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TECHNICAL UPDATE

Chemically inducible gene expression in seeds before testa rupture

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Abstract

Impermeability of the testa hinders efficient penetration of some small chemicals, such as transcriptional inhibitors, through the endosperm and the embryo during seed experiments. In *Arabidopsis* seeds, 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid, a substrate for β-glucuronidase, did not permeate through the endosperm and embryo efficiently at the stages before testa rupture. The *Arabidopsis* testa also limited efficient entry of methoxyfenozide, a chemical ligand that was used for inducible gene expression experiments, into seeds. While the detection of a reporter gene at the early imbibitional stages could be replaced by reverse transcription-polymerase chain reaction (RT-PCR), the interference of entry of the chemical ligand into seeds by the testa was still problematic to gene induction experiments. To develop an efficient inducible expression system for gene function analysis in seeds, an inducible expression system with nitrate, which is a testa-permeable ligand, was examined. The vector containing the 2.1-kb upstream sequence of *NITRITE REDUCTASE 1* was able to cause expression of a test gene (long non-coding RNA) in imbibed seeds at the stage before testa rupture in a nitrate-dependent manner. This system can be used not only for characterization of genes associated with seed dormancy and germination in basic research, but also for the development of germination recovery or enhancement technologies for agricultural applications.

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Introduction

An inducible gene expression system, which allows gene induction in a temporal and spatial manner, is a useful tool for gene function analysis. It is also used for the analysis of biological roles of seed dormancy- or germination-associated genes (Piskurewicz *et al.*, 2008; Martinez-Andujar *et al.*, 2011). Chemically inducible gene expression provides an excellent experimental system and also offers great potential for technology development to control seed germination (Martinez-Andujar *et al.*, 2011). However, there is a problem of impermeability of the testa to chemical ligands. Some chemical ligands may not penetrate the testa efficiently. While it is possible to deliver those ligands through the endosperm and the embryo by particularly targeting the stages after testa rupture, dormant seeds do not exhibit testa rupture and cannot take in chemical ligands efficiently, which hinders examination of the function of genes potentially associated with dormancy release or germination induction.

An inducible gene expression system with a permeable ligand is also important for the development of seed technologies that enable germination recovery from hyperdormant seeds. Different systems to cause hyperdormancy in seeds have been developed for prevention of preharvest sprouting (PHS) (Martinez-Andujar *et al.*, 2011; Nonogaki, 2014; Nonogaki *et al.*, 2014). However, the next important question is: how can hyperdormancy be released when PHS-resistant seeds need to be germinated? This question can be addressed by inducible expression of positive regulators of germination with a permeable ligand. In this paper, problems associated with

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impermeability of the *Arabidopsis* seed testa and the potential of an inducible gene expression system with a permeable ligand will be discussed.

Materials and methods

Seed germination

Arabidopsis seeds were plated on filter papers moistened with 3.5 ml of deionized water or test solutions, which were contained in 9-cm-diameter Petri dishes, and were incubated at 22°C under light. Germination percentages after 7 d, with or without 7-d pre-chilling (indicated for individual experiments), were recorded.

β-Glucuronidase staining

β-Glucuronidase (GUS) staining was performed using 100 mM sodium phosphate buffer (pH 7.0) containing 0.1% (v/v) Triton X-100 and 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid; RPI Co., Mount Prospect, Illinois, USA). Staining was examined after overnight incubation at room temperature in the dark.

Reverse transcription-polymerase chain reaction

For reverse transcription-polymerase chain reaction (RT-PCR) to analyse gene expression, RNA was extracted from seeds using a standard phenol-sodium dodecyl sulphate (SDS) extraction. Two micrograms of total RNA were reverse transcribed with Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, Wisconsin, USA). The conditions for PCR were: one cycle at 94°C (1 min), touchdown cycles (94°C for 15 s, 66°C → 59°C for 15 s and 72°C for 30 s) (one cycle for each annealing temperature) and 30 cycles at 94°C (15 s), 59°C (15 s) and 72°C (30 s), followed by extension at 72°C (7 min). Twenty cycles, instead of 30 cycles, were used for semi-quantitative RT-PCR. For the expression of the At2g48160 gene, the forward (5′- ACAGAGGAGAGGAAGCAATC-3′) and reverse (5′-CTGAGAGCTTAGCAGACACT-3′) primers were used. Ex Taq DNA polymerase (Takara, Mountain View, California, USA) was used for RT-PCR.

*Nine-*cis*-epoxycarotenoid dioxygenase induction*

The details about the 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) inducible system were described previously (Martinez-Andujar *et al.*, 2011). Briefly, seeds carrying the inducible *NCED* were incubated in the presence or absence of Intrepid2F (Dow AgroSciences, Indianapolis, Indiana, USA), a chemical inducer which contained 62 μM methoxyfenozide as an active ingredient.

Nitrate-inducible gene expression

The 5′ upstream sequence (2092 bp) of the *Arabidopsis NITRITE REDUCTASE 1* (*NIR1*) was amplified using the *Arabidopsis* Columbia (Col-0) genomic DNA and the forward (AtNIR-Pro-F3: 5′-AGTAGGGATGT-GTCGTGTGT-3′) and reverse primers (AtNIR-Pro-R: 5′-GATGATGGCGGAAGAAGGAGT-3′). The isolated fragment was re-amplified for InFusion (Takara) reaction with the forward (pNIR InFusion[*Hin*dIII]-F: 5′- GGCCAGTGCCAAGCTTAGTAGGGATGTGTCG-3′) and reverse (pNIR InFusion[*Bam* HI]-R: 5′- CGGTAC-CCGGGGATCCGATGATGGCGGAAGA-3′) primers. PrimeSTAR, a high-fidelity DNA polymerarse (Takara), was used for this purpose.

The fragment prepared with the InFusion primers was cloned to the pCambia 1301 vector (courtesy of Tony Chen, Department of Horticulture, Oregon State University) digested with *Hin*dIII and *Bam* HI. Then, the *Bam* HI site in the vector was re-digested to insert a test gene (long non-coding RNA) using an InFusion reaction. The complete vector was used for *Agrobacterium*-mediated plant transformation as described previously (Liu *et al.*, 2005a, b). Transgenic plants carrying the nitrate-inducible system were selected using hygromycin.

For induction experiments, 10 mg of seeds produced from the hemizygous T1 plants were plated with 10 mM potassium nitrate or potassium chloride (control) solution. RNA was extracted from seeds as described previously (Liu *et al.*, 2005a, b). For leaf induction, a rosette was cut at the petiole, which was soaked in a test solution contained in a 0.5-ml tube and incubated for 5 h. RT-PCR was performed as described above, except that different forward (#1847: 5′-GAACGTGGAACCCTTAGTTAC-3′) and reverse (#1828: 5′-ATGAGGAAGCCAAACTCCAA -3′) primers were used in this experiment.

Results and discussion

Impermeability of the testa

It has been suggested that the testa of *Arabidopsis* seed is impermeable to some small chemicals (Debeaujon *et al.*, 2000; Rajjou *et al.*, 2004). We have also observed a similar issue for the penetration of 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid, a substrate for GUS, into seeds before testa rupture. When an enhancer-trap line exhibiting GUS expression in the cotyledon tips was isolated, its expression was also detected in seeds (Fig. 1) (Liu *et al.*, 2005a, b). The excised embryos showed the GUS signals at the tip of cotyledons. The GUS signals were also visible in the intact seeds (Fig. 1). However, the signals seemed to be limited to only the

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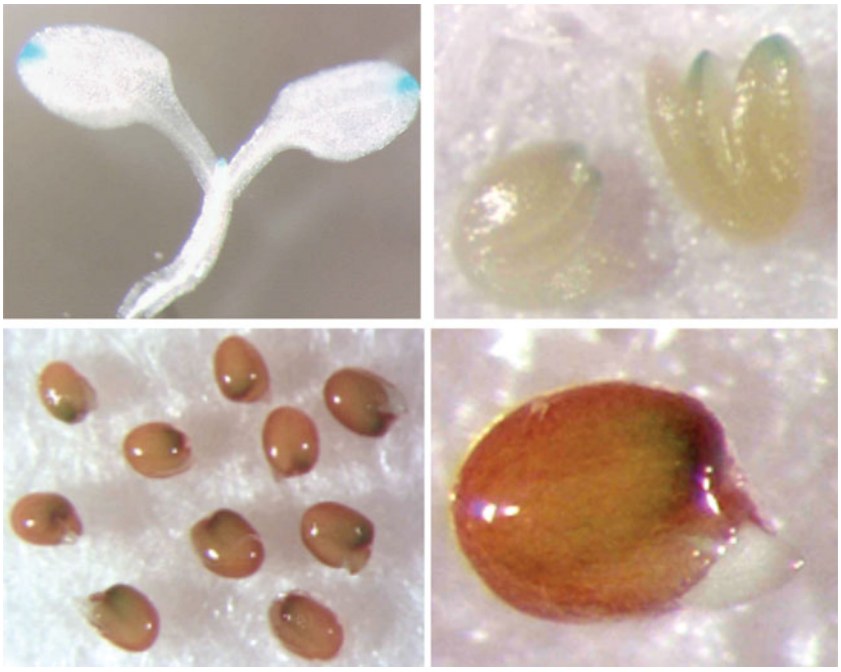


Figure 1. An *Arabidopsis* enhancer-trap line exhibiting GUS signals in the cotyledon tips. The signals were detected in a seedling (top, left), excised embryos (top, right) and seeds (bottom). The signals in seeds were detected after testa rupture.

stages after testa rupture and were not observed at the early stages of imbibition.

In contrast, the analysis of the expression of the corresponding gene (At2g48160) in imbibed seeds using RT-PCR indicated that its expression peaked around 6 h after the start of imbibition (Fig. 2a). We speculated that the lack of the GUS signals at the early imbibitional stages in the enhancer-trap seeds was probably because the endosperm and the embryo were not exposed to the substrates, due to the impermeability of the testa.

To test this possibility, the enhancer-trap line was crossed to the *transparent testa3* (*tt3*) *transparent testa glabra* (*ttg*) double mutant (as a female). All resultant seeds exhibited a pale-coloured testa, which is known to have better permeability (Debeaujon *et al.*, 2000; Rajjou *et al.*, 2004). When these seeds were examined for GUS expression, the signals were detected at the imbibitional stages much earlier than testa rupture, with the peak expression around 6–12 h after the start of imbibition (Fig. 2b). The GUS signals localized exclusively to the cotyledon tip regions as observed before. The timing of the GUS expression in imbibed seeds with the transparent testa (Fig. 2b) was reasonably consistent with the results of RT-PCR using the enhancer-trap seeds with the wild-type testa (Fig. 2a). These results suggest that the pigment proanthocyanidins contained in the testa (Nesi *et al.*, 2001) cause limited permeability to the GUS substrate.

A similar issue was observed for Intrepid2F (ingredient: methoxyfenozide), a chemical ligand used for the Plant Gene Switch System, which is a chemically inducible gene expression system (Padidam, 2003; Koo *et al.*, 2004; Tavva *et al.*, 2006; Martinez-Andujar *et al.*, 2011). Induction of *NCED*, a rate-limiting abscisic acid (ABA) biosynthesis gene, by this system was sufficient to suppress seed germination (Martinez-Andujar *et al.*, 2011). However, almost all *NCED*-induced seeds were suppressed only after the testa rupture in this system (examples shown in Fig. 3). Since ABA is synthesized and functions inside seeds to maintain dormancy in the native system and dormant seeds do not exhibit testa rupture after imbibition, it is not conceivable that *NCED* and ABA play their roles particularly in the narrow window of the stages between testa and endosperm rupture. The fact that almost all of the seeds were arrested after testa rupture in the *NCED*-inducible system would rather suggest that *NCED* induction and ABA synthesis did not occur until the rupture of the testa, which prevented the entry of the chemical ligand methoxyfenozide into the endosperm and the embryo. These results exemplify that testa permeability is also an issue for chemically inducible gene expression systems with this type of ligand.

The need for an inducible expression system with a permeable ligand

The impermeability of the testa may not be an issue when a germination suppressing gene is induced, because sooner or later radicle growth will be inhibited

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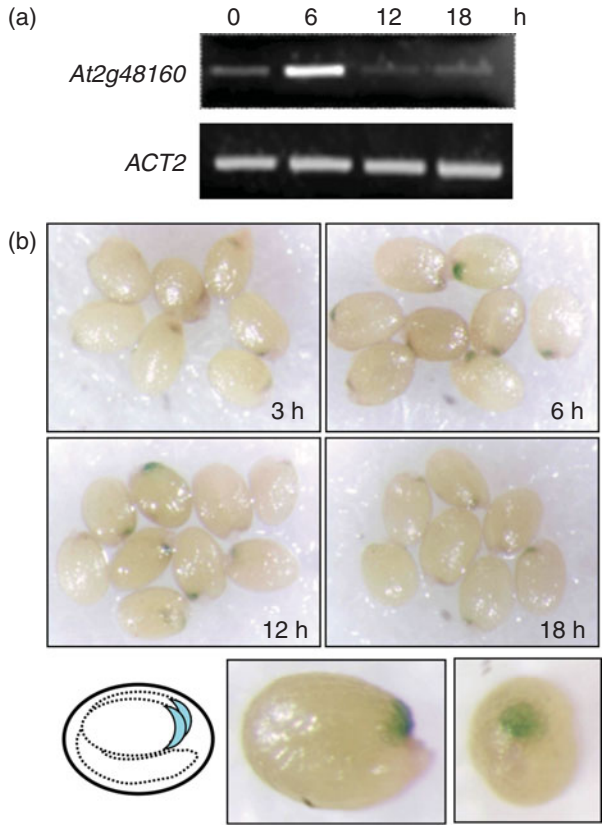


Figure 2. Expression of the At2g48160 gene corresponding to the enhancer-trap line shown in Fig. 1. (a) Expression of the enhancer-trapped gene in seeds at the different imbibitional stages (0, 6, 12 and 18 h), which was examined by RT-PCR; *ACT2, ACTIN2*. (b) Expression of the enhancer-trapped gene in the seeds with the *transparent testa3 (tt3) transparent testa glabra* (*ttg*) background. The line was created by crossing the *tt3 ttg* line as female with the pollen from the enhancer-trap line, which produced pale-coloured seeds. GUS expression at the cotyledon tips is highlighted in the schematic representation and the close-up views at bottom.

by the expression of the negative regulator, as observed for the *NCED*-inducible system above. However, inducible gene expression systems with chemical ligands, which cannot penetrate through the endosperm and the embryo, will not operate well when germination recovery is intended. Dormant seeds do not exhibit testa rupture and therefore will not provide an opportunity for the chemical ligand to trigger gene expression inside seeds, even when a positive regulator of germination is successfully incorporated into the system. This could be a problem when genes with dormancy-releasing potential are examined for their function in basic research. Therefore, it is necessary to develop an inducible gene expression system with a testa-permeable ligand.

Inducing positive regulators of seed germination is also important for seed enhancement or germination recovery technologies. An advanced system, which imposes hyperdormancy to developing seeds through amplification of *NCED* expression and the ABA biosynthesis and signalling, has been developed for the prevention of PHS (Nonogaki *et al.*, 2014) [called '*NCED*-amplified lines (*NCED*∞)' hereafter]. The *NCED*-amplified lines do not need chemical induction but increase ABA levels in seeds during the maturation stages in a spontaneous manner, through a positive-feedback mechanism. The dormancy imposed by this system is extremely deep (Fig. 4a). While this phenotype is preferable for PHS prevention, it is necessary to establish strategies for seed germination recovery to make a comprehensive technology for germination control. We found that the hyperdormancy imposed by this system can be released partially by a combination of seed storage, prolonged prechilling treatments and dormancy-releasing chemicals (Fig. 4b). However, full recovery of germination needs to be achieved by more progressive approaches.

Induction of germination-promoting factors, such as gibberellin (GA) biosynthesis genes, ABA deactivation genes and *NCED* antisense or RNA interference (RNAi), has great potential to recover germination. However, the inducible system mentioned above may not function well for this purpose because the chemical ligand does not seem to penetrate the testa efficiently and the *NCED*-amplified hyperdormant seeds do not exhibit testa rupture. Therefore, it is necessary to develop a new inducible gene expression system with a chemical ligand that could permeate through the endosperm and the embryo, particularly for germination recovery purposes.



Figure 3. Typical appearance of the *NCED*-induced *Arabidopsis* seeds by the chemcal ligand Intrepid2F (methoxy fenozide). While near-complete suppression of germination was observed, almost all seeds still reached testa rupture, suggesting that induction occurred only after testa rupture and that the testa is impermeable to methoxyfenozide.

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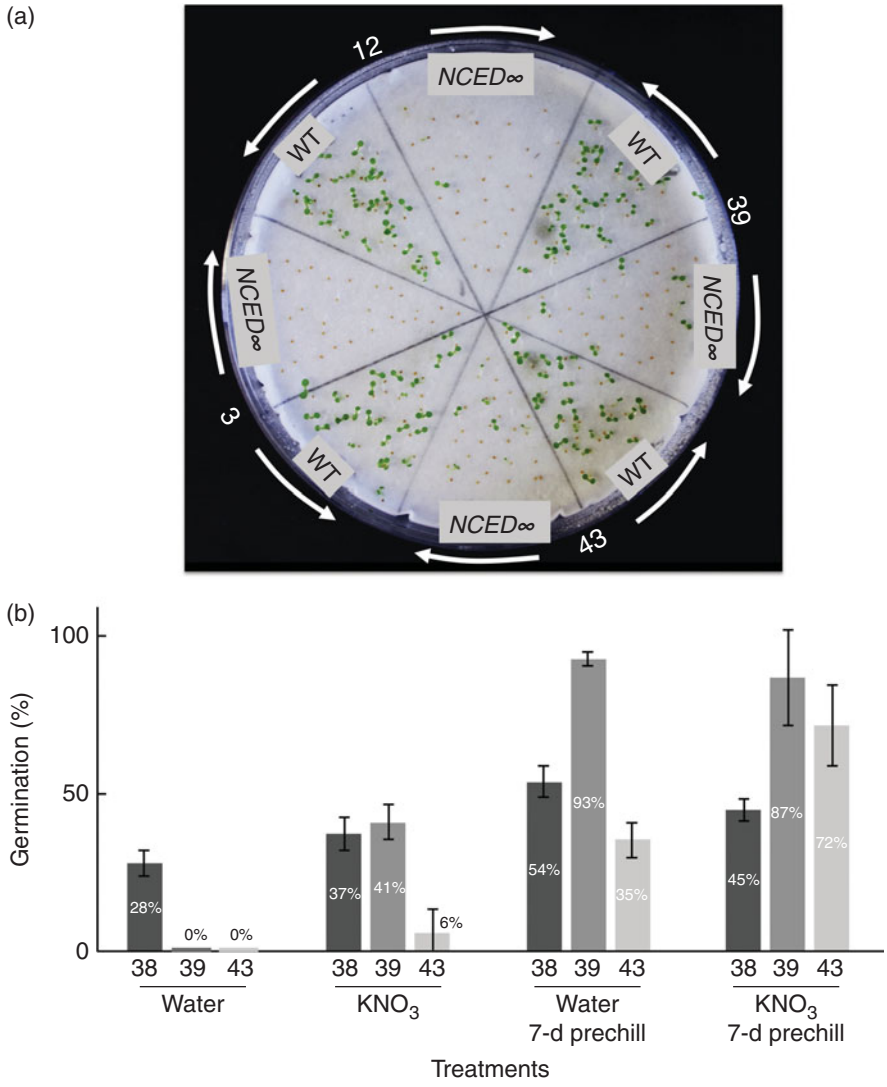


Figure 4. Suppression and recovery of seed germination in the *NCED*-amplified *Arabidopsis* lines (*NCED*∞), in which ABA biosynthesis and signalling were amplified through a positive-feedback mechanism and hyperdormancy was induced in a spontaneous manner. (a) Examples of germination suppression in 3-month-old seeds of the four independent *NCED*∞ lines (3, 12, 39, 43). Wild-type (WT) and the *NCED*-amplified (*NCED*∞) seeds from the same maternal plant were compared for each line in water [modified from Nonogaki *et al.* (2014) *The Plant Journal* **78**, 529–539]. (b) Recovery of germination by a combination of 50 mM potassium nitrate (KNO_3) and 7-d prechilling treatments. One-month-old seeds of the three independent *NCED*∞ lines (38, 39, 43) were examined. Note that full germinaiton recovery was not observed, even with the most efficient treatment (KNO_3 plus 7-d prechilling).

The potential of the nitrate-inducible gene expression system

To explore an inducible gene expression system in imbibed seeds before testa rupture, we focused on a gene promoter responsive to nitrate, which is a soluble chemical known to be permeable through the testa and also as a dormancy-releasing reagent (Fig. 4b; discussed below). The promoter of the *Arabidopsis NIR1* has been characterized (Konishi and Yanagisawa, 2010). Approximately 3-kb upstream and 2-kb downstream regions of this gene enable induction of a GUS reporter gene in *Arabidopsis* seedlings in a nitrate-responsive manner. The promoter region contains the nitrate-responsive *cis*-element (NRE). A synthetic promoter, which contains the four copies of a 43-bp sequence including NRE and the 35S minimal promoter, is capable of directing nitrate-responsive transcription (Konishi and Yanagisawa, 2010).

Based on this information, we have isolated a 2.1-kb region of the promoter containing NRE together with part of the 5′ untranslated region (UTR) of this gene and cloned the fragment to a transformation vector (Fig. 5a), which was used to prepare transgenic plants.

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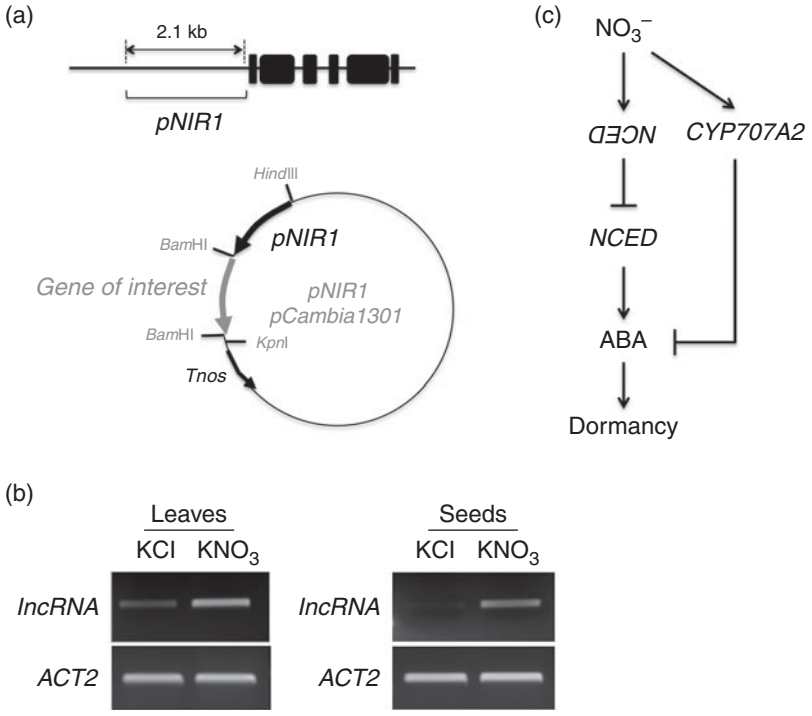


Figure 5. Nitrate-inducible gene expression in *Arabidopsis* seeds. (a) Schematic representaitons of the structure of the *NITRITE REDUCTASE 1* (*NIR1*), including the promoter region (*pNIR1*) used for the experiments (top) and the transformation vector containing *pNIR1*, to which a germination recovery gene of interest can be inserted (bottom). The major restriction sites (*Bam*HI, *Hin*dIII, *Kpn*I) are indicated in the scheme. *Tnos*, nos (nopaline synthase) terminator. (b) Semi-quantitative RT-PCR showing examples of induction of a gene of interest [long non-coding RNA (lncRNA)] in leaves and 15-h-imbibed seeds by 10 mM potassium nitrate (KNO_3) with a control of potassium chloride (KCl); *ACT2*, *ACTIN2*. (c) Schematic representation of the concept of dual effects of ABA reduction by antisense *NCED*-induction by the nitrate-inducible system. Since nitrate induces *CYP707A2*, an ABA deactivation gene in the native system, antisense *NCED* induction by nitrate could have dual effects of decreasing ABA biosynthesis and increasing ABA deactivation, both of which reduce ABA levels in seeds and release dormancy.

A test transcript [long non-coding RNA (lncRNA)] was examined in the transgenic *Arabidopsis* leaves. The accumulation of lncRNA, which was also observed in the control leaves at a moderate level, was strongly enhanced in a nitrate-responsive manner (Fig. 5b), verifying the responsiveness of the *NIR1* promoter to nitrate. In seeds, this lncRNA was expressed at a very low level; however, it was clearly induced by nitrate (Fig. 5b). The seeds used for RNA extraction for that experiment were harvested at 15 h of imbibition, at which no testa rupture was observed. Clear induction of gene expression in the seeds by nitrate demonstrates the utility of the inducible system with a permeable ligand for gene induction at the relatively early stage of imbibition before testa rupture. It is known that nitrate induces *CYP707A2*, an ABA deactivation gene in seeds (Matakiadis *et al.*, 2009). Therefore, if *NCED*-suppressive genes, such as antisense *NCED* or *NCED RNAi*, are induced by nitrate, it could have a dual effect of reducing ABA biosynthesis and increasing ABA deactivation, both of which are expected to reduce ABA levels in seeds and promote seed germination (Fig. 5c). Thus, the *NIR1* system has great potential for inducible gene expression in seeds.

There are many systems that can be used for chemically inducible gene expression (Padidam, 2003). Antibiotic-based inducible systems, such as tetracycline repressor (TetR)-based inducible system or TetR-based tetracycline-inactivatable (tTA) system, have been developed and used successfully for plants (Gatz, 1997; De Veylder *et al.*, 2000; Love *et al.*, 2000). There are also the glucocorticoid receptor (GR)-based dexamethasone-inducible system (Lloyd *et al.*, 1994; Aoyama *et al.*, 1995) and the oestrogen receptor (ER)-based oestradiol-inducible system (Bruce *et al.*, 2000; Zuo *et al.*, 2002). Even a combination of GR-based and TetR-based systems in a dual-control system has been established (Böhner *et al.*, 1999). However, the antibiotics and steroid hormones used in these systems are not ideal ligands for applications, especially in seed-production fields or seed-processing factories. The ecdysone receptor (EcR)-based Plant Gene Switch System with methoxyfenozide, a non-steroidal ecdysteroid agonist (Mosallanejad *et al.*, 2010), has addressed this application issue. However, we experienced the permeability issue, particularly for seeds, and addressed that issue in this study. The nitrate-inducible gene expression system examined in

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this work met the requirements for both the regulatory aspect (field applicable) and induction efficiency (testa permeable). A similar system to the nitrate-inducible system, which uses a testa-permeable ligand, is the ethanol-inducible gene expression system (Caddick *et al.*, 1998; Roslan *et al.*, 2001). Since a low concentration of ethanol, which may not affect seed germination, is sufficient for induction of gene expression, this system can also be applied to gene expression in seeds. However, it has been suggested that care must be taken not to expose plants (and seeds) to ethanol vapour so that induction is not triggered inadvertently (Padidam, 2003). Basal expression of reporter genes driven with the ethanol-inducible system was observed in seedlings grown on agar in the absence of exogenous inducer (Padidam, 2003). It is also possible to have leaky expression in the nitrate-inducible system. These issues need to be considered carefully during experimental design and data interpretation.

It should also be noted that a precaution is required for the use of the nitrate-inducible gene expression system in seed research, especially when it is used to characterize function of genes with germination-inducing potential. The dormancy releasing effects of nitrate could make it difficult to separate the positive effects of the gene examined and of nitrate itself. In that case, it will be necessary to include a good control, such as nitrate-treated wild-type seeds, which are produced under the same conditions as transgenic seeds, and examine germination differences between the control and nitrate-induced transgenic seeds. In contrast, when putative negative regulators of seed germination are induced by this system and germination suppression phenotypes are observed despite the positive role of nitrate in seed germination, that would be even more convincing for functional characterization of the negative regulators of germination.

While there are both advantages and disadvantages for the use of the nitrate-inducible gene expression system in basic seed research, from a technology development point of view, the system, which allows gene induction at the relatively earlier stages of imbibition before testa rupture will be a robust tool for seed germination recovery and enhancement technologies. The combination of this system with the PHS prevention systems will provide a comprehensive technology for the future.

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Conflicts of interest

One of the technologies described in this manuscript has been filed for a patent. One of the authors is a trainee from a seed company that is not related to the patent.

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