Biology in the Dry Seed: Transcriptome Changes Associated with Dry Seed Dormancy and Dormancy Loss in the Arabidopsis GA-Insensitive sleepy1-2 Mutant

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Plant embryos can survive years in a desiccated, quiescent state within seeds. In many species, seeds are dormant and unable to germinate at maturity. They acquire the capacity to germinate through a period of dry storage called after-ripening (AR), a biological process that occurs at 5–15% moisture when most metabolic processes cease. Because stored transcripts are among the first proteins translated upon water uptake, they likely impact germination potential. Transcriptome changes associated with the increased seed dormancy of the GA-insensitive sly1-2 mutant, and with dormancy loss through long sly1-2 after-ripening (19 months) were characterized in dry seeds. The SLY1 gene was needed for proper down-regulation of translation-associated genes in mature dry seeds, and for AR up-regulation of these genes in germinating seeds. Thus, sly1-2 seed dormancy may result partly from failure to properly regulate protein translation, and partly from observed differences in transcription factor mRNA levels.

Two positive regulators of seed dormancy, DELLA GAI (GA-INSENSITIVE) and the histone deacetylase HDA6/SIL1 (MODIFIERS OF SILENCING1) were strongly AR-down-regulated. These transcriptional changes appeared to be functionally relevant since loss of GAI function and application of a histone deacetylase inhibitor led to decreased sly1-2 seed dormancy. Thus, after-ripening may increase germination potential over time by reducing dormancy-promoting stored transcript levels. Differences in transcript accumulation with after-ripening correlated to differences in transcript stability, such that stable mRNAs appeared AR-up-regulated, and unstable transcripts AR-down-regulated. Thus, relative transcript levels may change with dry after-ripening partly as a consequence of differences in mRNA turnover.

Keywords: SLY1, Arabidopsis, dormancy, dry after-ripening, germination, seeds, transcriptome, DELLA

INTRODUCTION

Plant colonization of dry land was made possible by the evolution of seeds as a means of propagation. The plant embryo encapsulated in orthodox seeds can survive long periods in a desiccated, quiescent state, allowing time for dispersal (reviewed in Bewley et al., 2013). Osmoprotectants like LEA (Late Embryogenesis Abundant) proteins and non-reducing sugars...
protect desiccated seeds from cellular damage due to destabilization of membranes and proteins. Non-reducing sugars and compatible solutes replace water in dry seeds at 5–15% moisture, resulting in a “glassy state” that allows only gradual molecular movement (Buitink and Leprince, 2004). Ribosomes are inactive in dry seeds, but form polysomes without de novo translation during water uptake or imbibition (Spiegel and Marcus, 1975; Rajjou et al., 2004). mRNAs transcribed during seed maturation are stored in dry seeds, and likely play an important role in determining whether or not a seed can germinate because they encode the earliest proteins translated during seed germination (Marcus and Feeley, 1964; Dure and Waters, 1965; Waters and Dure, 1965, 1966; Chen et al., 1968; Gordon and Payne, 1976; Ishibashi et al., 1990; Almoguera and Jordano, 1992).

Seed dormancy is an adaptation that prevents seed germination even when immediate environmental conditions are favorable (Finch-Savage and Leubner-Metzger, 2006). Seed dormancy prevents germination out of season, allows time for seed dispersal, and increases the variation in the timing of germination (reviewed in Koornneef and Alonso-Blanco, 2000; Venable, 2007; Poisot et al., 2011). Seed dormancy is established during embryo maturation, the final stage of seed development. Dormancy can be relieved through a period of dry storage called after-ripening, through moist chilling (cold stratification), or through seed coat scarification. The after-ripening time required for dormancy loss depends on genotype, and can be perturbed through altered function of dormancy-regulating genes (Ariizumi and Steber, 2007; Chiang et al., 2011; Kendall et al., 2011; reviewed in Koornneef and Alonso-Blanco, 2000; reviewed in Nonogaki, 2014). This genetic variation is particularly important in cereal crops where lack of seed dormancy can lead to problems with preharvest sprouting, the germination of grain on the mother plant when cool and rainy conditions occur before harvest (reviewed by Rodríguez et al., 2015). Informed genetic strategies may allow us to increase seed dormancy sufficiently to prevent preharvest sprouting without causing problems with poor germination and emergence when winter crops are planted in the fall with little after-ripening.

The word “germination” refers both to a process and an event. The germination process has been divided into three phases (reviewed in Bewley et al., 2013). During Phase I, rapid water uptake (imbibition) leads to cellular rehydration associated with expression of genes involved in seed maturation and desiccation tolerance such as LEAs, small heat shock proteins (smHSPs) and oxidoreductases. During Phase II, water uptake plateaus and the seed undergoes essential processes, including DNA repair, initiation of transcription and translation, mitochondrial repair, respiration, initiation of stored nutrient mobilization, DNA synthesis, and cell expansion. Phase III begins with germination the event (germination per se), defined by embryonic root emergence. Phase III also includes post-germinative events such as completion of nutrient mobilization, cell division, and seedling growth. Living dormant seeds do not reach Phase III, but they do imbibe water and enter Phase II. This paper will refer to ungerminated seed in Phase I or II as “imbibing seeds” to distinguish them from seeds undergoing germination per se.

Understanding how dormancy loss through after-ripening occurs in a dry and metabolically quiescent seed is one of the great mysteries of plant science (reviewed in Koornneef and Alonso-Blanco, 2000; Bewley et al., 2013). Changes during dry seed storage regulate germination potential once the seed is imbibed, yet the severe water deficit in dry seeds likely inhibits most biological processes, including transcription and translation. Transcriptome studies have observed differential accumulation of stored dry seed mRNAs with after-ripening of multiple species (Comai and Harada, 1990; Bove et al., 2005; Leubner-Metzger, 2005; Cadman et al., 2006; Leymarie et al., 2007; Oracz et al., 2007; Bazin et al., 2011; Chittnis et al., 2014; Meimoun et al., 2014). The changes in transcript levels with dry seed after-ripening may result from transcription or differential mRNA turnover. Based on inhibitor studies, protein translation, but not gene transcription, is required for seed germination (Spiegel and Marcus, 1975; Rajjou et al., 2004). This emphasizes the importance of stored mRNAs, since translation of stored mRNA is necessary and sufficient for seed germination.

Some have hypothesized that localized moisture conditions may allow active transcription in dry seeds, while others maintain this is unlikely. Hydrogen proton NMR microimaging of dry seeds detected possible moisture pockets proposed to make dry seed transcription possible (Leubner-Metzger, 2005). Polysome profiles of nuclei isolated from dry seeds of Brassica napus suggested active transcription, albeit at 8% of the rate observed during seed maturation (Comai and Harada, 1990). However, non-transcriptional processes likely cause apparent changes in relative transcript abundances during dry seed after-ripening (reviewed in Bewley et al., 2013). Differential RNA turnover may be triggered by mRNA oxidation resulting from oxygen diffusion into dry seeds (Oracz et al., 2007). Dry seed after-ripening of sunflower (Helianthus annuus) was associated with differential transcript levels, including 24 after-ripening-down-regulated mRNAs preferentially targeted for destruction by mRNA oxidation (Bazin et al., 2011). Oxidative reactions have also been implicated in dormancy regulation through lipid peroxidation, carbonylation of specific proteins, or oxidation of disulfide bonds to alter protein structure (Alkhalfioui et al., 2007a,b; Oracz et al., 2007). Regardless of the mechanisms causing changes in the dry seed transcriptome with after-ripening, it is important to consider whether changes can impact germination capacity.

The plant hormones abscisic acid (ABA) and gibberellin (GA) act antagonistically to regulate seed dormancy and germination (reviewed in Finkelstein et al., 2008). While ABA promotes seed dormancy, GA stimulates germination. ABA establishes dormancy during seed maturation (Koornneef and van der Veen, 1980; Steber et al., 1998; Iuchi et al., 2007; Willige et al., 2007; Hauvermale et al., 2015). ABA-insensitive or biosynthesis mutants rescue the failure...
to germinate in GA biosynthesis or GA-insensitive mutants (Karssen and Lačka, 1986; Steber et al., 1998). Thus, GA acts upstream of ABA to stimulate germination.

Gibberellin stimulates seed germination, stem elongation, and flowering by negatively regulating the DELLA (Asp-Glu-Leu-Leu-Ala) repressors of GA responses (reviewed in Hauvermale et al., 2012). GA-binding stimulates the protein–protein interaction between the GID1 (GA-INSSENSITIVE DWARF1) GA receptors and DELLA protein. Formation of the GID1-GA-DELLA complex causes either DELLA inactivation or destruction via the ubiquitin-proteasome pathway (McGinnis et al., 2003; Dill et al., 2004; Ariizumi et al., 2008, 2011, 2013; Wang et al., 2009; Ariizumi and Steber, 2011). The Arabidopsis SLEEPY1 (SLY1) gene encodes the F-box subunit of an SCF (Skp, Cullin, F-box) E3 ubiquitin ligase that directly binds to and ubiquitinates DELLA upon formation of the GID1-GA-DELLA complex. Thus, GA causes SBF of SLY1 to polyubiquitinate, and thereby, target DELLA for destruction by the 26S proteasome. Arabidopsis has five DELLA proteins, RGA (REPRESSOR OF GA1-3), GAI (GA-INSSENSITIVE1), RGL1, RGL2, and RGL3 (RGA-LIKE). The failed seed germination of the GA biosynthesis mutant gai-1-3 in the light was strongly rescued by loss of the DELLA RGL2 (Cao et al., 2005). However, rescue of gai-1-3 germination in the dark, also required loss of DELLA’s RGA and GAI. The GA-insensitive gain-of-function mutation gai-1 was associated with reduced GA sensitivity during germination in the dark, and reduced germination on ABA in the ABA-insensitive ABI1-1 mutant background (Koornneef et al., 1985; Ariizumi et al., 2013). DELLA’s are thought to repress GA responses through transcriptional regulation via interaction with DNA-binding proteins such as PHOTOMORPH-INTERACTING FACTORS, PIF3, PIF4, and PIF1.

Loss of SLY1 leads to overaccumulation of DELLA repressors of seed germination associated with increased seed dormancy (Steber et al., 1998; McGinnis et al., 2003; Ariizumi and Steber, 2007). The Arabidopsis GA-insensitive sly1-2 mutation is a 2-bp deletion leading to loss of the last 40 amino acids of the 151 amino acid protein. Seeds of sly1-2 have strong initial seed dormancy, but acquire the ability to germinate either with GID1 gene overexpression (GID1-OE) or with 1-2 years of dry after-ripening (Ariizumi and Steber, 2007; Ariizumi et al., 2013). In contrast, Landsberg erecta (Ler) wild-type seeds fully after-ripen within 2 weeks. Neither after-ripening nor GID1-OE result in reduced accumulation of DELLA repressors of seed germination. Thus, GA signaling can occur without DELLA-proteolysis leading to increased germination potential. There are three GA receptor genes in Arabidopsis, GID1a, GID1b, and GID1c. GID1b protein has higher affinity for GAs and for DELLA protein than GID1a and GID1c (Nakajima et al., 2006; Yamamoto et al., 2010). This is likely the reason that GID1b-OE rescues sly1-2 seed germination and plant height phenotypes better than GID1a-OE and GID1c-OE (Ariizumi et al., 2008, 2013; Hauvermale et al., 2014).

This paper examines the pattern of transcript accumulation in dry seeds associated with increased seed dormancy and dormancy loss in the GA-insensitive sly1-2 (sleepy1-2) mutant of Arabidopsis. Transcripts involved in protein translation were sly1-up-regulated in dry seeds, and sly1-down-regulated upon seed imbibition. Thus, it appears that SLY1 may be needed both to down-regulate protein translation during seed development, and to up-regulate translation during germination. The importance of protein translation during seed germination has been well characterized (Galland et al., 2014; Layat et al., 2014). This agrees with our previous research showing that increasing germination capacity with after-ripening is associated with increased abundance of protein translation-associated genes (Nelson and Steber, 2017). In that study, the transcriptional changes associated with sly1-2 dormancy and dormancy loss were quite different during early and late Phase II of seed imbibition. Based on this result, we postulated that earlier transcriptome differences most likely regulate whether a seed can or cannot germinate. By this rationale, transcriptome differences in dry seeds should play key roles in dormancy and dormancy loss since the stored transcripts in dry seeds are likely the first transcripts to impact germination potential. Consistent with this notion, mutations in two genes showing down-regulation with dry seed after-ripening, the DELLA GAI and the histone deacetylase HDA6, led to decreased seed dormancy. This suggests that GAI and histone deacetylation may establish and maintain seed dormancy.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Landsberg erecta (Ler) wild-type and mutant lines used in this study including ga1-3, sly1-2, sly1-2 GID1b-OE, gai-1, gai-16, sly1-2 gai-16, and sil1 all in the Ler background were described previously (Peng and Harberd, 1993; Peng et al., 1997; Furner et al., 1998; Steber et al., 1998; Ariizumi et al., 2008). All lines were grown under fluorescent lights in a Conviron® growth chamber according to McGinnis et al. (2003). Harvested seeds were stored at room temperature and low humidity (∼15–30%) in open tubes for dry after-ripening treatments.

The standard practice of harvesting seeds after the entire plant has turned brown (fully desiccated) was used in all cases, except where indicated that harvest occurred at “near maturity.” Since all parts of a plant do not turn brown simultaneously, harvesting fully brown plants means that some portion of the seeds collected have been after-ripening on the plant for up to a few weeks. In order to obtain dormant seeds for wild-type or when expecting germination rates higher than wild-type, seeds were harvested when the mother plants were partially brown and partially green. By collecting only seeds that fell freely from dry siliques and sifting seeds through a fine mesh, we ensured that only brown (desiccated) seeds were collected for use in assays.

Microarray Seeds

This study used the same seed batches examined previously during imbibition to investigate starting state transcriptomes of Ler wt, sly1-2(D), sly1-2(AR), and sly1-2 GID1b-OE (Nelson and Steber, 2017). Two-week-old Ler wt, sly1-2, sly1-2 GID1b-OE were grown side-by-side, while 19-month-old sly1-2 was
grown in advance to allow comparison of dormant to non-dormant sly1-2. All seeds for microarray analysis were collected from fully brown plants. The GID1b-overexpression allele in the sly1-2 background is a translational fusion of HA:GID1b on the 35S cauliflower mosaic virus promoter. Growth and storage conditions are described further in Nelson and Steber (2017).

**Ler After-ripening Time Course**
A single batch of Ler wt seeds was harvested “near maturity” to collect dormant seeds for an after-ripening time course. Freshly harvested seeds were stored in open tubes overnight before collecting dormant, 0 week after-ripened (0wkAR), seeds for germination and RT-qPCR assays. Seeds from the same batch were collected for RT-qPCR and germination assays each day for 14 days.

**GAI Mutant Germination Assays**
Seeds of Ler wt, gai-1, gai-16, and sly1-2 gai-16 were grown side-by-side and harvested at near maturity. Freshly harvested seeds were stored in open tubes overnight before collecting dormant 0wkAR seeds for germination assays.

**sill1/ha6 Mutant Germination Assay**
The hda6 loss of function mutant in the Ler background, sill1 was a kind gift from Dr. Jong-Myong Kim at the RIKEN Plant Science Center in Yokohama, JAPAN. Ler wt and sill1 seeds used for germination assays were grown side-by-side and harvested at near maturity to obtain dormant seeds. Freshly harvested seeds were stored in open tubes overnight before collecting dormant 0wkAR seeds for the germination assay. Seeds were stored for an additional 14 days in open tubes then collected for the 2wkAR germination assay.

**Germination on Tricostatin A**
Seeds of Ler wt, gai-3, and sly1-2 seeds were harvested from fully brown plants. Seeds were stored for 2 weeks with the exception of long after-ripened sly1-2, which was stored for more than 1 year.

**Germination Experiments**
For all germination screens, seeds were sterilized with 70% ethanol and 0.01% SDS for 5 min followed by 10% bleach and 0.01% SDS for 10 min, washed, and plated on 0.8% agar plates containing 0.5× MS salts (Sigma–Aldrich) and 5 mM MES [2-(N-morpholino)ethanesulfonic acid], pH 5.5 (referred to as MS-agar plates). Germination was scored daily. Germination of the same batch of seeds used for microarray analysis was performed as in Nelson and Steber (2017). For the Ler after-ripening time course germination of at 0wkAR, 1wkAR, and 2wkAR was scored for three replicates of 100 seeds each after cold stratification for 4 days at 4°C in the dark. After-ripening time course seeds were the seeds used for the RT-qPCR time course for AHB1 gene expression in Ler wt. For the comparison of GAI mutants, germination was scored for three replicates of 70–100 seeds each with both with and without cold stratification for 4 days at 4°C in the dark. For sill1 mutants, because we expected higher germination efficiency than wild-type would be difficult to capture, each plate was divided into two halves with Ler wt plated on one side and sill1 plated on the other for side-by-side comparison. For the same reason, three replicates of 70 seeds each for each of three biologically independent batches of Ler wt and sill1 at 0 and 2 weeks of after-ripening were scored both with and without cold stratification for 4 days at 4°C in the dark. The tricostatin A (TSA) dose response experiments were performed for 2–4 replicates of about 30–90 seeds each. Tricostatin A (TSA) was added to plates at 0, 0.5, 1, 2, 4, and 6 µM concentrations and germination was recorded for 2–4 replicates of about 30–90 seeds.

**Total RNA Isolation from Dry Seeds**
RNA extractions for microarray and RT-qPCR were performed as in Nelson and Steber (2017). Briefly, 20 mg of dry seed per sample were flash frozen in liquid nitrogen and RNA was isolated using a phenol-chloroform based extraction method optimized for extraction from tough tissues, such as dry seeds (Nelson and Steber, Unpublished). The extraction method is based on the Oñate-Sánchez and Vicente-Carbajosa (2008) with additional steps to prevent phenol contamination and increase yield. RNA quantity and quality were determined using a NanoDrop ND-2000c spectrophotometer (Thermo Scientific) and gel electrophoresis using RNA denatured at 70°C for 5 min in a formaldehyde dye. For six samples selected from RNA used in the Ler after-ripening time course RT-qPCR experiment, quality and quality were also determined using the Agilent 2100 bioanalyzer with the RNA 6000 Nano Kit [RNA integrity number (RIN) = 9.0–9.3].

**Microarray and Data Analysis**
Microarray analysis of RNA from dry seeds was performed in triplicate using the Affymetrix ATH1 oligonucleotide-based DNA microarray chip (22,810 genes represented). For each replicate of Ler wt (stored dry for 2 weeks), dormant sly1-2 (stored dry for 2 weeks), after-ripened sly1-2 (stored dry for 19 months), and sly1-2 GID1b-OE (stored dry for 2 weeks), 2 µg of RNA was processed by the Molecular Biology and Genomics Core Laboratory at Washington State University biotin-labeled CRNA synthesis, ATH1 chip hybridization, and chip scanning. The LIMMA package as part of the Bioconductor suite of tools in the R was used for data analysis as described previously (Gentleman et al., 2004; Smyth, 2005; R Core Team, 2016; Nelson and Steber, 2017). Raw data files are available at ArrayExpress. Raw data files were analyzed using the same methods as above to facilitate fair comparison. The raw dataset from Finch-Savage et al. (2007) was obtained from NASCArrays, and dataset from Kendall et al. (2011) was obtained from ArrayExpress. In Finch-Savage et al. (2007) dry seeds of freshly harvested and 120 days after-ripened Cv1

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1. http://crb.wsu.edu/core-laboratories/molecular-biology-and-genomics-core
2. http://www.ebi.ac.uk/arrayexpress
3. http://arabidopsis.info/affy
wild-type from independent seed batches were analyzed. The Kendall et al. (2011) study compared dry seeds of Ler wt and ft-1 collected from dehisced siliques. When referring to the differential regulation in A relative to B, or AvsB, up in AvsB means up-regulated in A (or down-regulated in B), whereas down in AvsB means down-regulated in A (or up-regulated in B).

Gene Ontology, Gene Family, and TAGGIT Ontology Analyses
Analysis for enrichment in gene categories was performed by (1) looking for global enrichment of genes in standard gene ontology (GO) categories, (2) looking for global enrichment of genes in specific gene families (GF), and (3) looking for enrichment of genes within a specific set of seed dormancy and germination related gene categories (TAGGIT). GO biological process and GF enrichment was performed using the BioMaps tool as part of the VirtualPlant 1.3 suite of online tools for analysis of genomic data1 (Katari et al., 2010). Enrichment was determined for a list of differentially regulated genes against the whole genome using a Fisher Exact Test with FDR correction for multiple comparisons using a p-value cutoff of $p < 0.01$ (Fisher, 1922). For each significantly enriched category a value for enrichment expected by chance (Expected), was presented for comparison to observed enrichment values (Observed).

For seed germination and dormancy specific GO classifications, the TAGGITontology and TAGGITplot R functions that we developed previously on the Carrera et al. (2007) TAGGIT categorizations were used (Nelson and Steber, 2017). These functions are publicly available through github as part of the microarrayTools R package2. TAGGIT uses 26 categories defined for their involvement in seed dormancy and germination and matches genes to categories based on lists of AGI locus identifiers in combination with a gene description search for specific keywords. For simplicity, “more up-regulation” or “more down-regulation” in a category refers to a higher degree of enrichment in either the up-regulated gene fraction, or in the down-regulated gene fraction, respectively.

One of the concerns about comparisons of dry seed gene datasets is that differential regulation may be random background due to differences in seed batches. To confirm that the differences in category enrichment identified by TAGGIT could not emerge from a random dataset due to unexpected bias in the computational algorithm, a non-overlapping random set of 330 up- and 430 down-regulated genes was analyzed by TAGGIT (Supplementary Figure 1). This random dataset showed low category enrichments and only small changes between up- and down-regulation datasets, indicating that the differential enrichment in TAGGIT categories observed for sly1-2 and Cvi dry seed datasets were non-random.

Transcription Factor Gene Identification in R
To determine the number of transcription-factor-coding mRNAs (TF-mRNAs) in a given geneset a list of Arabidopsis transcription factors was compiled based on the combined databases of PlnTFDB8, AtTFDB9, and PlantTFDB10, since each database contained some unique entries (Supplementary Table 1; Davuluri et al., 2003; Palaniswamy et al., 2006; Pérez-Rodríguez et al., 2009; Zhang et al., 2014). This list contains both true DNA-binding transcription factors and transcription co-factors. In order to categorize a list of TF-mRNAs into transcription factor families, an R function called countTFs was written for this study (Supplementary Figure 2). countTFs is available for public use as part of the microarrayTools R package through github9.

PlantGSEA Transcription Factor Target Analysis
The web-based Plant GeneSet Enrichment Analysis toolkit (PlantGSEA10) with the Transcription Factor Targets (TFT) dataset was used to determine enrichment for known targets of transcription factors within differentially regulated genesets (Yilmaz et al., 2010; Lai et al., 2012; Yi et al., 2013). This toolkit uses published ChIP-seq or ChIP-chip data to identify “Confirmed” or “Unconfirmed” transcription factor targets. Targets that are “unconfirmed” were only identified by a single experimental approach, while “confirmed” targets were identified by two or more approaches with in vivo evidence. The “All” category includes both confirmed and unconfirmed targets. Enrichment of transcription factor targets was determined using a Fisher statistical test with the Yekutieli (FDR under dependency) correction for multiple testing adjustment with $\alpha = 0.05$ (Fisher, 1922; Benjamini and Yekutieli, 2001). To prevent falsely high enrichment for transcription factors with few known targets a 5 hit minimum cutoff was used.

RT-qPCR Analysis
RT-qPCR analysis was performed using gene-specific primers for GAI, HDA6, DOG1, SLY1, MFT, HSFA9, and AHb1 for comparison to microarray results. RT-qPCR was also performed for Ler wt dry seeds at 0, 2, and 4 weeks of after-ripening to determine if an increase in AHb1 mRNAs could be seen with after-ripening. Primers for SLY1 were selected to allow binding of both the sly1-2 mutant and native SLY1 transcript, since the ATH1 chip cannot distinguish between SLY1 and sly1-2 transcripts. The ProScript® M-MuLV First Strand cDNA synthesis kit (New England Biolabs) was used for cDNA synthesis from 1 µg of total RNA and the LightCycler FastStart DNA Master SYBR Green I kit (Roche) was used for qPCR. The QuantPrime online tool11 was used for primer design with the exception of the previously published DOG1, GAI, and HSFA9 (Zhang and Zhu, 2011; Nomoto et al., 2012; Guan et al., 2013). Primer sequence and annealing temperatures are presented in Supplementary Figure 3. Dilution curves were used to calculate reaction efficiencies; all efficiencies were

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1. www.virtualplant.org
2. https://github.com/bakuhatsu/microarrayTools
3. http://www.quantprime.de
4. http://plntfdb.bio.uni-potsdam.de/v3.0/
5. http://arabidopsis.med.ohio-state.edu/AtTFDB/
6. http://planttfdb.chi.pku.edu.cn
7. https://github.com/bakuhatsu/microarrayTools
8. http://structuralbiology.cau.edu.cn/PlantGSEA/
9. http://www.quantprime.de
within 10% of each other and ±10% of 100% efficiency. qPCR conditions were: 10 min at 95°C (initial denature), then 45 cycles of 10 s at 95°C (denaturation), 5 s at the primer-specific annealing temperature (see Supplementary Figure 3), and 10 s at 72°C (extension). Data was analyzed using the Delta–Delta Ct method with three replicates per gene or timepoint using the AKR2B (ANKYRIN REPEAT-CONTAINING 2B; At2g17390) reference gene (Livak and Schmittgen, 2001; Hruz et al., 2011). Statistical testing was performed by pairwise t-test with Bonferroni–Holm correction for multiple comparisons with α = 0.07 (Supplementary Figure 4; Holm, 1979).

RESULTS

Strategies for Examining Mechanisms of sly1-2 Dormancy and Dormancy Loss in Dry Seeds

In order to ask specific questions regarding the initial transcriptome state of dormant and non-dormant sly1-2 seeds, an Affymetrix® oligonucleotide-based microarray transcriptome analysis was conducted on dry seeds of: (a) wild-type Ler (WT) stored for 2 weeks, (b) dormant sly1-2 stored for 2 weeks [sly1-2(D)], (c) after-ripened sly1-2 stored for 19 months [sly1-2(AR)], and d) sly1-2 GID1b-overexpressed (sly1-2 GID1b-OE) stored for 2 weeks (Figure 1C). Ler WT reached 96% germination after 1 day, whereas sly1-2(D) did not germinate even after 7 days of imbibition (Figure 1B). sly1-2 germination was rescued by long after-ripening for 19 months (51% germination by 7 days), and by GID1b-OE (73% by 7 days). The same seed stocks were previously used in an imbibed seed microarray study, including a “0h” timepoint taken immediately after cold stratification for 4 days at 4°C in the dark, and a “12h” timepoint (4 days at 4°C, followed by 12h at 22°C in the light) (Figure 1A; Nelson and Steber, 2017). Time points examined and comparisons made between this and previous studies are summarized in Figures 1C,D.

Stored mRNA Transcriptome Differences Associated with the sly1-2 Dormancy Phenotype

The sly1-2(D) to wild-type Ler (sly1-2 DvSWT) comparison identified 794 transcript differences associated with the sly1-2 seed dormancy phenotype (Figure 2A). Since the comparison of another mutation affecting germination, ft-1 (flowering locus t-1), to Ler wt dry seeds detected no transcriptome differences (Chiang et al., 2009; Kendall et al., 2011), these changes in dry seed transcript levels were likely effects of the sly1 mutation during seed development, maturation, or during the 2 weeks of dry after-ripening. The sly1-2 DvSWT comparison had more negative log2-fold changes (logFCs) (517 sly1-down-regulated) than positive (277 sly1-up-regulated) (Figure 2A), resulting in an adjusted Fisher-Pearson standardized moment coefficient skewed toward down-regulation (G1 = −0.56, vs.

FIGURE 1 | Microarray experimental design. (A) Seeds in this study were examined at the “dry” (orange) timepoint in dry seeds. Comparisons were also made to Nelson and Steber (2017) “0h” (blue) timepoint after cold stratification in the dark for 4 days at 4°C and the “12h” (green) timepoint with cold stratification and 12h in the light at 22°C. (B) Germination of seeds used for microarray analysis. The same batches of seeds as in Nelson and Steber (2017) were imbibed on MS-agar plates for 4 days at 4°C, then moved to the light at 22°C and scored for germination. (C) Ler wt, sly1-2(D), and sly1-2 GID1b-OE seed batches were 2 weeks old, while sly1-2(AR) was 19 months old seed. (D) Experimental comparisons made in this paper, including comparisons from reanalysis of data from Finch-Savage et al. (2007) and Kendall et al. (2011).
FIGURE 2 | Genome wide expression plots. Plots indicate skew, chromosomal distribution, and magnitude of (A) dry seed sly1-regulated transcriptome differences, (B) dry seed sly1-2 GID1b-OE vs. sly1-2(D) differences, (C) differences after-ripened (AR) and dormant (D) sly1-2 dry seeds and (D) differences between after-ripened (AR) and dormant (D) Cvi. Genes with significant differences are indicated in orange (based on FDR \( p < 0.05 \)). Up-regulation is indicated by positive log2-fold change (logFC) and down-regulation with negative. Shaded area mark the ±2 and ±3 logFC to allow comparison of magnitude and skew between genesets.

\[ G1 = 0 \text{ if symmetrical} \] (Joanes and Gill, 1998). Plots comparing normalized intensities showed transcriptome differences across a wide range of signal intensities, indicating that significance was not an artifact of small changes at low intensities (Supplementary Figure 5A). The sly1-2 F-box mutation results in an inability to degrade DELLla transcriptional regulators (Nelson and Steber, 2016). Thus, negative DELLla regulation in sly1 mutants may directly or indirectly cause the reduced accumulation of many transcripts during dry seed development. Not surprisingly, some of the top 50 differentially regulated genes were seed-related genes such as a LEA and seed storage proteins (Figure 3A). Of the top 50 DELLla/sly1-regulated genes in dry seeds, 21 were similarly regulated at the previously published 0h and 12h imbibed timepoints (Nelson and Steber, 2017).

The differentially abundant genes in the dry seed sly1-2 DvsWT comparison were characterized using BioMaps GO and gene family (GF) to look for biological process enrichment\(^\text{12}\) (Supplementary Figures 6A–C; Katari et al., 2010). There was significant up-regulation of two ribosomal GF, and down-regulation of the glycosyltransferase gene family, including genes involved in auxin and ABA hormone signaling (Supplementary Figure 6A; Yonekura-Sakakibara, 2009). Many sly1-up-regulated GO categories were also related to protein translation, ribonucleoprotein complex and ribosome biogenesis (Supplementary Figure 6B). The sly1-down-regulated GO categories included stress or stimuli responses related to seed dormancy such as response to ABA, abiotic stress, and oxidation/reactive oxygen species (Supplementary Figure 6C; reviewed in Graeber et al., 2012).

**Transcriptome Differences Associated with Rescue of sly1-2 Germination by Long After-ripening and GID1b-OE**

The fact that sly1 mutants have increased seed dormancy suggests that SLY1-directed DELLla destruction is needed for dormancy loss and germination. However, the germination of sly1-2 seeds is partly rescued by GIDI overexpression and by long after-ripening without any decrease in DELLla protein accumulation.

\(^{12}\)www.virtualplant.org
(Figure 1B; Ariizumi and Steber, 2007; Ariizumi et al., 2008). We previously learned that sly-1 rescue by GID1b-OE was associated with far fewer changes in expression than rescue by long after-ripening in imbibing seeds (Nelson and Steber, 2017). We made a similar observation in dry seeds (Figures 1B,C and Table 1). There were 770 genes with different transcript abundances between D and AR sly-1 dry seed, 330 up-regulated and 430 down-regulated with after-ripening of sly-1 (sly-1 ARvsD). In contrast, only 7 genes showed differential accumulation with GID1b-overexpression in sly-1 (GIDvsD) dry seeds (Figure 2B).

While more transcripts showed decreased rather than increased levels with after-ripening, the dataset was slightly skewed toward AR-up-regulation (G1 = 0.35), likely due to stronger up-regulation of fewer transcripts (Figure 2C). For example, there were 20 up-regulated transcripts with logFCs from 2 to 4.3, whereas only 4 of the down-regulated transcripts had logFCs greater than 2. This is consistent with observations made during dry after-ripening of the dormant ecotype Cvi; where there were 777 up- and 1426 down-regulated transcripts in the Cvi ARvsD comparison (Figure 2D). Since the plotted normalized intensities of sly-1 ARvsD showed significant differences (red) over a wide range of intensities, the small number of transcripts highly up-regulated do not appear to be artifacts of comparing low intensity values (Supplementary Figure 5B). Many of the sly-1 ARvsD transcriptome changes observed in dry seeds were also seen at 0h and 12h of imbibition, but with lower logFCs (Figure 3B). The most up-regulated gene was the AHB1 (Arabidopsis nonsymbiotic Hemoglobin1; Abbruzzetti et al., 2011) gene involved in oxidative stress response, whereas the most down-regulated gene was the DELLA GAI. It is interesting that GAI was up-regulated in the sly-1 DvsWT dry seed comparison and down-regulated with dry after-ripening (Figure 3B). This suggests that GAI plays a role in sly-1 dormancy that is reversed with long after-ripening. BioMaps gene family analysis and GO analysis showed that many of the dry seed sly-1-regulated terms were oppositely AR-regulated (Supplementary Figures 6A–C; Katari et al., 2010). The sly-1 down-regulated stimuli response terms, including ABA and abiotic stress, were AR-up-regulated in dry sly-1 seeds (Supplementary Figure 7A). Only translation and terms related to cellular/metabolic processes were sly-1 up- and AR-down-regulated (Supplementary Figures 6A,B, 7B).
The significant overlap between AR-regulated genes in Cvi and sly1-2, despite the fact that sly1-2 is in the Ler ecotype, suggests that these changes are biologically relevant (Figure 4A). The direct overlap of sly1-2 and Cvi AR-regulated transcriptome changes identified a list of genes associated with both Cvi wt and sly1-2 dormancy loss (Supplementary Table 2). There were 38 up- and 101 down-regulated transcripts in sly1-2 and Cvi with after-ripening. This smaller dataset included genes that are AR-regulated in both sly1-2 and Cvi wt. This dataset included many genes related to ABA or GA signaling and germination. Among them, the DELLA GAI, 5 members of the ABA PP2C (Protein Phosphatase Type 2C) family genes, MFT (MOTHER OF FT AND TFL), and HDA6 (HISTONE DEACETYLASE6) were all AR-down-regulated.

GID1b-OE rescue of sly1-2 germination was associated with only seven differentially abundant transcripts in dry seeds, 5 up- and 2 down-regulated (Table 1). Since GID1b is overexpressed on the 35S promoter, it was not surprising that the most up-regulated gene was GID1b itself. Among the remaining 6 genes, 3 were similarly regulated at 0h and 12h of imbibition, including: the up-regulated At1g21630 (EF hand family) gene, and down-regulated At2g46250 (myosin heavy-chain related) and BAG6 (BCL-2-Associated Anthogene6) genes. When the dataset was compared to the dry seed transcriptome changes with after-ripening of sly1-2, LTP4 and LEAS/SAG21 were GID1b-OE- and AR-up-regulated (Figure 4B). LTP4 encodes a phospholipid transfer protein localized to the cell wall, while LEAS/SAG21 encodes a senescence-associated protein with a role in oxidative stress tolerance (Arondel et al., 2000; Hundertmark and Hincha, 2008). Both LTP4 and LEAS/SAG21 are also ABA-induced transcripts.

**Protein Translation and Gene Transcription Are Major Gene Categories Regulated by SLY1 and After-ripening**

TAGGIT seed-related ontology analysis was used to compare gene enrichment in seed-specific categories for genes differentially regulated in DvsWT, sly1-2 ARvsD (current study, Ler ecotype), and ecotype Cvi ARvsD dry seed comparisons (Figures 1D, 5; Carrera et al., 2007; Finch-Savage et al., 2007; Nelson and Steber, 2017). It is interesting that the protein translation category accounted for 25% of the sly1-1-up-regulated genes (DvsWT; Figure 5A) given that the translation category

**TABLE 1 | Complete table of sly1-2 GID1b-OE vs. sly1-2(D) differentially regulated genes across all three imbibition timepoints.**

| ID       | Gene          | drya | 0ha | 12ha |
|----------|---------------|------|-----|------|
| At3g83010| GID1b         | 8.32 | 8.58 | 8.28 |
| At5g59310| LTP4          | 4.40 | –    | –    |
| At4g02380| LEAS          | 2.34 | –    | –    |
| At1g21630| EF hand family| 1.65 | 2.77 | 2.95 |
| At1g44575| NPT4          | 0.89 | –    | –    |
| At5g46050| PTR3          | –    | 1.26 | –    |
| At5g54070| HSFA9         | –    | –    | 1.34 |
| At4g09610| GAS6A2        | –    | –    | 1.29 |
| At3g45970| EXPL1         | –    | –    | 1.01 |
| At2g34740| A PP2C        | –    | –    | 0.97 |
| At3g22490| A LEA         | –    | –    | 0.91 |
| At5g46690| Unknown protein| –  | –    | 0.83 |
| At2g46240| BAG6          | –2.13 | –2.69 | –3.19 |
| At2g46250| Myosin heavy chain related | –1.31 | –1.80 | –3.39 |
| At1g17430| 1/3 hydroxlate fold family | –  | 1.10 | –    |
| At5g01740| NTF2 family   | –    | –1.07 | –    |
| At5g48860| SDI1          | –    | –1.06 | –    |
| At5g58860| HORST         | –    | –1.00 | –    |
| At1g09200| Histone H3.1  | –    | –0.33 | –    |
| At1g22760| PAB3          | –    | –0.90 | –    |
| At5g66580| ANQ1/MKK6     | –    | –0.83 | –    |
| At1g66190| Phosphoglycerate kinase | –   | –0.79 | –    |
| At5g15230| GASA4β        | –    | –    | –1.51 |
| At5g07480| KUOX1         | –    | –1.28 | –    |
| At2g48800| Oxidoreductase| –    | –1.13 | –    |
| At2g40880| CYSA          | –    | –0.77 | –    |
| At2g16060| AHB1/QLB1     | –    | –0.73 | –    |

*a sly1-2 GID1b-OE vs. sly1-2(D) log2 fold changes.  
b log2 fold change of –1.51 ± 0.36, significant based on RT-qPCR.

**FIGURE 4 | Comparisons between dry transcriptome datasets. (A) The overlap between sly1-2 and Cvi dry seed after-ripening regulated datasets. Cvi dataset is from Finch-Savage et al. (2007). (B) The overlap between dry seed GID1b-OE-regulated and after-ripening regulated mRNAs.**
FIGURE 5 | TAGGIT gene ontology analysis of *sly1-* and after-ripening-regulated transcriptome differences in dry seeds. (A) *sly1-2 DvsWT* dry seed transcriptome differences. (B) Differences with after-ripening of *sly1-2* dry seeds. (C) Differences with after-ripening of *Cvi* dry seeds. The value on the x-axis shows the percentage of either the total up-regulated or total down-regulated genes within a dataset.

was among the most highly *sly1*-down-regulated at 0h and 12h of seed imbibition in our previous study (Supplementary Figure 8; Nelson and Steber, 2017). The translation category was also strongly down-regulated with after-ripening of both *sly1-2* and *Cvi* dry seeds (Figures 5B,C). In contrast, the translation category showed strong up-regulation with after-ripening of imbibed Ler wt but not *sly1-2* seeds (Nelson and Steber, 2017). Thus, it appears that the *SLY1* gene is needed both to down-regulate protein translation-associated genes during seed development and to up-regulate protein translation genes during seed germination.

It appears that dry after-ripening involves similar mechanisms in *sly1-2* and *Cvi* since many TAGGIT categories, such as auxin, ethylene, LEAs, inhibition of protein degradation, cell wall, and cell cycle, showed similar regulation in both experiments (Figures 5B,C). TAGGIT analysis of a randomly generated dataset confirmed that TAGGIT profiles similar to those observed for *sly1-2* ARvsD and *Cvi* ARvsD were unlikely to happen by chance, suggesting that this agreement has functional relevance (Supplementary Figure 1). However, there was not perfect agreement in all *sly1-2* and *Cvi* categories. For example, ABA was strongly up-regulated in *sly1-2*, but slightly down-regulated in *Cvi*, while the cytoskeleton category was up-regulated in *sly1-2* but down-regulated in *Cvi*. Since these categories were similarly regulated in *sly1-2* and Ler during late Phase II, they may result from either the *sly1* mutation or ecotype differences (Nelson and Steber, 2017).

The first proteins translated from stored mRNAs may activate or block transcriptional cascades leading to germination. Thus, we examined if differentially expressed transcription-factor-encoding mRNAs (TF-mRNAs) are among the AR-regulated genes in dry seeds using a combined list of Arabidopsis transcription factors compiled from the PlnTFDB, AtTFDB, and PlantTFDB databases (Davuluri et al., 2003; Palaniswamy et al., 2006; Pérez-Rodriguez et al., 2009; Jin et al., 2013). This analysis revealed 27 transcription-factor-encoding mRNAs (TF-mRNAs) up-regulated and 42 TF-mRNAs down-regulated with dry after-ripening (Supplementary Figure 9C). Categorization of genes by transcription factor families using the *countTFs* R function, written for this study (see Section "Materials and Methods"), revealed that transcription factor families strongly regulated with *sly1-2* after-ripening included AP2-EREBP, ARF (Auxin Response Factors), C3H (Cys3His zinc fingers), GRAS, and MYB-related families (Supplementary Figure 9D).

Since 2 weeks of dry after-ripening is sufficient to stimulate wild-type Ler but not in *sly1-2* germination, we examined changes in TF-mRNA accumulation in the *sly1-2* DvsWT dry seed comparison. Of the 794 *sly1*-regulated transcripts, 53 TF-mRNAs were *sly1*-down-regulated, while only 10 TF-mRNAs were up-regulated (Figure 6 and Supplementary Figure 9A). Thus, a major effect of the *sly1* mutation appears to be loss of TF-mRNAs that may be translated during imbibition. When these TF-mRNAs were examined at 0h and 12h, most of the dry seed *sly1*-down-regulated genes were not similarly regulated at 0h or 12h, while 7 of the 10 *sly1*-up-regulated genes were similarly regulated at 0h or 12h of imbibition (Figure 6). The *sly1*-down-regulated TF-mRNAs families included AP2-EREBP (APETALA2 and ethylene-responsive element binding proteins), bHLHs (basic helix-loop-helix), C2H2 zinc fingers, and MYB-related family...
transcription factors (Supplementary Figure 9B). The DELLA GAI was among the sly1-up-regulated TF-mRNAs. Thus, DELLA accumulation in sly1-2 may promote GAI expression, possibly through feed-forward regulation (Zentella et al., 2007).

In addition to TF-mRNAs, the Plant GeneSet Enrichment Analysis (PlantGSEA) tool was used to look for enrichment of known transcription factor targets within the dataset of stored mRNA differences in the sly1-2 DvsWT dry seed comparison (Yilmaz et al., 2010; Lai et al., 2012; Yi et al., 2013). Targets of the bHLH transcription factor PIF1/PIF5 (PHYTOCHROME INTERACTING FACTOR1/PIF3-LIKE5) were strongly enriched in the sly1-down-regulated geneset, representing 9% of the sly1-down-regulated genes in dry seeds (Supplementary Figure 10). Thus, PIF1/PIF5 may represent a SLY1-dependent regulator of seed dormancy.

An Association between mRNA Stability and Changes in Relative Transcript Levels with Dry After-ripening

Seed dormancy is relieved by after-ripening during dry storage. Little metabolic activity is possible in a dry seed, suggesting that differences in transcript turnover rates rather than active transcription may cause the changes in transcript abundances observed with dry after-ripening. Data analysis was used to explore whether apparent up- or down-regulation of stored mRNA was associated with differences in transcript stability. If a small number of stable or protected mRNAs degrade more slowly than the ribosomal RNA, microarray of apparently equal RNA amounts would indicate that these stable genes were up-regulated. A previous study identified genome-wide mRNA stabilities for 13,012 transcripts by measuring transcriptome changes over time after Ler cell cultures were treated with the transcriptional inhibitor Actinomycin D (Narsai et al., 2007). This included mRNA half-life values for 99 of the 139 sly1-2 and Cvi AR-regulated transcripts. A heatmap of these 99 AR-regulated transcript changes was plotted in decreasing order of mRNA half-life to examine whether lower intrinsic mRNA stability was associated with decreasing mRNA levels with dry after-ripening (Figure 7A). Although mRNA stability alone cannot account for all up- and down-regulation, shorter half-life mRNAs appeared more AR-down-regulated and longer half-life mRNAs appeared more AR-up-regulated. Similarly, when the AR-regulated transcripts were categorized by half-life range, a larger percentage of stable mRNAs (12–24 h or 6–12 h half-life) were up-regulated, whereas more unstable mRNAs (1–3 h half-life) were down-regulated (Figures 7B,C). This trend for high stability mRNAs to be up-regulated and lower stability mRNAs to be down-regulated was not seen at sly1-2 ARvsD 0h and 12h timepoints, indicating that mRNA stability is not the major determinant of transcript levels in imbibing seeds (Supplementary Figures 11A–C). The dry transcriptome counterexamples where mRNA stability was high, yet transcript levels were low or vice versa may be transcripts subject to more active regulation, such as protection by an RNA-binding proteins or targeted mRNA oxidation.

Comparison of Differential Regulation of Stored mRNAs by RT-qPCR and Microarray

RT-qPCR analysis was used to validate transcript level differences identified by microarray in the sly1-2 ARvsD 0h and 12h comparisons (Figure 8). For comparison, both RT-qPCR and microarray expression were plotted relative to the
FIGURE 7 | Transcriptome differences categorized based on inherent mRNA stability. (A) Heat map of genes differentially regulated in dry seeds with after-ripening of both sly1-2 and Cvi wt. Genes are plotted in order of high to low mRNA stability determined based on the half-life scores from Narsai et al. (2007). (B,C) Plots of fractions of after-ripening-up- and down-regulated genes in each half-life range stability category. (B) For sly1-2 dry seed transcriptome changes. (C) For Cvi dry seed transcriptome changes. There was a correlation of higher stability with up-regulation and lower stability with down-regulation in dry seed datasets. Both datasets had few genes with half-life in the 0–1 h range.

constitutively expressed control gene AKR2B (ANKYRIN REPEAT-CONTAINING 2B, At2g17390) (Hruz et al., 2011). RT-qPCR confirmed that GAI, HDA6, MFT, and HSFA9 (HEAT SHOCK FACTOR A9) were AR-down-regulated, while GAI and MFT were sly1-up-regulated in dry seeds (Figure 8A). As in imbibed seeds, the SLY1/sly1-2 transcript was AR-up-regulated and sly1-down-regulated in dry seeds (Nelson and Steber, 2017). The dormancy-associated DOG1 (DELAY OF GERMINATION1) gene was AR-up-regulated in the sly1-2 microarray analysis, but just outside of statistical significance (p = 0.071) by RT-qPCR. Conversely, DOG1 was AR-down-regulated in Cvi wt (Finch-Savage et al., 2007). Finally, the AHb1 transcript was highly AR-up-regulated based on microarray and RT-qPCR (p = 8 × 10^{-4}) analysis in sly1-2 (Figure 8B).

Since AHb1 was not significantly up-regulated with ecotype Cvi dry after-ripening, it may be the case that AR-up-regulation of AHb1 is dependent on the Ler ecotype. Thus, an after-ripening time course examined if AHb1 was up-regulated with dry after-ripening of wild-type Ler. RNA was isolated from dry Ler seeds immediately after harvest at maturity (0 weeks after-ripened, 0wkAR), then after-ripened for 1 (1wkAR) and 2 weeks (2wkAR). AHb1 mRNA levels showed an increasing trend with AR, and a significant increase from 0wkAR to 2wkAR by RT-qPCR analysis (Figure 8C and Supplementary Figure 12). Thus, AHb1 is up-regulated with dry after-ripening in the Ler ecotype, both in WT and sly1-2 seeds.

Functional Analysis of DELLA GAI and HDA6, Genes Down-regulated with Dry After-ripening

Dormancy loss due to dry seed after-ripening may result from degradation of transcripts encoding strong negative regulators of seed germination. For example, DELLA family genes are known to negatively regulate Arabidopsis seed germination. Both DELLA GAI and the histone deacetylase HDA6 were down-regulated with dry after-ripening of both sly1-2 and Cvi seeds. In addition, GAI was up-regulated in the sly1-2 DvsWT dry seed
comparing, indicating that GAI mRNA expression is associated with seed dormancy and negatively regulated by SLY1 and after-ripening. To examine whether the down-regulation of these mRNAs with dry after-ripening is functionally relevant, the effect of mutant alleles on seed dormancy and dormancy loss were examined.

Based on double mutant studies with ga1-3, DELLA GAI was believed to play a less important role in repressing seed germination than DELLA RGL2 (Lee et al., 2002; Tyler et al., 2004; Gao et al., 2005). While RGL2, RGL3 and GAI transcript levels were high in imbibing WT, sly1-2(D), sly1-2(AR), and sly1-2 GID1b-OE seeds, the fact that only GAI and RGL3 transcript levels were high in dry seeds suggests that GAI may be more important in dry seed after-ripening (Supplementary Figure 13). Furthermore, GAI was the only DELLA transcript differentially regulated with after-ripening in dry sly1-2 seeds, showing AR-down-regulation in both sly1-2 and Cvi wt seeds. Consistent with the notion that GAI regulates seed dormancy, gai-t6 had a higher and gai-1 a lower germination rate than wild-type Ler seeds when seed germination was examined in highly dormant fresh seeds harvested at near maturity (Figures 9A,B). Cold stratification improved germination for all lines, but gai-t6 consistently germinated faster than wild-type, while gai-1 germinated slower. If elevated GAI mRNA levels in sly1-2 seeds stimulate dormancy, then we would expect gai-t6 to rescue sly1-2 seed germination. Indeed, while dormant sly1-2 seeds failed to germinate even with cold stratification, the sly1-2 gai-t6 double mutant germinated without cold stratification reaching 25% with 16 days of incubation (Figures 9C,D). Taken together, these results suggest that GAI plays an early role in the negative regulation of seed germination.

If HDAR6 stimulates seed dormancy in wild-type Ler, then we would expect hda6 mutants to be less dormant than wild-type. The germination phenotype of the HDA6 allele in the Ler background called sil1 (modifiers of silencing1) was examined in seeds harvested near maturity to maximize dormancy. Seeds of sil1 germinated more efficiently than wild-type Ler in three biologically independent batches of seeds at 0 and 2 weeks of after-ripening, both with and without cold stratification (Figures 10A,B and Supplementary Figures 14A,B). This suggests that histone deacetylation by HDA6 stimulates seed dormancy, presumably by inhibiting the expression of genes needed for germination.

If histone deacetylation stimulates the seed dormancy of GA mutants, then inhibition of histone deacetylation should rescue the germination of GA-insensitive sly1-2 and of the
FIGURE 9 | Examining the role of GAI in the regulations of seed germination based on germination screens using freshly harvested seeds collected at near maturity. (A,B) Comparing of Ler wt, gai-1 (gain of function allele), and gai-t6 germination: (A) with cold stratification for 4 days at 4°C, before moving to the light at 22°C where germination was scored daily (“Cold”), and (B) without cold, seeds placed directly at 22°C and germination scored daily (“No Cold”). Loss of GAI function leads to an increase in germination and gain of GAI function leads to increased dormancy. (C,D) Comparing sly1-2 and sly1-2 gai-t6 germination (C) with cold stratification, and (D) without cold stratification. Loss of GAI function caused partial rescue of sly1-2 seed germination.

GA biosynthesis mutant ga1-3. This was examined using a specific inhibitor of histone deacetylases called tricostatin A (TSA) (Yoshida et al., 1995). TSA rescued the germination of dormant and after-ripened sly1-2 in a dose-dependent manner (Figure 11). Interestingly, TSA also stimulated the germination of ga1-3 seeds, suggesting that GA functions in part by relieving transcriptional repression by histone deacetylases. TSA rescued germination most efficiently at 2 μM (76%), and showed decreasing germination at 4 and 6 μM TSA. It may be that histone deacetylation and TSA alter the expression of other positive or negative regulators of germination at different concentrations.

DISCUSSION

DELLA-Directed Seed Dormancy in sly1-2

There are many mechanisms contributing to seed dormancy. The sly1 mutant has increased dormancy due to overaccumulation of DELLA proteins, the negative regulators of GA responses and seed germination. Thus, comparing sly1-2 vs. WT (DvsWT) defined transcriptome differences associated with DELLA-imposed seed dormancy.

The majority (65%) of these genes were down-regulated in sly1-2, suggesting that a major effect of sly1 loss/increased DELLA is decreased transcript abundance (Figure 2A). DELLA proteins act in concert with DNA-binding proteins to regulate transcription (Oh et al., 2004, 2006, 2007; Gallego-Bartolomé et al., 2010). Thus, it is interesting that the DELLA-interactor PIF1/PIL5 is a regulator of many highly sly1-down-regulated transcripts (Supplementary Figure 10). PIF-regulated genes were expected to be among SLY1/DELLA-regulated genes because DELLA proteins bind PIF3 and PIF4, inhibiting PIF DNA-binding and transcriptional activation while promoting PIF3 protein degradation by the 26S proteasome (de Lucas et al., 2008; Feng et al., 2008; Li et al., 2016). PIF1/PIL5 is a known DELLA interactor whose negative regulation of germination is relieved by light (Oh et al., 2004, 2006, 2007; Gallego-Bartolomé et al., 2010). Thus, it appears that DELLA overaccumulation in sly1-2 seeds during development or maturation may cause transcriptional repression of PIF1/PIL5-regulated gene targets accounting for some of the down-regulation of stored mRNAs in dry seeds.

Transcription factors produced early in seed imbibition are ideal candidates to initiate the transcriptional cascades leading to or blocking germination per se. There were 5-times more sly1-down-regulated than sly1-up-regulated TF-mRNAs (Supplementary Figure 9). This suggests that
DELLA overaccumulation in sly1 leads to lower expression of transcription factors. Known regulators of germination, ABI5 (ABA-INSENSITIVE5) and DELLA GAI are examples of major sly1-regulated TF-mRNAs (Figure 6; Koornneef et al., 1985; Lopez-Molina et al., 2002). Thus, different levels of germination-promoting or -inhibiting TFs in sly1-2 and WT may be one mechanism allowing wild-type Ler, but not sly1-2, seeds to germinate at 2 weeks of after-ripening.

While it is tempting to believe that dry seed transcriptional differences in sly1-2(D) compared to WT arise entirely during development or maturation, these differences may also arise during 2 weeks of dry storage. For example, transcripts may be degraded at different rates in different genotypes, either faster or slower in the sly1-2 mutant than in WT. Since sly1-2 requires 1–2 years to reach a germination rate similar to WT after-ripened for 2 weeks, it is possible that some germination-inhibiting transcripts require more time to degrade or oxidize in sly1-2 than in WT. It could also be the case that germination-promoting transcripts are less protected in sly1-2. Investigation of DvsWT transcriptome differences during development and maturation might help to differentiate transcriptome differences arising during development from those arising during dry storage.

**Evidence for the Functional Relevance of Dry Seed Transcriptome Changes**

While it may be argued that changes in the dry seed transcriptome are merely artifacts of mRNA oxidation/damage over time, the results of this study provide circumstantial evidence that some of these changes are of regulatory importance in dormancy loss. First, similar changes occurred with dry after-ripening in two different ecotypes. Second, transcription factors known to function in dormancy, dormancy loss, and GA signaling were among the AR-differentially regulated genes. And third, mutations in two of these differentially regulated genes resulted in altered seed dormancy and germination.

The overlap in the sly1-2 and Cvi ARvsD comparisons suggested that dry seed transcriptome changes are not due to random degradation of transcripts as seeds age, but may represent dormancy-loss mechanisms. Of the 770 stored mRNAs that were differentially regulated with after-ripening in dry sly1-2 seeds, 12% of the AR-up-regulated and 23% of the AR-down-regulated were similarly regulated in Cvi wt (Figure 4A). Since sly1-2 is a mutation in the Ler rather than the Cvi ecotype, differences between these two ARvsD comparisons may result either from ecotype differences or the sly1-2 mutation. Interestingly, the regulation of TAGGIT gene categories was similar in sly1-2 and Cvi wt dry seed after-ripening (Figures 5B,C). The partial overlap in the sly1-2 and Cvi
ARVsD comparisons may simply suggest that the seed dormancy of the two genotypes results from only partially overlapping mechanisms. In other words, there are multiple ways to acquire and to lose seed dormancy.

Even transcripts that are AR-regulated in sly1-2 but not Cvi may function in after-ripening of the Ler ecotype. For example, the AHB1 transcript was not AR-up-regulated in Cvi, but was strongly AR-up-regulated transcript in dry seeds of sly1-2 and Ler. AHB1 (also called Arabidopsis class 1 phytooglobin or pgb1) protects roots from severe oxidative stress (Hill et al., 2016; Mira et al., 2017). Thus, it may play a similar role in dry seeds. There appears to be a link between class 1 phytooglobin expression and seed dormancy/germination in barley (Ma et al., 2016). Dormancy can also be rescued without a large change at the transcriptome level, as evident in barley (Ma et al., 2016). Dormancy can also be rescued without a large change at the transcriptome level, as evident in barley (Ma et al., 2016).

Transcription factors produced early in seed imbibition are ideal candidates to initiate the transcriptional cascades leading to or blocking germination per se. Thus, it is interesting that transcription factors known to control dormancy and dormancy loss were among the AR-regulated genes. ABA hormone establishes dormancy, ethylene can break dormancy in ga1-1, and auxin has been implicated in dormancy and dormancy release (Finkelstein et al., 2008; Karssen et al., 1989). In light of this, it is interesting that TAGGIT ontology analysis found that 9% of TFs were ABA-related, 12% were ethylene-related, and 7% were auxin-related (Supplementary Figure 9C). For example, ABA related protein phosphatase genes, HAB2 (HOMOLOGY TO ABI2), AHG3 (ABA-HYPERSENSITIVE GERMINATION3), and HA13 (HIGHLY ABA-INDUCED PP2C GENE3) were among transcripts down-regulated with sly1-2 after-ripening (Supplementary Table 2; Finkelstein et al., 2008). Moreover, the negative regulator of germination and GA signaling, DELLA GAI was also AR-down-regulated in dry sly1 seeds (Figure 6; Koornneef et al., 1985). Examination of mutations in two sly1 AR-downregulated genes resulted in altered seed dormancy, allowing us to conclude that the decreased transcript levels of GAI and HDA6 are likely to increase germination.

**GAI Regulation of Seed Dormancy**

The DELLA GAI was the most AR-down-regulated gene in dry sly1-2 seeds, suggesting a more important role in seed germination than previously believed. The DELLA RGL2 is considered the major DELLA repressing seed germination, since rgl2 mutations best rescue ga1-3 germination in the light (Tyler et al., 2004; Cao et al., 2005). DELLA GAI also functions as a negative regulator of germination, since the ga1-3 gai-t6 rgl2-1 triple but not the ga1-3 rgl2-1 double mutant can germinate in the dark. DELLA GAI functions as a negative regulator of germination, since the ga1-3 gai-t6 rgl2-1 double mutant can germinate in the dark. DELLA GAI functions as a negative regulator of germination, since the ga1-3 gai-t6 rgl2-1 double mutant can germinate in the dark. DELLA GAI functions as a negative regulator of germination, since the ga1-3 gai-t6 rgl2-1 double mutant can germinate in the dark. DELLA GAI functions as a negative regulator of germination, since the ga1-3 gai-t6 rgl2-1 double mutant can germinate in the dark. DELLA GAI functions as a negative regulator of germination, since the ga1-3 gai-t6 rgl2-1 double mutant can germinate in the dark. DELLA GAI functions as a negative regulator of germination, since the ga1-3 gai-t6 rgl2-1 double mutant can germinate in the dark. DELLA GAI functions as a negative regulator of germination, since the ga1-3 gai-t6 rgl2-1 double mutant can germinate in the dark. DELLA GAI functions as a negative regulator of germination, since the ga1-3 gai-t6 rgl2-1 double mutant can germinate in the dark. DELLA GAI functions as a negative regulator of germination, since the ga1-3 gai-t6 rgl2-1 double mutant can germinate in the dark. DELLA GAI functions as a negative regulator of germination, since the ga1-3 gai-t6 rgl2-1 double mutant can germinate in the dark. DELLA GAI functions as a negative regulator of germination, since the ga1-3 gai-t6 rgl2-1 double mutant can germinate in the dark.

**Control of Seed Dormancy by Histone Modification**

Chromatin modifications regulate developmental processes including dormancy by altering gene transcription (reviewed in Nonogaki, 2014). Since 65% of the differentially-regulated transcripts in sly1-2 (DvsWT) were down-regulated, it was interesting that rescue of sly1-2 seed germination by long after-ripening was associated with down-regulation of the HDA6 histone deacetylase because histone deacetylases repress gene transcription. Histone deacetylation represses gene expression through heterochromatin formation, whereas histone acetylation promotes gene expression and has been implicated in seed dormancy release by stimulating gene expression needed for seed germination. Our hypothesis was that HDA6 down-regulation with after-ripening of sly1-2 and Cvi breaks dormancy through increased expression of germination-promoting transcripts. The notion that HDA6 stimulates seed dormancy was supported by the observation that loss of HDA6 in the sil1 mutant decreased seed dormancy in freshly harvested seeds (Figure 10). In addition to the hda6/sil1 mutant, the histone deacetylase mutants hda9 and hda19 also exhibited reduced seed dormancy (Wang et al., 2013; van Zanten et al., 2014). HDA9 is down-regulated with imbibition, but neither HDA9 nor HDA19 were down-regulated with sly1-2 after-ripening. HDA6 also appears to function in ABA and salt stress response, as hda6 and hda19 mutants were hypersensitive to ABA and salt inhibition of germination (Chen and Wu, 2010; Chen et al., 2010; Luo et al., 2012).

The increased seed dormancy associated with reduced GA signaling appears to be partially due to gene repression by histone deacetylation. The GA biosynthesis mutant ga1-3 fails to germinate, and never regains the ability to germinate through after-ripening. Interestingly, the inhibitor of histone deacetylase...
activity TSA partly rescued the germination not only of sly1-2 but of gal-1-3 seeds (Figure 11). The increased seed dormancy in sly1-2 is rescued by long after-ripening, whereas the seed dormancy of the GA biosynthesis in gal-1-3 is not. No GA signaling can occur in gal-1-3, whereas some GA signaling can occur in sly1-2 mutants that cannot trigger DELLA destruction (Ariizumi and Steber, 2007; Ariizumi et al., 2013). Thus, DELLA-proteolysis independent GA signaling may be sufficient for HDA6 down-regulation with sly1 after-ripening. Taken together, this suggests that histone deacetylation maintains dormancy in GA mutants and that TSA-treatment may bypass GA signaling to relieve seed dormancy by allowing histone acetylation. This is consistent with previous studies suggesting that histone deacetylation stimulates and TSA relieves seed dormancy (Yoshida et al., 1995; Yano et al., 2013; van Zanten et al., 2014). Future work will need to examine whether down-regulation of HDA6 with after-ripening is associated with altered histone acetylation of HDA6 targets.

**SLY1 and GA Signaling Regulate Protein Translation**

Our sly1-2 transcriptome studies indicate that regulation of translation-associated gene expression is one of the major roles of GA signaling in seeds (Nelson and Steber, 2017). Inhibitor studies showed that translation, not gene stimulation, is required for seed germination per se (Rajjou et al., 2004). Thus, regulation of translation-associated genes is an excellent strategy for determining whether or not a seed can germinate. Consistent with this notion, previous studies found that translation-associated genes were strongly up-regulated with seed imbibition and Cvi after-ripening (Nakabayashi et al., 2005; Dekkers et al., 2016). Differentially regulated translation-associated genes in this and other studies included ribosomal subunits and translation initiation and elongation factors. The translation-associated category was strongly AR-up-regulated in imbibing Ler wild-type seeds, but not well AR-up-regulated in imbibing sly1-2 seeds (Dekkers et al., 2016; Nelson and Steber, 2017). The positive regulator of GA signaling, SLY1, was needed to up-regulate translation-associated genes with after-ripening of imbibed seeds (Nelson and Steber, 2017). Moreover, protein translation-associated transcripts were strongly GA-up-regulated and DELLA-down-regulated, indicating that regulation of translation-associated genes is a general function of GA signaling (Nelson and Steber, 2017). Previous work showed that after-ripening was associated with higher protein translation after 24h of imbibition in H. annuus (Layat et al., 2014). After-ripening can also be associated with increased translation of specific transcripts (Layat et al., 2014; Basbouss-Serhal et al., 2015). One possibility is that the increased mRNA accumulation of specific translation initiation factors with after-ripening is responsible for recruitment of specific transcripts. Future work will need to determine if dormant gal-1-3 and sly1-2 seeds have either a general defect in protein translation or an inability to translate specific transcripts.

In contrast to imbibed seeds, translation-associated genes were strongly AR-down-regulated in dry sly1-2 and Cvi seeds (Figures 5B,C). Although not as much as in DvsWT, translation-associated mRNAs accounted for 12% of the up-regulated transcripts in the sly1-2 ARvsWT dry seed comparison (Figure 5A). This indicates that SLY1 is not a requirement for this decrease with after-ripening, but that loss of SLY1 resulted in a higher starting-point during seed maturation. Thus, it appears that SLY1 is needed for down-regulation of translation-associated transcripts during seed maturation, since the translation-associated category accounted for 25% of the sly1-up-regulated genes in dry seeds (Figure 5A). This suggests that SLY1 may serve as a kind of shutdown signal to down-regulate translation associated genes during seed maturation to prepare for the quiescent state. In this context, it is interesting to note that sly1-2 mutant seeds exhibit a mild decrease in survival of long-term storage (Ariizumi and Steber, 2007). Future work should examine the early imbibition proteome to determine if translation-associated proteins over-accumulate in sly1-2 seeds during early imbibition. If too much of early translation is devoted to translation-associated gene expression, there may be limited amino acids available for protein synthesis of other important early-translated transcripts.

**Differences in mRNA Stability Correlate to Changes in Transcript Levels with Dry After-ripening**

If changes in the dry seed transcriptome increase germination potential, then how can a quiescent, dry seed differentially regulate these changes in transcript levels? If we assume that de novo transcription is very unlikely in dry seeds, then such changes must be regulated through degradation that preferentially targets certain mRNAs over others. Genes that are up-regulated in transcriptome analyses may be those that are more stable or well protected than the majority of the transcriptome, while those that are down-regulated are those that are less stable or otherwise more prone to degradation (i.e., targeted for degradation via mRNA oxidation or other mechanisms) than the majority. Consistent with this notion, comparison of dry seed AR-regulation with Arabidopsis mRNA stability, showed a correlation between AR-up-regulation and higher mRNA stability, as well as AR-down-regulation and lower mRNA stability (Figure 7). This is consistent with a previous study showing RNA degradation during dry after-ripening of sunflower seeds and Arabidopsis (Bazin et al., 2011; Basbouss-Serhal et al., 2017). Imbibed seeds did not show a correlation between mRNA stability and AR-regulation (Supplementary Figures 11A–C). In fact, in early Phase II (0h) there appeared to be a negative correlation between mRNA stability and AR-regulation, possibly indicating increased transcription of mRNAs that were not present in dry seeds at the time of imbibition due to lower stability.

Novel mechanisms may control those transcripts whose dry seed accumulation cannot be explained by differences in mRNA stability. Such genes may be regulated by other factors that increase or reduce the chances of degradation in a real seed. Future work should examine whether the subcellular localization
of transcripts or RNA-binding proteins determine whether transcripts appear to be AR-up- or AR-down-regulated in dry seeds, as opposed to de novo transcription. Genes like At3g23090 that have low stability mRNAs, but are up-regulated with after-ripening would be good candidates for such studies.

CONCLUSION

How dormancy is lost in dry, metabolically inactive seeds is a fascinating question. This study took some first steps toward addressing this question by identifying transcriptional mechanisms underlying dormancy and dormancy loss in dry seeds of the GA-insensitive mutant, sly1-2. Our general model is that dry after-ripening of seeds leads to down-regulation of transcripts that negatively regulate seed germination. Loss of function mutations in two of these strongly AR-down-regulated transcripts, GA1 and HDA6, resulted in increased germination potential (Figures 9, 10). The AR-down-regulation of these two transcripts and of other transcription factors suggests that the control of gene transcription and of histone acetylation is one major mechanism controlling dormancy and after-ripening of dry seeds. The sly1 seed dormancy phenotype was strongly associated with decreased abundance of transcription factor mRNAs, and generally skewed toward transcriptome down-regulation. Thus, it appears that over-accumulation of DELLA repressors has the general effect of down-regulating dry seed transcript abundances. There is one major counterexample to this observation; genes associated with protein translation were strongly up-regulated in dry dormant sly1-2 seeds compared to wild type accounting for 25% of the sly1-up-regulated transcripts. Translation-associated genes are the major class of GA and SLY1-regulated transcripts in seeds (Figures 5A,B; Nelson and Steber, 2017). Ribosomes are inactive in dry seeds, and must be reactivated in order to germinate (Bewley et al., 2013). SLY1 is needed to down-regulate protein translation-genues during seed maturation and to up-regulate protein translation-genues with after-ripening during seed imbibition. Future work will need to examine if the increased dormancy of sly1-2 and ga1-3 results largely from inability to efficiently up-regulate protein translation.

AUTHOR CONTRIBUTIONS

CS provided the initial research design and obtained funding. TA performed the TSA experiments for Figure 11. SN performed all remaining experiments and bioinformatics analyses. Both CS and SN contributed to the research and analysis design, and to the writing of this article.

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SUPPLEMENTARY MATERIAL

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