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Adaptive molecular evolution of a defence gene in sexual but not functionally asexual evening primroses

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Abstract

Theory predicts that sexual reproduction provides evolutionary advantages over asexual reproduction by reducing mutational load and increasing adaptive potential. Here, we test the latter prediction in the context of plant defences against pathogens because pathogens frequently reduce plant fitness and drive the evolution of plant defences. Specifically, we ask whether sexual evening primrose plant lineages (Onagraceae) have faster rates of adaptive molecular evolution and altered gene expression of a class I chitinase, a gene implicated in defence against pathogens, than functionally asexual evening primrose lineages. We found that the ratio of amino acid to silent substitutions (K_a/K_s = 0.19 vs. 0.11 for sexual and asexual lineages, respectively), the number of sites identified to be under positive selection (four vs. zero for sexual and asexual lineages, respectively) and the expression of chitinase were all higher in sexual than in asexual lineages. Our results are congruent with the conclusion that a loss of sexual recombination and segregation in the Onagraceae negatively affects adaptive structural and potentially regulatory evolution of a plant defence protein.

Introduction

Sex is the dominant mode of reproduction among eukaryotic life, but why sex prevails over asexual reproduction is among the most important unresolved and contested questions in biology (Bell, 1982; Barton & Charlesworth, 1998; Salathé *et al.*, 2008b; Otto, 2009; Becks & Agrawal, 2010; Lively, 2010). Despite being less common, asexual reproduction is widespread across the tree of life and is associated with many ecological and evolutionary advantages over sexual reproduction. For example, asexual individuals can avoid ecological and genetic costs of sex by reproducing faster ('cost of males', Maynard Smith, 1978), and they also exhibit greater relatedness to offspring ('cost of meiosis', Williams, 1975; Lively & Lloyd, 1990). In contrast, the prevalence of sex is attributed to evolutionary advantages associated with recombination and segregation of alleles, which are greatly suppressed or completely absent in asexual individuals. For example, evolutionary theory predicts that recombination and segregation enable more effective purging of deleterious mutations (Muller, 1964; Lynch & Gabriel, 1990) and promote beneficial (adaptive) allele combinations (Muller, 1932; Agrawal, 2006; Otto, 2009). There has been some empirical work in animal systems, showing that asexual lineages have the propensity to accumulate more deleterious mutations than related sexual lineages (Paland & Lynch, 2006; Johnson & Howard, 2007; Neiman *et al.*, 2010), but we are unaware of any studies that examine whether asexual lineages also experience slower rates of adaptive molecular evolution (positive selection) than related sexual lineages.

Natural enemies of plants are ubiquitous in nature and can have major negative fitness consequences for host species. Under such circumstances, natural enemies are expected to impose selection on host species to evolve adaptive defence traits that help them avoid attack and/or minimize negative fitness consequences of attack

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(Ehrlich & Raven, 1964; Zangerl & Berenbaum, 2005; Stenberg *et al.*, 2006). Understanding the effects of recombination and segregation on the molecular evolution of host defence genes could provide key insights into whether sexually reproducing hosts have an advantage over asexually reproducing hosts when responding to natural enemies and whether sex promotes adaptive molecular evolution. For example, if plants adapt to natural enemies by altering proteins that recognize or attack natural enemies, then sexual lineages should exhibit stronger signatures of positive selection on defence proteins than asexual lineages. Such adaptive changes might not only involve structural changes to proteins (e.g. amino acid replacements in coding regions) but might also rely on changes in regulatory regions or a combination of these factors (Doebley, 1993; Purugganan, 1998; Hoekstra & Coyne, 2007).

Here, we first show that pathogens can potentially exert selection on Onagraceae species and then we test whether a loss of sexual recombination and segregation in plants negatively impacts adaptive molecular evolution in a plant gene involved in defence against pathogens. We focus on a class I basic chitinase (*chiB*) because plant chitinases are well studied and have a clear function in plants. Specifically, plant chitinases are disease resistance enzymes that are present in virtually all plants, and they aid in both the recognition and attack of fungal pathogens (Collinge *et al.*, 1993; Kasprzewska, 2003; Ferreira *et al.*, 2007). *ChiB* also offers an excellent model to test whether recombination and segregation (two fundamental components of sex) promote adaptive molecular changes within a plant defence gene because previous work on plants, without regard to variation in sexual reproduction, showed that specific amino acid sites within *chiB* are under positive selection (Bishop *et al.*, 2000; Tiffin, 2004). We test our hypotheses by examining species belonging to the evening primrose family (Onagraceae). This family is an ideal plant system for testing the effects of sex on plant defences because it contains many sexual and functionally asexual species, where a breakdown in sex arises because of a genetic system called permanent translocation heterozygosity (PTH). PTH species retain fertilization but experience a near-complete suppression of both recombination and segregation such that all seeds produced by a maternal plant are genetically identical (Stebbins, 1950; Cleland, 1972; Rauwolf *et al.*, 2008, 2011). Furthermore, there have been many (> 20) independent transitions between sexual and functionally asexual (PTH) reproduction in the Onagraceae (Holsinger & Ellstrand, 1984; Johnson *et al.*, 2009, 2011), which allows for a naturally replicated and robust test of the effects of sex on plant defence evolution.

Based on evolutionary theory, we predicted that the repeated losses of recombination and segregation in the Onagraceae cause decreased rates of adaptive molecular evolution in *chiB*. To test this prediction, we applied maximum-likelihood comparative phylogenetic approaches to infer rates of structural molecular evolution of the *chiB* enzyme across 16 sexual and 16 asexual evening primrose (Onagraceae) plant lineages. We further compared *chiB* gene expression across a subset of these lineages to determine whether a loss of sex is also associated with changes in regulatory evolution.

Materials and methods

Pathogen severity and plant fitness

We examined plant fitness and the severity of damage by a generalist foliar pathogen, powdery mildew (*Erysiphe* sp.), on different genotypes of *Oenothera biennis* (a functionally asexual PTH species) to determine whether pathogens could select on genetic variation in resistance. We measured the severity of damage on 12 different genotypes of *O. biennis* planted at a single field site (University of Toronto's Koffler Scientific Reserve) 50 km north of Toronto, Canada, a relatively high-latitude site for this and other *Oenothera* species. These plots were established for a separate study, and detailed methods of the experimental design are provided in this previous study (Johnson *et al.*, 2006). Pathogen severity was not previously reported but was measured as percentage leaf area covered with *Erysiphe* spp. on the upper surface of the first 15 leaves, counting from the base of the plant, where area covered was scored between 0 and 4 (0 = no mildew, 1 = 1–25%, 2 = 25.1–50% covered, 3 = 50.1–75% and 4 = 75.1–100%). Plant fitness was measured as lifetime total number of fruits produced.

Plant and genetic material

Total genomic DNA was extracted from 30 *Oenothera* and 2 *Gayophytum* spp. (representing 16 PTH and 16 sexual species; Fig. 1) following standard CTAB protocols. For RNA extraction and cDNA synthesis, we first grew replicate plants of 25 *Oenothera* spp. (Fig. 1) at the same time and under controlled environmental conditions in a randomized design. Then, for all plants, we harvested leaf tissue from the tips of rapidly expanding true leaves of seedlings when they reached the four-leaf stage by immediately placing tissue in liquid nitrogen. Total RNA was extracted using Qiagen's RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with minor modifications to remove interfering mucilage and polyphenols (Greiner, 2008). RNA concentration and purity were determined spectrophotometrically (Nanodrop 2000c, Thermo Scientific, Wilmington, DE, USA), and 150 ng of total RNA from each sample was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen, Karlsruhe, Germany) and an Oligo$(dT)_{24}$ primer (GeneRacer Kit; Invitrogen). Residual RNA complements were removed from the synthesized cDNA by incubation with RNase H (Invitrogen, Carlsbad, CA, USA). RNA extraction and cDNA synthesis were performed for all samples simultaneously.

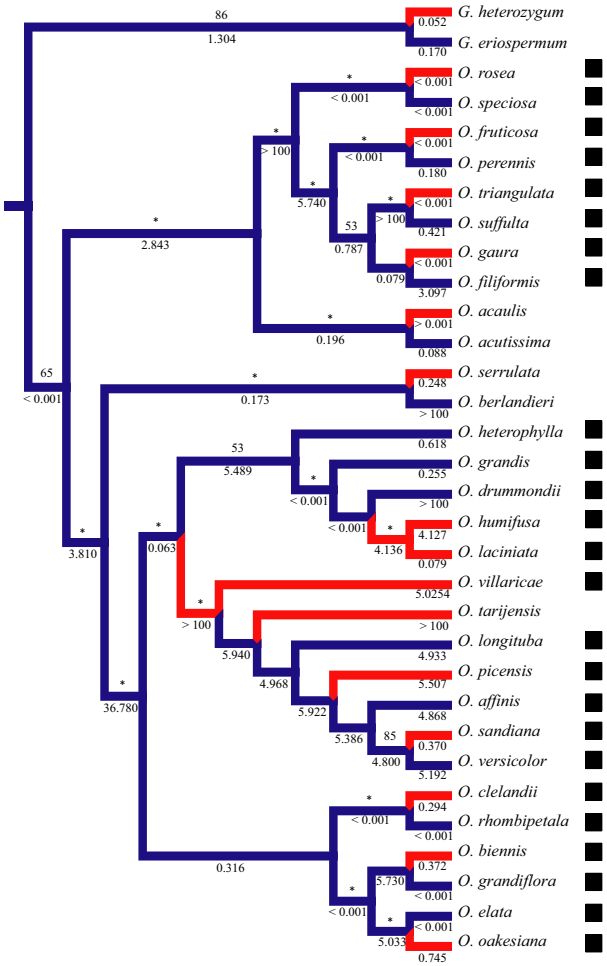

Fig. 1 Evolutionary history of sexual and functionally asexual permanent translocation heterozygote (PTH) reproduction in the Onagraceae, adapted from the study of Johnson *et al.* (2009). Transitions between sexual (blue/dark grey in print edition) and PTH (red/mid-grey in print edition) reproduction are shown, and Bayesian posterior probabilities are shown above branches and are indicated when > 50%; *posterior probability > 95%. Rates of molecular evolution ($\omega = K_a/K_s$) are displayed below each branch and were estimated in CODEML from a model that allowed each branch to evolve independently (lnL = −3172.75, d.f. = 63). Squares show species used in gene expression analyses.

Molecular sequence data

We used a partial *Oenothera chiB* sequence (Mráček *et al.*, 2006, GenBank accession FG122686.1) and the *Arabidopsis* class I orthologous chitinase sequence (Samac *et al.*, 1990, GenBank accession M38240) to design primers to amplify a 756-bp coding region corresponding to class I basic chitinase (*chiB*) from our samples (Table S1). PCR products were cloned into a pGEM®-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* grown on LB with ampicillin to facilitate blue-white screening of successfully transformed colonies. Cloned products were PCR-amplified with M13 forward and reverse primers and sequenced in both directions. Initial sequencing of eight clones from each of a phylogenetically diverse set of eight species showed that *chiB* is likely present as a single copy gene within our species (no individual showed > 2 alleles/locus). We therefore amplified and sequenced the forward and reverse sequences of four colonies per species for all 32 species. All sequencing was performed using an ABI 3730xl capillary sequencer following the ABI Big Dye terminator sequence protocol (ABI Biosystems, Foster City, CA, USA). Raw sequence data were edited using SEQUENCHER 4.10.1 software (Gene Codes Corporation, Ann Arbor, MI, USA), and consensus sequence data were generated for each species. Consensus sequences of the coding region for all 32 species (756–777 bp) were aligned with CLUSTALW followed by manual manipulations in BioEdit (Hall, 1999); the nucleotide alignment used for subsequent analysis is provided (Table S2), and sequences are available as GenBank accessions and in Dryad (doi:10.5061/dryad.sk94j). Lastly, to compare our results for *chiB* with a gene not involved in defence, we also examined molecular evolution of cytosolic phosphoglucose isomerase (*PgiC*), a gene that is known to play a highly conserved role in plant metabolism of glucose into pyruvate during ATP synthesis. The sequence data used for this analysis were taken directly from the study of Johnson *et al.* (2009), where 459 bp of coding sequence was available for 10 sexual and 10 PTH species.

RT-qPCR expression data

A single primer pair was designed from a highly conserved region of *chiB* to amplify the gene across all species (Table S1). After screening several potential reference control genes, we selected elongation factor 1a (*EF1α*) as our control gene because analysis showed that it gave the most consistent amplification and expression results for the species considered in this study (Supporting information). For each species and gene, we assayed expression from two to four biological replicates (individual plants) and two technical replicates per biological replicate. We also included 'no-template' controls to detect contamination. All RT-QPCRs were performed in a Stratagene Mx 3000P (Stratagene, La Jolla, CA, USA) as a 25-μL reaction, which contained 2× Brilliant II SYBR Green QPCR mastermix (with low ROX; Stratagene), 0.2 μM of each forward and reverse primers and 20 ng RNA equivalent of cDNA. PCR cycling conditions followed normal protocols (Table S1).

Statistical analysis

Selection by pathogens

To determine whether pathogens affect plant fitness and could act as selective agents, we first analysed pathogen

severity and plant fitness data separately to obtain mean values for each genotype using generalized linear models with type III error of SAS (Proc GLM; SAS Institute, Cary, NC, USA) as described in the study of Johnson *et al.* (2006). We then correlated mean genotype pathogen damage and relative plant fitness values to calculate a standardized selection gradient on resistance to the pathogen (Lande & Arnold, 1983).

Molecular sequence evolution

We tested for selection on *chiB* using maximum-likelihood methods implemented in CODEML of the PAML package (Yang, 2007). The input tree for all analyses was an unrooted tree generated using protein coding and noncoding sequences from five genes (Johnson *et al.*, 2009) and does not include sequence information from *chiB* analysed here. We chose this phylogeny because relationships between taxa were well supported (e.g. high Bayesian posterior probabilities) and the mode of reproduction (sexual or PTH) could be reliably mapped onto the phylogeny. This ancestral state reconstruction showed at least 12 independent transitions between sex and PTH, as published in the study of Johnson *et al.* (2009) and shown in Fig. 1. Comparisons of the ratio of nonsynonymous (K_a) to synonymous (K_s) substitution rates ($\omega = K_a/K_s$) among lineages and codon sites were used to infer the type of selection acting on *chiB*, where $\omega < 1$ corresponds to purifying selection, $\omega = 1$ corresponds to neutral evolution and $\omega > 1$ corresponds to positive selection (Li *et al.*, 1985; Nei & Gojobori, 1986; Yang & Bielawski, 2000). We used three different types of models to address the questions of our study: 'branch models', 'site models' and 'branch-site models', and the goodness of fit of different nested models was assessed using likelihood ratio tests.

Branch models test whether different lineages are evolving at different rates by allowing for variable ω among branches in the tree, but invariable ω among different amino acid sites (Yang, 1998; Yang & Nielsen, 1998). We used a one-ratio model as the null model to determine the average overall selection pressure acting upon *chiB* (H0, one estimate of ω), and then we compared this model with two alternative models (tests 1 and 2), both of which allowed for variable ω between PTH (ω_{PTH}) and sexual (ω_{SEX}) lineages. The alternative model in test 1 was a two-ratio model (H1, two estimates of ω: ω_{PTH} and ω_{SEX}), in which the character states (PTH or SEX) of internal branches of the phylogeny were estimated as shown in Fig. 1. To determine whether the estimated character state of the internal branches influenced overall estimates of ω between PTH and sexual lineages, we also compared H0 (test 2) with a three-ratio model (H2, three estimates of ω: ω_{PTH}, ω_{SEX} and $\omega_{internal}$). Although branch models test whether ω varies among lineages, it is not clear whether any observed differences are due to difference in K_a, K_s or both. To address this limitation, we obtained estimates of nonsynonymous (K_a) and synonymous (K_s) substitutions for each lineage using a model in CODEML that allowed each branch to evolve independently, and then we examined the effects of sex on these rates with analysis of variance (ANOVA) tests.

Site models account for different functional or structural constraints experienced by individual amino acid sites within a protein by allowing for variable ω among sites, but invariable ω among branches (Nielsen & Yang, 1998; Yang *et al.*, 2000). These models estimate the strength of selection and the proportion of amino acid sites that experience purifying (ω_0), neutral (ω_1) and/or positive (ω_2) selection. Following Yang *et al.* (2000), we made several comparisons to test for rate heterogeneity among amino acid sites. First, we compared the one-ratio model (H0) with a discrete model (M3, three estimates of ω), which allows for purifying, neutral and positive selection to act on sites (test 3). Next, because we detected variation in selection among sites, we made two additional comparisons. First, we compared the nearly neutral model M1a (two estimates of ω for purifying and neutral selections) with the positive selection model M2a (three estimates of ω for purifying, neutral and positive selections), which also allows for positive selection (test 4). Second, we compared the M7 model that assumes a β distribution of class sites that range from purifying selection to neutral evolution ($0 < \omega < 1$) with the continuous M8 model, which has an additional class of sites with $\omega > 1$ that are under positive selection (test 5).

We used branch-site models to determine whether certain sites are under positive selection in only PTH or sexual lineages, because these models simultaneously account for heterogeneity in selection between different lineages and across amino sites (Yang & Nielsen, 2002; Zhang *et al.*, 2005). In all branch-site models, we used a phylogeny with the topology and mode of reproduction (PTH or sexual) mapped, as published in the study of Johnson *et al.* (2009) and shown in Fig. 1, because likelihood ratio tests between various models showed that this character state topology best fits the data (Table 1; tests 1 and 2). We used the branch-site model A (Zhang *et al.*, 2005) as an alternative hypothesis with both PTH and sexual lineages set as foreground branches because only the foreground branches are allowed to experience positive selection in CODEML. We tested these alternative hypotheses against two null hypotheses: the nearly neutral model (M1a) and the same branch-site model A, but with ω_2 fixed at 1 (Zhang *et al.*, 2005; tests 6–9). We also compared the new clade model C, which estimates ω_2 separately for different lineages without any constraints (e.g. ω_2 can be any value equal to or greater than zero), with the nearly neutral model (Yang, 2007; test 10), to determine whether any sites that are under positive selection in sexual lineages are also under positive selection in PTH lineages and vice versa. To assess whether any patterns of selection observed for *chiB* are specific to *chiB* or to genes

Table 1 Likelihood values and parameter estimates from PAML analyses of *chiB* across 32 Onagraceae species. The phylogeny and mapped character states used for these analyses are reported in the study of Johnson *et al.* (2009) and do not use sequence information from *chiB*. Results from comparative analyses that use a phylogeny derived from *chiB* sequence information are reported in Supporting information and Table S4.

Model	*fp*	lnL	Estimates of parameters†	Positively selected sites‡
H0: one-ratio	1	−3213.62	$\omega = 0.17$	None
Branch-specific models				
H1: two-ratio	2	−3211.34	$\omega_{PTH} = 0.11$, $\omega_{SEX} = 0.20$	NA
H2: three-ratio	3	−3211.06	$\omega_{PTH} = 0.10$, $\omega_{SEX} = 0.22$, $\omega_{internal} = 0.18$	NA
Site-specific models				
M3: discrete	5	−3091.25	$p_0 = 0.73$, $p_1 = 0.22$ ($p_2 = 0.05$) $\omega_0 = 0.02$, $\omega_1 = 0.40$, $\omega_2 = 3.12$	13*, 32**, 37**, 126**, 145**, 154**, 207**, 208**
M1a: nearly neutral	2	−3108.97	$p_0 = 0.87$ ($p_1 = 0.13$), $\omega_0 = 0.04$	NA
M2a: positive selection	4	−3093.65	$p_0 = 0.88$, $p_1 = 0.09$ ($p_2 = 0.03$) $\omega_0 = 0.05$,$\omega_2 = 3.96$	32**, 207**
M7: β	2	−3112.47	$p = 0.09$, $q = 0.44$	NA
M8: $\beta + \omega > 1$	4	−3091.05	$p = 0.19$, $q = 1.44$ $p_0 = 0.96$ ($p_2 = 0.04$) $\omega_2 = 3.30$	32**, 37*, 126*, 145*, 154*, 207**, 208*
Branch-site models				
Model A_null (PTH)	3	−3107.69	$p_0 = 0.86$, $p_1 = 0.12$ ($p_2 = 0.02$) $\omega_0 = 0.04$ ($\omega_1 = \omega_2 = 1$)	NA
Model A_alt (PTH)	4	−3106.77	$p_0 = 0.87$, $p_1 = 0.12$ ($p_2 = 0.01$) $\omega_0 = 0.04$, $\omega_2 = 2.89$	None
Model A_null (SEX)	3	−3106.15	$p_0 = 0.86$, $p_1 = 0.06$ ($p_2 = 0.08$) $\omega_0 = 0.04$ ($\omega_1 = \omega_2 = 1$)	NA
Model A_alt (SEX)	4	−3082.82	$p_0 = 0.88$, $p_1 = 0.09$ ($p_2 = 0.03$) $\omega_0 = 0.05$, $\omega_2 = 5.65$	32**, 126*, 154*, 207**
Clade Model C	5	−3082.48	$p_0 = 0.88$, $p_1 = 0.09$ ($p_2 = 0.03$) $\omega_0 = 0.05$, $\omega_{2PTH} = 0.05$, $\omega_{2SEX} = 5.69$ ($\omega_1 = 1$)	None

fp, number of free parameters; lnL, log-likelihood value for each model.

†Parameters estimated in models: In branch-specific models, ω_{PTH} and ω_{SEX} are the K_a/K_s ratios for permanent translocation heterozygote (PTH) and sexual lineages, respectively; In site-specific and branch-site models, p_0, p_1 and p_2 equal the proportion of sites with K_a/K_s ratios less than one (ω_0), roughly equal to one (ω_1) and greater than one (ω_2), and the ω values reflect the estimates of K_a/K_s for purifying (ω_0), neutral (ω_1) and positive (ω_2) selection, respectively. In branch models M7 and M8, site classes are drawn from a beta distribution with $\beta(p,q)$, where p and q are the shape parameters. Values in parentheses are not directly estimated but are determined by the relationship that $p_0 + p_1 + p_2 = 1$ or they are fixed in the model.

‡Positively selected sites, where $*P > 95\%$; $**P > 99\%$, following Bayes empirical Bayes analysis (M2a, M8, Model A_alt) or naïve empirical Bayes (M3). Final determination of significance followed likelihood ratio tests (Table 2).

showing positive selection, we repeated similar analyses in CODEML for a 'control' gene, *PgiC*, which a previous study had generated coding sequence data (459 bp) for 10 of the sexual and 10 PTH species included here (Johnson *et al.*, 2009). To determine whether sites that we identified to be under positive selection in the above analyses were also implicated to be under positive selection in different plant species with divergent histories, we aligned the *chiB* sequence from *O. grandiflora* (sexual species) to homologous sequences published for *Boechera* and *Zea* (Bishop *et al.*, 2000; Tiffin, 2004) to compare sites under positive selection.

Lastly, we performed several additional analyses to further determine whether (i) the topology and character state assignment of our phylogeny, (ii) variation in the rate of nonsilent substitutions (K_s) or (iii) recombination influenced the results of CODEML (Supporting information). These additional analyses are not as comprehensive as the analyses discussed above, but they have the advantage of allowing us to examine potential confounding effects that could arise from an incorrect phylogeny or from variation in the rates of nonsilent substitutions (K_s) or recombination; CODEML does not account for the latter. The results of these additional analyses were largely consistent with our analyses discussed above, indicating that our results are robust to assumptions made about the specific phylogeny and assumptions made by CODEML (see Supporting information). Therefore, we report the full results from these additional analyses online.

ChiB expression

Expression was quantified as the number of cycles needed for the amplification to reach a threshold fixed

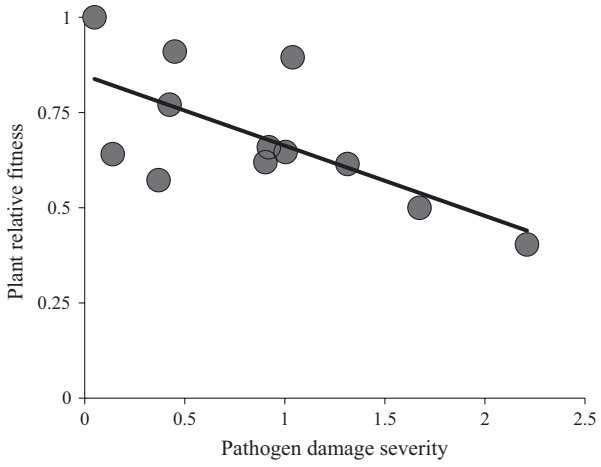

Fig. 2 Plant relative fitness as a function of mean foliar damage by powdery mildew (*Erysiphe* sp.) for 12 different genotypes of *Oenothera biennis*, a functionally asexual permanent translocation heterozygosity (PTH) species ($R^2 = 0.45$; $P < 0.02$).

in the exponential phase of the PCR (C_T value). For each species and each biological replicate, *chiB* expression was calculated as the average C_T value of *chiB* relative to the average C_T value of *EF1α*. Because preliminary analyses of the expression data (following methods of Blomberg *et al.*, 2003; Ives *et al.*, 2007) found that *chiB* trait evolution was independent of the species phylogeny (results available upon request), we compared expression levels between species with a standard one-way ANOVA.

Results and discussion

Selection by a pathogen on a PTH *Oenothera*

Both the severity of pathogen damage by *Erysiphe* sp. and plant fitness varied among the 12 *O. biennis* genotypes planted in the field. Plant fitness was significantly negatively genetically correlated with pathogen damage (Fig. 2), and in comparison with other studies of natural selection (Kingsolver *et al.*, 2001; Hersch & Phillips, 2004), the strength of genotypic selection imposed by pathogens was moderate to strong ($S' = ß' = -0.11$).

Adaptive evolution within sexual and asexual Onagraceae

When we averaged over all sites and lineages, we detected purifying selection acting on *chiB* (H0: $\omega = 0.17 < 1$, Table 1). However, when we allowed functionally asexual and sexual lineages to evolve separately, we found clear differences in molecular evolution of *chiB* between asexual PTH and sexual lineages. Specifically, the average rate of nonsynonymous to synonymous substitutions ($\omega = K_a/K_s$) was significantly lower for PTH lineages ($\omega_{PTH} = 0.11$) than for sexual lineages ($\omega_{SEX} = 0.19$; test 1, $P = 0.033$; Tables 1 and 2). Higher values of ω, even when less than one, can arise

Table 2 Likelihood ratio tests between different CODEML models in PAML (Table 1).

Test	Compared models (alt vs. null)	d.f.	−2ΔlnL	*P* value
1	H1 vs. H0	1	4.56	0.033
2	H2 vs. H0	3	5.12	0.077
3	M3 vs. H0	4	244.74	< 0.0001
4	M2a vs. M1a	2	30.65	< 0.0001
5	M8 vs. M7	2	42.84	< 0.0001
6	ModelA_ null vs. ModelA_alt (PTH)	1	1.84	0.1750
7	M1a vs. ModelA_alt (PTH)	2	4.40	0.1108
8	ModelA_ null vs. ModelA_alt (Sex)	1	46.66	< 0.0001
9	M1a vs. ModelA_alt (Sex)	2	52.30	< 0.0001
10	Clade Model C vs. M2a	3	52.98	< 0.0001

Test 1: tests for differences in ω between sexual and permanent translocation heterozygote (PTH) lineages, where character state of internal branches is estimated and shown in Fig. 1.
Test 2: tests for differences in ω between sexual and PTH lineages (tips), where the character state and ω of the internal branches are treated as an unknown and separate character state as the tips.
Test 3: tests whether there is rate heterogeneity among amino acid sites.
Test 4: tests whether rate heterogeneity among amino acid sites includes positive selection at some sites.
Test 5: tests whether positive selection, not purifying selection, occurs at some sites.
Test 6: tests whether positive selection occurs in PTH lineages; null model fixes $\omega_2 = 1$.
Test 7: tests whether positive selection occurs in PTH lineages; null model does not include ω_2.
Test 8: tests whether positive selection occurs in sexual lineages; null model fixes $\omega_2 = 1$.
Test 9: tests whether positive selection occurs in sexual lineages; null model does not include ω_2.
Test 10: tests whether rate heterogeneity among amino acid sites, which includes positive selection at some sites, differs between lineages.

from positive selection acting on a small number of specific amino acid sites. Consistent with this interpretation, we detected significant positive selection acting on four sites in sexual lineages ($\omega_2 = 5.65$, $P < 0.0001$ for tests 8 and 9; Tables 1 and 2), whereas zero sites exhibited significant positive selection in PTH lineages ($\omega_2 = 2.89$, $P > 0.1$ for tests 6 and 7; Tables 1 and 2). Furthermore, the sites identified to be under positive selection in sexual lineages appear to be under purifying selection in asexual lineages ($\omega_{2PTH} = 0.05$ and $\omega_{2SEX} = 5.69$, test 10). Among the four positively selected sites in sexual lineages, one was in the active-site cleft region of the protein, which is thought to be important in pathogen recognition (Bishop *et al.*, 2000). These results are congruent with the prediction that a loss of recombination and segregation severely constrains adaptive molecular evolution of a plant gene involved in defence against pathogens.

Differences in ω between asexual and sexual lineages may not be entirely dependent on differences in the frequency of positive selection on amino acid substitutions (Rausher *et al.*, 2008). For instance, higher overall ω in sexual lineages could also arise from differences in synonymous substitutions (K_s) with or without changes in nonsynonymous substitutions (K_a). We tested this possibility by examining variation in K_s and K_a among species. We found no evidence that K_s differed between PTH and sexual lineages ($F_{1,30} = 0.113$, $P = 0.739$; Table S3). Furthermore, additional phylogenetic analyses that explicitly incorporate variation of K_s into estimates of ω identified similar patterns of selection acting on *chiB* as our analyses in CODEML, which does not allow K_s to vary (Supporting information). In contrast, we found that K_a marginally differed between sexual and PTH lineages ($F_{1,30} = 3.102$, $P = 0.088$, Table S3) where K_a was three times higher in sexual species (0.006 ± 0.002) than in asexual PTH species (0.002 ± 0.002). Variation in K_a among lineages could be due to both the accumulation of beneficial (positively selected) and deleterious mutations (Kimura, 1977; Rausher *et al.*, 2008). The presence of four positively selected amino acid sites and the fact that deleterious mutations are predicted and observed to accumulate disproportionately within asexual lineages (e.g. Muller, 1964; Paland & Lynch, 2006; Neiman *et al.*, 2010), not within sexual lineages, both suggest that the latter explanation is less plausible than differences in positive selection. Cumulatively, our results are consistent with differences in positive selection between functionally asexual PTH and sexual lineages.

A nonmutually exclusive alternative explanation is that differences in the ecology between sexual and asexual species, aside from differences in adaptive constraint, contribute to the observed differences in protein evolution. For example, PTH species tend to occur at slightly higher latitudes than sexual species (Johnson *et al.*, 2010), and it has been argued that biotic selection pressures might be weakest at high latitudes (Schemske *et al.*, 2009; but see Moles *et al.*, 2011). Such a scenario could cause selection by pathogens to be stronger on sexual than on asexual populations. Although we acknowledge that ecological factors can influence molecular evolution, there are several reasons why this ecological mechanism is unlikely to fully explain our results. First, closely related sexual and PTH species often co-occur in the same habitat and location (Johnson *et al.*, 2010), where they are likely to be exposed to similar selection pressures by pathogens. Moreover, a complex assemblage of pathogens, including powdery mildews (*Erysiphe* spp.), leaf-spot fungus (*Septoria oenotherae*) and rusts (*Puccinia* spp.; Farlow & Seymour, 1888; Klaphaak & Bartlett, 1922; Dickey & Levy, 1979), attacks both sexual and PTH taxa throughout their ranges, including at high latitudes. Finally, we found that even at relatively high-latitude sites, PTH populations can experience strong selection by pathogens. It is not possible to test whether all ecological factors are roughly equal between lineages, but there is no *a priori* reason to suggest that such differences are consistent and explain our findings of positive selection within sexual but not within asexual lineages. For example, Onagraceae includes annual, biennial and perennial species, and there is no association between these different life history traits and rates of synonymous or nonsynonymous substitutions within *chiB* in our data set (Table S3). We therefore conclude that the most parsimonious answer to explain our results are that differences in recombination and segregation directly influenced the ability of populations to respond to selection on *chiB*.

Comparisons of the effects of sexual reproduction on genes expected to be under purifying selection

For genes under strong purifying selection, the opposite pattern of selection is expected where asexual lineages accumulate more deleterious mutations than sexual lineages, indicated by higher rates of nonsynonymous substitutions. The influence of sexual reproduction on the molecular evolution of the nonrecombining mitochondrial genome has been examined in three recent studies involving *Daphnia* and snails (Paland & Lynch, 2006; Johnson & Howard, 2007; Neiman *et al.*, 2010). These studies all reported that a loss of sex was associated with stronger purifying selection on proteins in sexual as opposed to asexual lineages ($\omega_{SEX} < \omega_{ASEX} < 1$), consistent with relaxed purifying selection and an accumulation of deleterious mutations in asexual lineages. This result is expected because mitochondria are essential for cellular respiration and the function of mitochondrial genes is highly conserved among eukaryotes. Therefore, virtually all nonsynonymous mutations are likely to be deleterious, and these mutations are predicted to be more efficiently purged by sexual than by asexual reproduction.

Although defence against pathogens can also be critical for a plant's survival, coevolution between plants and their parasites should cause selection on nuclear defence genes to be more dynamic than selection on mitochondrial genes. Moreover, nonsynonymous mutations of defence genes may be either beneficial or deleterious, and theory predicts that a loss of recombination and segregation (asexuality) negatively affects the fixation of beneficial mutations and thus adaptive molecular evolution of plant defence genes (Howard & Lively, 1994; Salathé *et al.*, 2008a). To assess whether asexual PTH Onagraceae lineages are also more likely than sexual Onagraceae lineages to accumulate deleterious mutations in genes under strong purifying selection and/or that are not directly related to defence, we ran similar analyses in CODEML for *PgiC* (data from Johnson *et al.*, 2010; Table S4). Overall, we found that $\omega = 0.17$ (identical to *chiB*), but there was no evidence for positive selection and ω did not significantly differ between lineages (Table S4). Furthermore, we have also conducted similar analyses for four genes in the flavonoid biosynthetic pathway, which overall are under much stronger purifying selection ($0.04 \leq \omega \leq 0.09$; E. I. Hersch-Green, H. Myburg & M. T. J. Johnson, unpublished data). Interestingly, for three of the genes, we found that ω was higher in the PTH than in the sexual lineages, a pattern that would be expected if deleterious mutations accumulate disproportionately in functionally asexual PTH lineages (E. I. Hersch-Green, H. Myburg & M. T. J. Johnson, unpublished data). Our results combined with previous studies provide empirical support for theoretical predictions that recombination and segregation have a potential adaptive benefit for organisms by both increasing the fixation of beneficial mutations and eliminating deleterious mutations.

Evolution of chitinase among plant lineages

Molecular evolution of chitinases has been studied in other plant lineages but without regard to variation in sexual reproduction. When we ignore differences in reproductive mode (asexual vs. sexual), we found that between two (M2a), seven (M8) or eight (M3) amino acid sites were evolving under significant positive selection in the Onagraceae [ω_2 (M3) = 3.12, ω_2 (M2a) = 3.96, ω_2 (M8) = 3.30; tests 2–4, $P < 0.0001$; Tables 1 and 2]. To determine how the pattern of positive selection that we observed compared with other plant lineages, we aligned a 230-AA (690 bp) region of our Onagraceae *chiB* sequence to orthologous sequences in dicot (*Boechera* chiB, from Bishop *et al.*, 2000) and monocot lineages (*Zea* chiI, from Tiffin, 2004; Fig. 2). Our alignment started after the protein's hinge region because there were few similarities across genera in the hinge region and *N*-terminal domain, making it difficult to align across genera. Within this 230-AA region, approximately 3% (7/230, Fig. 2) of the amino acid sites within Onagraceae were under strong positive selection [ω_2 (M3) = 3.12 or ω_2 (M8) = 3.30, Table 1], as compared to approximately 4% (9/230, Fig. 3) in *Boechera* ($\omega_2 = 6.8$, Bishop *et al.*, 2000) and approximately 8% (19/230, Fig. 3) in *Zea* ($\omega_2 = 1.2$, Tiffin, 2004). All three genera exhibited signatures of positive selection in the active-site cleft region of the protein, where two of the seven positively selected sites in Onagraceae were in the active-site cleft region, compared to six of the nine sites in *Boechera* and four of the 19 sites in *Zea*. Differences in the occurrence, strength and location of positively selected sites across plant genera and lineages suggest that adaptive responses might be lineage-specific, depending on differences in mode of reproduction,

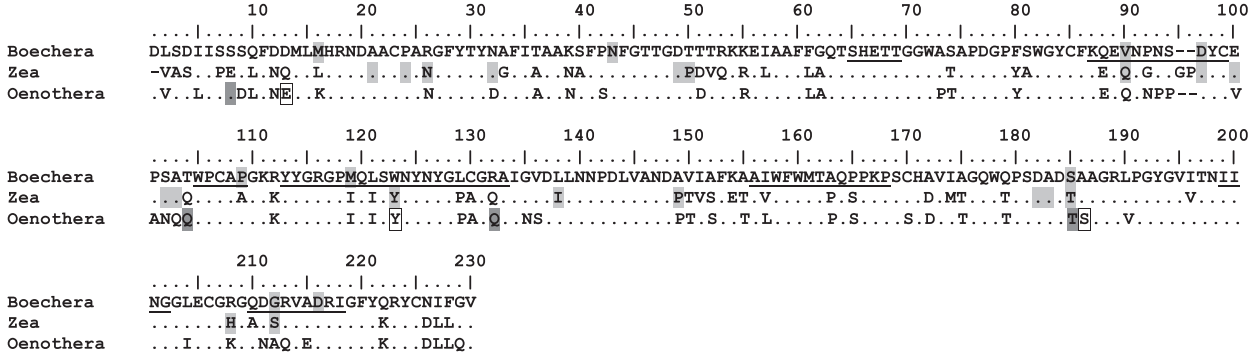

Fig. 3 Aligned orthologous amino acid sequences of mature chitinase proteins from *Boechera* (*chiB*, GenBank AAF69783), *Zea* (*chiI*, GenBank AY532743) and *Oenothera* (*chiB*, Dryad: doi:10.5061/dryad.sk94j). Amino acids under positive selection identified in *Boechera* (Bishop *et al.*, 2000) and *Zea* (Tiffin, 2004) are shaded light grey, amino acids under positive selection identified in sexual *Oenothera* lineages (branch-site models, Table 1) are shaded darker grey, and three additional sites identified as being positively selected under models that allow evolution to vary among amino acid sites but not among lineages (site-specific models M3 and M8, Table 1) are outlined. Active-site residues are underlined.

demographic history, pathogen pressure, pathogen virulence and other extrinsic and intrinsic factors (Bishop *et al.*, 2000; Stotz *et al.*, 2000; de Meaux & Mitchell-Olds, 2003; Tiffin, 2004; Siol *et al.*, 2010).

Effects of recombination and segregation on chitinase expression

We found that a loss of recombination and segregation was also associated with significant differences in *chiB* expression ($F_{1,165} = 18.14$, $P < 0.0001$). For example, constitutive *chiB* expression (C_T value of *chiB*/C_T value of *EF1α*) was 8% higher in sexual lineages (1.53 ± 0.02) than in asexual PTH lineages (1.41 ± 0.02). This suggests that sexual lineages maintain slightly higher basal levels of chitinase than asexual lineages, which could aid in the initial defence against pathogens. Presently, we do not know whether the observed differences in gene expression arise from adaptive changes in regulatory regions (Garfield & Wray, 2010) or are due to an overall accumulation of deleterious mutations (higher genetic load) within these regions. The relative role of structural vs. regulatory changes in adaptive evolution is vigorously being debated in evolutionary and developmental biology (Doebley & Lukens, 1998; Hoekstra & Coyne, 2007; Wagner & Lynch, 2008; Wray, 2008; Martin *et al.*, 2010), and this system represents an exciting avenue for future research to address this problem.

Conclusions

Interactions between plants and their natural enemies are ubiquitous in nature, and evolutionary dynamics between them are thought to be fundamental to plant evolution and the origin and maintenance of biodiversity (Ehrlich & Raven, 1964; Agrawal, 2011). Here, we show that a breakdown in recombination and segregation (sex) is negatively correlated with adaptive structural molecular evolution and could potentially affect regulatory evolution of a plant protein involved in defence against pathogens. Specifically, we show that sexual lineages have a higher incidence of positive amino substitutions and elevated constitutive expression of a chitinase pathogen resistance protein (*chiB*). Our results suggest that sexual reproduction (recombination and/or segregation) influences the evolution of a defence gene and more specifically that a loss of sex in plants might constrain adaptive evolutionary responses to pathogens.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Selection of *EF1α* as a reference gene for RT-QPCR. Effects of phylogeny, nonsynonomous substitutions, and recombination on molecular evolution.

Table S1 Primers and PCR cycling conditions.

Table S2 FASTA sequence files for the *chiB* coding regions of 32 Onagraceae species.

Table S3 Mode of reproduction, rates of non-synonymous (K_a) and synonymous (K_s) substitutions within *chiB* gene, and life history traits for 32 Onagraceae species.

Table S4 Likelihood values and parameter estimates for CODEML analyses in PAML of *PgiC* gene (459 bp) for 20 Onagraceae species (10 asexual PTH and 10 sexual lineages).

Table S5 (a) Likelihood values and parameter estimates and (b) likelihood ratio tests for CODEML analyses in PAML of *chiB* gene for 32 Onagraceae species.

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