (i.e. within the pen^{lyc} tissue class), we first sought to exclude loci that are expressed in normal mature *S. pennellii* styles, including those pollinated with intraspecific pollen. Within the stylar tissue, we also aimed to differentiate transcripts being expressed solely by the pollen vs. solely by the style during the UI response (see Supporting information).

To distinguish between genes being expressed by the style and by the pollen in tissue composed of cells from both, we used aligned nucleotide sites with species-specific allele differences to determine pollen- and style-specific reads in the pen^{lyc} sample. Even with very low genetic divergence between these two species (~1.4%), we were able to identify species-specific nucleotide differences in pollen- and style-specific reads for 7269 genes. Of the genes with species-diagnostic nucleotides, reads were pollen/style differentiable for 6664 genes in at least one pen^{lyc} sample and 2285 were differentiable in all three pen^{lyc} samples (see Materials and methods for these criteria). Our ability to discriminate the specific origin of reads was correlated with mean expression level in the pollen: the number of detectable pollen reads from mixed tissues was highly correlated with expression in germinated pollen ($\rho = 0.33$; $P = 4.1 \times 10^{-27}$, Pearson's rank test). This means that our ability to detect activity of pollen-specific genes likely depends on the absolute expression of pollen genes.

After eliminating expression contributed by pollen, we contrasted patterns of expression in UI-expressing pistil tissue (pen^{lyc}:pistil) to unpollinated pistil tissues that are UI competent but not expressing UI (pen^{S-}, pen^{S+}). We detected a relatively modest number of pistil-side loci with expression changes that specifically accompany the activation of the UI response (Table 4) with generally more modest fold changes than detected between UI-competent vs. UI-incompetent styles. Some of these expression changes likely reflect the regulation of genes involved in normal (nonrejecting) pistil functions that are activated during pollination. For example, whole genome arrays of similar reproductive tissues in *A. thaliana* (Boavida *et al.* 2011) detected differential expression of loci broadly involved in signalling and metabolic pathways in pollinated vs. unpollinated styles, as expected of interactions that involve coordinated growth of the pollen tube down the style. Similarly, transcriptome profiling of unpollinated and self-pollinated styles within one self-incompatible and one

Table 3 Top 20 genes with largest decrease in expression in unilateral incompatibility-competent tissues

Gene	Fold change	*P*-value	Annotation
Solyc03g020080	10226.3	9.10×10^{-18}	Proteinase inhibitor II
Solyc11g022590	2957.2	5.60×10^{-22}	Kunitz trypsin inhibitor 4 (proteinase inhibitor)
LycoD18798g1	2665.1	9.40×10^{-18}	Unknown *Solanum lycopersicum* gene
Solyc10x058194	1428.2	2.20×10^{-16}	Unknown
Solyc08g015650	1379.6	7.90×10^{-25}	La-related protein 7 (RNA-binding protein Lupus La)
Solyc08g065490	1360.6	1.40×10^{-26}	Serine hydroxymethyltransferase
Solyc06g035940	1217.7	1.20×10^{-23}	Homeobox-leucine zipper protein
Solyc08g065310	1176.3	3.70×10^{-21}	Unknown protein
Solyc06g082570	1160.1	1.50×10^{-23}	Unknown protein
Solyc09g084450	1144.1	2.20×10^{-24}	Chymotrypsin inhibitor-2
Solyc08g023530	1097.5	3.40×10^{-25}	Unknown protein
Solyc01g057460	1089.9	4.10×10^{-22}	CTV.22 (Mediator of RNA polymerase II transcription subunit 15a)
Solyc12g068080	1089.9	8.90×10^{-23}	Unknown protein
Solyc10x058616	1060.1	2.90×10^{-25}	Unknown
Solyc03g031600	1024.0	1.30×10^{-21}	Peroxisomal membrane protein PMP22
Solyc12g068060	1016.9	4.50×10^{-24}	Unknown protein
Solyc07g049140	1002.9	1.90×10^{-13}	Metallocarboxypeptidase inhibitor
Solyc12x071317	1002.9	2.20×10^{-24}	Unknown fragment (possibly associated with *Solyc12g068080*)
Solyc09g009650	955.4	9.60×10^{-25}	Unknown protein
Solyc07g008970	948.8	6.80×10^{-26}	Unknown Protein

SC wild tomato species (*Solanum chilense* and *Solanum pimpinellifolium*, respectively; Zhao *et al.* 2015) showed expression differences in cell–cell communication between pollen and style. Among genes with significant expression differences in style tissue during UI detected here (Table 4), the most dramatic increase was a kinesin-like gene (*Solyc01g100120*). While *Solyc01g100120* has overall higher expression in the pollen, it also shows strong expression in *pen* but not *lyc* unpollinated and pollinated pistils. This protein is highly similar to *OsKCH1* and *NtKCH1*, which have been implicated as having a role in mitotic cell elongation (Frey *et al.* 2010; Klotz & Nick 2012).

Expression patterns of a priori candidate genes previously associated with SI and/or UI responses

We examined expression patterns in 45 a priori candidates from specific genes and classes of loci previously shown to be associated with SI and/or UI responses in this or other Solanaceous relatives, to assess whether their expression patterns differed between UI-competent and UI-incompetent pistil tissues (Table S9, Supporting information). For known pistil-specific loci, only two loci showed expression patterns associated with UI. *HT-A* (*Solyc12x072278*)—one of two 'HT' proteins known to be important in pistil-side SI recognition systems (McClure *et al.* 1999; Kondo *et al.* 2002b)—showed significantly higher expression in UI-competent pistil tissues (Fig. 2). This is consistent with previous observations that *HT-A* protein is abundant in mature (UI-competent) *S. pennellii* LA0716 pistils but virtually absent in mature (UI-incompetent) *S. lycopersicum* pistils (Bedinger *et al.* 2011). In *S. lycopersicum*, this locus appears to produce a truncated protein that lacks an asparagine-rich domain and is presumed to be nonfunctional (Covey *et al.* 2010). Our data here indicate that transcriptional changes in *S. lycopersicum* have also lead to reduced RNA expression of this locus.

The second pistil-side locus, *Solyc02g078050*, shows a modest but significant decrease in expression in UI-competent conditions (1.9-fold, $P = 8.43 \times 10^{-7}$; Fig. 2). This gene is orthologous to the *Nicotiana* 120 kDa protein that has been implicated in tobacco as an important gene for pistil-side SI (McClure *et al.* 1999). This locus also appears in our allele-specific analysis as one of the few genes that show both a pollen and style response during the phenotypic expression of UI (Table S8, Supporting information). Interestingly, this locus occurs in a local cluster of several genes that all encode S-RNase binding proteins and that interact with pollen tubes (Cruz-Garcia *et al.* 2005; Hancock *et al.* 2005; Eberle *et al.* 2013), including two *TTS*-like proteins (*Solyc02g078040* and *Solyc02g078100*) and the new candidate *PELPIII*-like protein *Solyc02g078060*/*Solyc02g078070* (see above) that appears in our list of top 20 genes with significant increased expression in UI-competent tissues.

For completeness, we also examined expression in loci associated with pollen-mediated components of SI or UI. We found that most *S*-locus F-box (*SLF*) proteins tested showed almost negligible expression in both species (<2 cpm for all tissues) and those with significant

Table 4 Top 15 genes with largest style-specific increase or decrease in expression during unilateral incompatibility (UI)

Gene	Fold change	*P*-value	Annotation
Increased expression during UI			
Solyc01g100120	1287.18	1.00×10^{-11}	Kinesin-like
Solyc02g078390	33.36	3.30×10^{-16}	MutS2 protein
Solyc02g072540	7.31	2.70×10^{-5}	CBL-interacting protein kinase 16
Solyc01g007110	3.51	2.50×10^{-6}	NC domain-containing protein
Solyc06g066800	2.99	1.30×10^{-3}	Glycosyl transferase family 8
Solyc01g099780	2.87	2.40×10^{-6}	Translationally-controlled tumour protein homolog
Solyc12g006930	2.83	3.80×10^{-11}	Acyl-ACP thioesterase
Decreased expression during UI			
Solyc05g055980	7.73	5.00×10^{-3}	Phosphatase 2C family protein
Solyc08g080170	7.41	1.00×10^{-8}	Hydroxymethylglutaryl-CoA synthase
Solyc05g055810	6.06	1.80×10^{-3}	Catalytic/hydrolase
Solyc01g010650	5.94	2.30×10^{-9}	UDP-galactose transporter 3
Solyc01g080360	5.62	3.80×10^{-8}	MHD domain-containing death-inducing protein
Solyc07g005300	5.28	1.40×10^{-8}	Unknown protein
Solyc07g054540	4.32	1.40×10^{-7}	Lipoyl synthase
Solyc01g103410	3.66	1.60×10^{-8}	Nucleic acid binding protein

expression were not consistent with UI-specific genes (Table S9, Supporting information). Of 17 annotated *Cullin* and *Cullin*-like genes, another family implicated in pollen-side UI (Li & Chetelat 2010), 11 genes showed significant expression differences during UI, but all were found to be pollen-specific, and thus were filtered out of the final list of pistil-specific UI genes.

Finally, of the 136 ITAG2.4 reference proteins identified as CRPs based on structural criteria (see Materials and methods), 4 *CRP* genes showed significantly increased expression in UI vs. non-UI pistils and five showed decreased expression. Of these genes, *Solyc07g007240* (52-fold increase) and *Solyc07g007260* (12-fold decrease) are notable for: (i) being nearly adjacent, (ii) having expression differences change in opposite directions and (iii) being metallocarboxypeptidase inhibitors. Peptidase inhibitors have been previously implicated in *Nicotiana* SI pollen rejection (Busot *et al.* 2008; Jiménez-Durán *et al.* 2013).The interleaved gene *Solyc07g007250* is also a metallocarboxypeptidase inhibitor showing high expression in both *lyc* and *pen*. A syntenic cluster of all three genes is also found in *S. pennellii* reference genome (as *Sopen07g003300*, *Sopen07g003310* and *Sopen07g003320*). The three pairs of orthologs show modest differentiation (2–5 amino acids) compared to much stronger differentiation among paralogs, indicating a triplication prior to divergence of *lyc* and *pen*. As with many genes in our most significantly differentially expressed lists, these loci also have proteinase inhibitory functions and are related to Kunitz trypsin inhibitors. Other CRPs identified in our analyses might also be functionally involved in UI competence, but currently lack functional annotations.

Expression profiles exhibit strong tissue-specific identity

We also used our data to assess similarity of expression profiles between different developmental stages, under different pollination conditions within species, and at the same stage or condition between species (Fig. 3). In general, we found that gene expression profiles were strongly correlated among different developmental stages of the same tissue (i.e. among stylar tissues and among pollen tissues) within species and more weakly associated between species (Table S1, Supporting information). Within species, pollen expression profiles between germinated and nongerminated pollen showed somewhat weaker correlations than those observed among stylar tissues. Overall, interspecific similarity in expression was also lower for pollen than pistils, suggesting greater divergence in gene expression associated with male gamete function than female reproductive tracts. This is despite the fact that fewer loci overall were detected as expressed in pollen tissue than pistil tissues (Dryad: http://dx.doi.org/10.5061/dryad.j3s5r).

We also used these data to test the hypothesis that styles from the nonrejecting species fail to express UI because of a paedomorphic transition: that is, non-UI styles represent developmentally immature UI styles that are thereby unable to mount a UI response. If this were the case, then the expression profile of SC styles (here, lyc^{S-} and lyc^{S+}) would be expected to be more similar to the expression profile of immature *S. pennellii* styles (pen^{S-5}) than mature *S. pennellii* styles (pen^{S-} and pen^{S+}). However, the overall correlation observed in the comparison of lyc^{S-} or lyc^{S+} vs. pen^{S-5} ($\rho = 0.82–0.83$) is

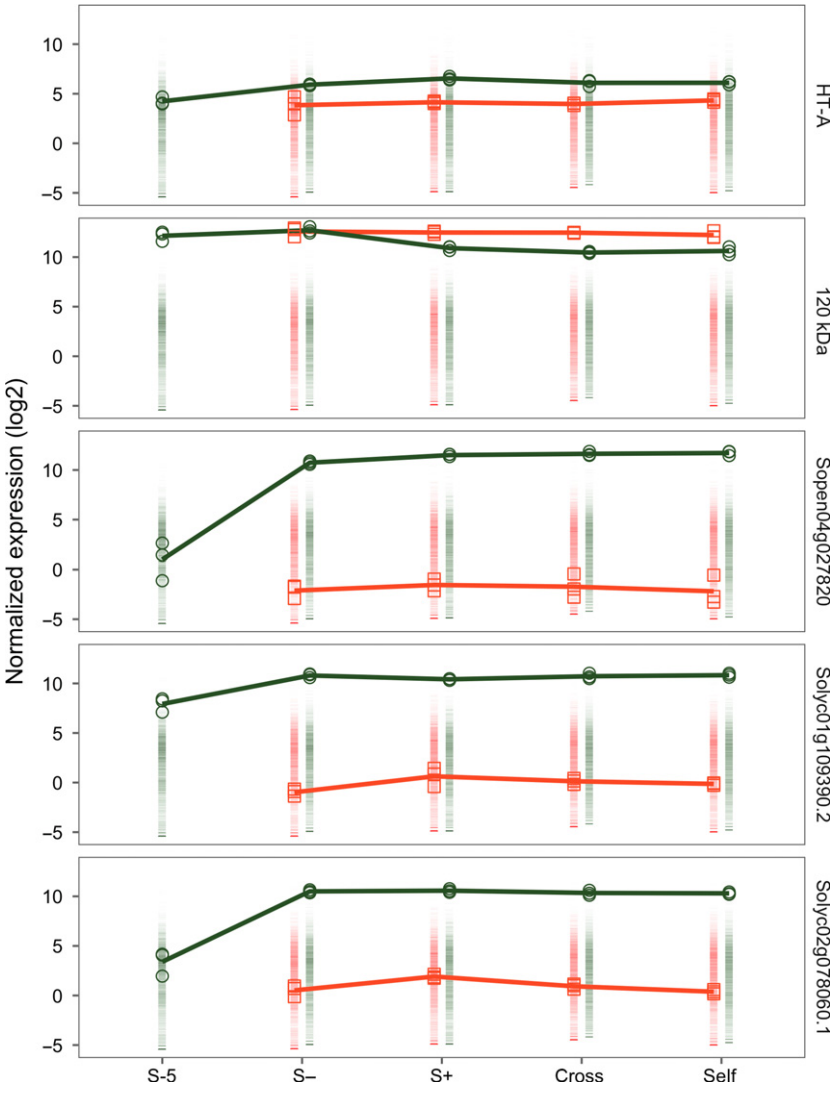

Fig. 2 Normalized expression (in log2 units) of five candidate genes in the styles of *Solanum lycopersicum* (orange squares) and *Solanum pennellii* (green circles) at five pistil developmental stages: unpollinated at 5, 1 and 0 days prior to anthesis [(S−5), (S−) and (S+), respectively], cross-pollinated ('cross') and self-pollinated ('self'). Lines are drawn between the mean expression levels at each stage. For comparison, horizontal dashes show mean expression levels of 2600 random genes from our data set (~10% of all genes analysed) in each tissue and species.

almost identical to lyc^{S-} or lyc^{S-} vs. lyc^{S+} or lyc^{S-} ($\rho = 0.81–0.82$). That is, mature non-UI styles do not show greater transcriptome-wide expression resemblance to immature UI styles. Instead, pistils from non-UI and UI species tend to resemble their own species' expression profiles throughout their development.

Discussion

Understanding the genetic mechanisms that underlie the formation of prezygotic reproductive barriers is a critical aspect of understanding how species barriers evolve and how they act during interspecific hybridization events. We used whole-transcriptome RNA-seq to investigate patterns of global gene expression that accompany competence for and expression of active heterospecific pollen rejection in the female reproductive tract, in part to evaluate known loci involved in reproductive behaviours, and to identify new candidate loci.

Whole-transcriptome analysis reveals strong candidate genes for postmating prezygotic isolation

Our analysis identified 1587 loci that exhibited significant changes in expression associated with UI competence. Many genes showing the strongest expression increases and decreases are consistent with a general mobilization of cell wall degradation enzymes and reduction in degradation enzyme inhibitors, respectively. This observation suggests that our whole-transcriptome analysis identified a diverse range of downstream effector molecules that might be responsible for pollen tube arrest, as well as some potential inhibitory regulators. In addition to these likely downstream expression changes, however, our analyses

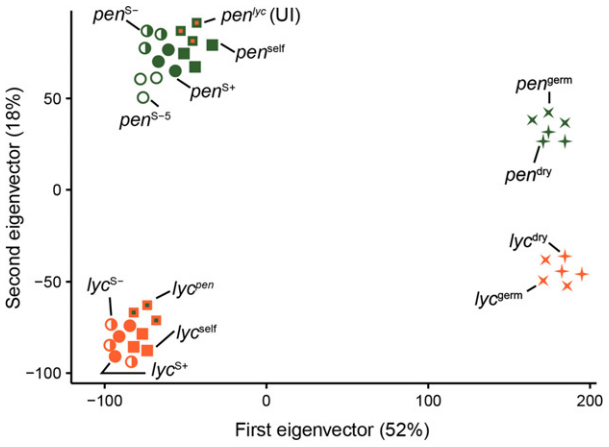

Fig. 3 Transcriptome-wide patterns of gene expression (summarized by a Principal Component Analysis) for *Solanum lycopersicum* and *Solanum pennellii* germinated and dry pollen (lyc^{germ}, pen^{germ}, lyc^{dry}, pen^{dry}), unpollinated pistils at 5 (*S. pennellii* only), 1 and 0 days before flower opening (pen^{S-5}, lyc^{S-}, pen^{S-}, lyc^{S+}, pen^{S+}), self-pollinated pistils (lyc^{self}, pen^{self}) and cross-pollinated pistils (lyc^{pen}, pen^{lyc}). Most variance in gene expression occurs between tissues (first eigenvector, horizontal axis), and secondarily between species (second eigenvector, vertical axis).

also presented the opportunity to evaluate the expression profiles of a set of a priori candidates and to identify new candidates for a direct functional role in enabling UI competence. We successfully identified several very strong causal candidates for pollen–pistil incompatibility, including genes known to be associated with both SI and UI responses, whose likely involvement in UI between this species pair is also bolstered by previous developmental, observational, and quantitative trait locus (QTL) mapping data on the UI phenotype.

Most notably, we found a significant increase in the expression of *HT-A*, a locus known to contribute to SI in wild tomato species and in other Solanaceous groups (Murfett *et al.* 1996; McClure *et al.* 1999; Hancock *et al.* 2005; Li & Chetelat 2010). Several lines of evidence have previously implicated *HT-A* in the expression of UI barriers, including in this specific species pair. First, the presence/absence of HT protein expression is associated with the presence/absence of UI responses among multiple wild tomato accessions, including SC accessions that lack functional S-RNase. For example, four SC wild tomato species that lack UI barriers also lack HT protein expression, whereas two SC species that have UI barriers also express *HT-A* protein (Kondo *et al.* 2002a,b); all tested SI tomato species express both *S-RNase* and *HT-A* (Covey *et al.* 2010). Second, the accumulation of *HT-A* protein in the style coincides spatially and temporally with the phenological development of UI competence in *Solanum pennellii* pistils (Chalivendra *et al.* 2013). Third, when functional *S-RNase* and *HT-A* are both transformed into *Solanum lycopersicum* (which lacks functional copies of both loci), this species is able to mount a UI rejection response against other species, whereas functional *S-RNase* is individually insufficient to restore UI (Tovar-Méndez *et al.* 2014). Fourth, *HT-A* (Chr. 12 at 62.53 Mb) colocalizes with a known pistil-side QTL for UI—named *ui12.1* (45.3–65.0 Mb)—previously mapped between *S. lycopersicum* and *Solanum habrochaites* (a wild tomato species that is sister to *S. pennellii*; Bernacchi & Tanksley 1997; Pease *et al.* 2016). Finally, in a segregating F_2 population between *S. lycopersicum* LA3475 and an SI accession of *S. pennellii* (LA3778; tgrc.ucdavis.edu), the presence of *HT* protein in the styles of F_2s is associated with a significantly more rapid UI rejection phenotype in these individuals (C. P. Jewell & L. C. Moyle, unpublished data). In conjunction with these lines of evidence, our RNA expression data here point strongly towards *HT-A* as a specific molecular player in the expression of postmating prezygotic UI in this species pair.

Apart from *HT-A*, in terms of pistil-side proteins, we also found a modest but significant decrease in the expression of the ortholog of the 120 kDa glycoprotein known to bind to S-RNases in *Nicotiana* (Hancock *et al.* 2005). Although this locus has not previously been investigated molecularly in tomato, RNAi experiments in *Nicotiana* hybrids indicate that suppression of 120 kDa in pistils will abolish an SI reaction, but RNAi individuals retain the ability to mount a UI rejection response (Hancock *et al.* 2005). In conjunction with our finding that 120 kDa has reduced expression specifically in UI-competent tissues, these observations suggest that 120 kDa might be contributing to the UI response in this species pair, although interpreting its possible functional role is currently less transparent than the case for *HT-A*.

We also identified several new gene candidates for involvement in UI. The most highly differentially expressed gene in UI-competent pistil tissues is an *S. pennellii*-specific PMEI (*Sopen04g027820*). This gene does not appear in the *S. lycopersicum* reference genome, but does appear in the genomes of other SI tomato species (Pease *et al.* 2016) along with SI potato and tobacco. As their name suggests, PMEIs act to inhibit pectin methylesterases (PMEs)—cell wall enzymes with critical roles in cellular adhesion and elongation. Moreover, as PMEs and PMEIs are known to interact within pollen tubes and ovaries in other angiosperms (Leroux *et al.* 2015; Levesque-Tremblay *et al.* 2015a,b), this gene represents a strong new candidate for investigation.

The second new candidate is a locus on chromosome 2 containing a cluster of four genes that all produce

glycoproteins that bind S-RNases and interact with growing pollen tubes. This locus includes one of our a priori candidates, 120 kDa, and two TTS (Transmitting Tract Specific)-like genes; however, the most suggestive gene encodes a *PELPIII*-like protein (*Solyc02g078060* + *Solyc02g078070*/*Sopen02g022900*) and appears in our top 20 loci with the largest expression increases in UI-competent tissues. *PELPIII* itself is known to be required for interspecific pollen rejection between some *Nicotiana* species (Eberle *et al*. 2013). These general functional features, including its specific involvement in interspecific pollen–pistil barriers in another Solanaceous system, its elevated rate of molecular evolution, and the likely presence of a premature stop codon in the *lyc* allele of this gene, make this candidate particularly intriguing for future functional analysis. In addition to these loci, other new candidates that we highlight above include a *PVR-like LTP* on chromosome 1—a protein from a class of genes with specific involvement in cell wall-loosening activity in mature *Nicotiana* styles (Nieuwland *et al*. 2005)—and a cluster of three *CRP*s on chromosome 7 that encode peptidase inhibitors, a class of genes previously implicated in *Nicotiana* SI pollen rejection (Busot *et al*. 2008; Jiménez-Durán *et al*. 2013).

Expression of different tissue types can be successfully differentiated in 'mixed' origin tissues

Our analysis also demonstrated that gene expression from tissues of different origin could be differentiated when assessed in a combined pool. Even with very low genetic divergence between species (~1.4%; Bolger *et al*. 2014)—and therefore few nucleotide positions differing within each read—we were able to differentiate pollen- and style-specific reads in stylar tissue containing growing pollen tubes. Pollen reads made up on average 44% of reads (IQR 17–71%) in the 1029 genes where both pollen and style read contributions to the mixed samples could be differentiated by sequence. This means that our ability to differentiate pollen from style expression for a given gene is driven in part expression levels in pollen. This is expected, since in mixed tissues where pistil cells represent the vast majority of tissue, pollen expression would have to be relatively high to make up a substantial proportion of overall transcripts sequenced.

Our study also demonstrates that the moderate sequencing depth (30–50×) at which our samples were sequenced is sufficient to determine the specific tissue source for thousands of reads and over 1500 genes. Given that expression was differentiable between pollen and style for all three pen^{lyc} samples in only 13.8% of genes with expression in any pollen sample (1029/7435 genes expressed in germinated pollen alone), clearly additional sequencing depth might recover additional pollen transcripts. However, the exact amount of additional depth required is difficult to predict given that diminishing returns are likely in attempts to capture additional pollen genes. Studies involving more divergent species would also likely have higher numbers of differentiable genes, as there are more diagnostic variants per read with which to determine expression.

Using gene expression data to identify the genetic basis of species barriers

Our study is not the first to examine patterns of gene expression in order to understand the genetic basis of species reproductive barriers. A suite of prior studies that examine gene expression in parental species and their dysfunctional hybrids (Ortíz-Barrientos *et al*. 2006; Artieri & Singh 2010; Good *et al*. 2010; Renaut & Bernatchez 2011; Barreto *et al*. 2015) have had variable success in terms of uncovering causal mechanisms of hybrid inviability and infertility. In some cases, consistent global patterns of regulatory disruption can reveal clear inferences about the genetic causes of hybrid problems (e.g. Barreto *et al*. 2015). For example, transgressive overexpression was found to localize to the X-chromosome in sterile but not fertile male mouse hybrids (Good *et al*. 2010), suggesting that sterility was due to disrupted postmeiotic sex-chromosome inactivation—a mechanism confirmed with further work (Campbell *et al*. 2013). However, when numerous genomewide expression changes are associated with low-fitness hybrid genotypes, differentiating the causal loci from expression changes that are the downstream consequences of disrupted molecular interactions becomes extremely challenging (Malone *et al*. 2007; Artieri & Singh 2010; Renaut & Bernatchez 2011). In these cases, inferences about the gene expression changes associated with species barriers can only be drawn at the most general level, especially in the absence of a priori candidate loci (Guerrero *et al*. 2016).

In contrast, here our analysis appears to have been successful in describing global patterns of gene expression associated with unilateral pollen–pistil incompatibility and in identifying specific causal candidates. In part, this was clearly facilitated by prior information on relevant developmental and phenotypic transitions, QTL mapping of UI phenotypes, and a list of suggestive a priori functional candidates that included proteins already known to contribute to this phenotype in other species. For example, prior information that the developmental appearance of UI competence is localized to later stage *S. pennellii* pistils (Bedinger *et al*. 2011) allowed us to explicitly contrast gene expression in tissues with and without the relevant competence. In

contrast, the relevant tissues and/or developmental stages are not always evident in cases of generalized hybrid dysfunction. Similarly, our analysis might have been facilitated by the fact that our reproductive isolating barrier is thought to be the product of active coordinated rejection, rather than the generalized product of dysfunctional or suboptimal interactions (as is likely in some postzygotic hybrid problems). Priori studies suggest that the molecular machinery involved in UI rejection is largely in place prior to (and even regardless of) this phenotypic response being triggered (Covey *et al.* 2010; Bedinger *et al.* 2011). Consistent with this, our transcript analysis indicates that specific activation of UI rejection (only in *S. pennellii* pistils pollinated with *S. lycopersicum* pollen) involves relatively modest changes in gene expression, functionally indicative of mostly downstream effects involved in arresting pollen tubes. This 'all rejection players present' behaviour indicates that pistils are actively primed for rejection responses, rather than pollen tube failure being the passive consequence of divergent pollen–pistil interactions.

Our analysis was also aided by data from both classical crossing studies (Lewis & Crowe 1958), and contemporary molecular and functional studies (see above) that have implicated molecular players from SI in the expression of UI, in both S-RNase-dependent and S-RNase-independent instances. These observations provide us with clear a priori candidates for our trait, some of which are strongly (e.g. *HT-A*) or more provisionally (e.g. 120 kDa) implicated in our study, and some of which are excluded from involvement in UI in this species cross (see Supporting information). They also provide an a priori expectation that some of the relevant trait differences between species might be due to changes (especially reductions/losses) in gene expression, the very changes that are most likely to be detected in an analysis of transcript abundance. For example, transitions from SI to SC are frequently accompanied by reduced or abolished expression of *S-RNase*, changes that are more likely be detected in gene expression analyses (provided that this loss is not entirely post-transcriptional) compared to structural alterations of protein function. Four of the five most promising candidates identified here (Fig. 2) also appear to involve a substantial reduction in gene expression in the SC lineage, mirroring trait losses that are typically associated with the loss of SI. Finally, these mechanistic connections between UI and SI indicate that our target reproductive isolating barrier shares similarities with some behavioural traits that are thought to contribute to species sexual isolation (e.g. Chung *et al.* 2014; Yeh & True 2014) in that intraspecific mate choice (in our case, via genetic SI) and species recognition (via UI) involve an overlapping suite of traits and/or mechanisms. Our postmating prezygotic isolating barrier might therefore be more analogous mechanistically to premating isolating traits (including those that mediate plant premating isolation, e.g. Hoballah *et al.* 2007; Hopkins & Rausher 2011; Xu *et al.* 2012), than to postzygotic isolation barriers.

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L.C.M., M.W.H, J.B.P., and N.A.S. designed the experiment; N.A.S. generated the experimental material; J.B.P. and R.F.G. conducted the bioinformatics and analyzed the data with input from M.W.H. and L.C.M; and J.B.P. and L.C.M. wrote the paper with contributions from R.F.G. and M.W.H.

Data accessibility

Data, Python scripts and R analysis scripts are available on Dryad (http://dx.doi.org/10.5061/dryad.j3s5r). Raw sequences are available in NCBI SRA under BioProject# PRJNA309342.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Supplementary Materials and Methods, and Results.

Appendix S2 Supplementary data files.

Fig. S1 Expression in the Top-20 UI upregulated genes by tissue.

Fig. S2 Expression in the Top-20 UI downregulated genes by tissue.

Fig. S3 Correlation in rank order of genes between full model and one comparing only UI-nonUI in unpollinated styles.

Table S1 Pairwise Spearman correlations between samples.

Table S2 Top-50 upregulated in UI-competent tissues.

Table S3 Top-50 downregulated in UI-competent tissues.

Table S4 Genes differentially expressed in pen^{lyc} samples vs. other UI-competent tissues.

Table S5 Genes differentially expressed in pen^{lyc} vs. non-UI pollinated styles.

Table S6 Top-20 UI vs. non-UI, for pollen-specific read counts.

Table S7 Top-20 UI vs. non-UI, for style-specific read counts.

Table S8 Genes that show a pollen and style response in UI cross.

Table S9 Expression in 45 a priori candidates.

Table S10 Top-50 upregulated in pollen of *Solanum pennellii* with respect to *Solanum lycopersicum*.

Table S11 Top-50 downregulated in pollen of *Solanum pennellii* with respect to *Solanum lycopersicum*.