Modulation of Nitrosative Stress by S-Nitrosoglutathione Reductase Is Critical for Thermotolerance and Plant Growth in Arabidopsis [W]

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Nitric oxide (NO) is a key signaling molecule in plants. This analysis of Arabidopsis thaliana HOT5 (sensitive to hot temperatures), which is required for thermotolerance, uncovers a role of NO in thermotolerance and plant development. HOT5 encodes S-nitrosoglutathione reductase (GSNOR), which metabolizes the NO adduct S-nitrosoglutathione. Two hot5 missense alleles and two T-DNA insertion, protein null alleles were characterized. The missense alleles cannot acclimate to heat as dark-grown seedlings but grow normally and can heat-acclimate in the light. The null alleles cannot heat-acclimate as light-grown plants and have other phenotypes, including failure to grow on nutrient plates, increased reproductive shoots, and reduced fertility. The fertility defect of hot5 is due to both reduced stamen elongation and male and female fertilization defects. The hot5 null alleles show increased nitrate and nitroso species levels, and the heat sensitivity of both missense and null alleles is associated with increased NO species. Heat sensitivity is enhanced in wild-type and mutant plants by NO donors, and the heat sensitivity of hot5 mutants can be rescued by an NO scavenger. An NO-overproducing mutant is also defective in thermotolerance. Together, our results expand the importance of GSNOR-regulated NO homeostasis to abiotic stress and plant development.

INTRODUCTION

Nitric oxide (NO) is a short-lived, endogenously produced radical that acts as a signaling molecule in all higher organisms (Lamattina et al., 2003; Wendehenne et al., 2004; Delledonne, 2005; Crawford, 2006; Besson-Bard et al., 2008). Despite its deceivingly simple structure, the rich chemistry of NO in biological systems gives rise to multiple secondary and tertiary reaction products, greatly complicating our mechanistic understanding of NO-related effects (Stamler and Hausladen, 1998; Mancardi et al., 2004; Ridnour et al., 2004). Directly and via its various chemical transformations, NO not only accomplishes signaling functions but also acts as a redox modulator with both antioxidant (by quenching other radical reactions) and pro-oxidant (through the production of reactive nitrogen species; RNS) properties. In addition to effects on redox status, the formation of RNS leads to nitrosation, nitrosylation, and nitration reactions with other molecules. Most of the regulatory effects of NO are thought to be mediated through posttranslational protein modifications, including heme nitrosylation, Tyr nitration, Cys nitrosation, and even glutathiolation (Lindermayr et al., 2005; Aracena-Parks et al., 2006; Wang et al., 2006b; West et al., 2006; Zaninotto et al., 2006).

In plants, NO is believed to be produced via two different enzymatic pathways (Guo et al., 2003; Crawford, 2006). In one pathway, it is generated by nitrate reductase through the successive reduction of nitrate to nitrite and further to NO. In the other pathway, L-Arg, plus oxygen and NADPH, is converted to NO and citrulline by the action of a NO synthase, although the actual existence and identity of plant NO synthase is currently unresolved (Crawford et al., 2006; Guo, 2006; Zemojtel et al., 2006). In some cases, NO is also produced by a nonenzymatic mechanism in which NO_2^- is converted to NO under acidic pH conditions in the plant apoplast (Bethke et al., 2004a). NO has been demonstrated to be involved in many different physiological processes in plants. These include seed germination (Beligni and Lamattina, 2000; Bethke et al., 2004b, 2006), plant defense responses (Zeidler et al., 2004; Zeier et al., 2004; Delledonne, 2005; Modolo et al., 2005; Mur et al., 2006), leaf senescence (Corpas et al., 2004; Guo and Crawford, 2005), stomatal movement (Garcia-Mata et al., 2003; Fan et al., 2004; Sokolovski et al., 2005), hormonal signaling (Guo et al., 2003; Huang et al., 2004), and flowering (He et al., 2004; Simpson, 2005). NO has also been implicated in responses to wounding and a number of abiotic stresses (Gould et al., 2003; Huang et al., 2004; Grun et al., 2006). Because of the multitude of possible chemical transitions and targets of NO, a precise determination of the mechanism of NO action in any of these important plant processes remains a challenge. Therefore, it is imperative to improve our understanding of NO metabolism in plants.

NO-derived RNS readily react with the major cellular antioxidant GSH to form S-nitrosoglutathione (GSNO). The main reaction of GSNO in biological systems involves the transfer of the NO

group to other cellular thiols to form longer-lived nitrosothiols (SNOs), an exemplary transnitrosation reaction. Endogenous GSNO has been proposed to be a significant player in NO regulatory mechanisms, particularly in the nitrosation of protein thiols, a process termed S-nitrosylation (Ji et al., 1999; Liu et al., 2001). This modification is sometimes referred to as "the new phosphorylation," although it is not known to be enzymatically catalyzed or otherwise protein-mediated. Increasing numbers of plant proteins are reported to be reversibly nitrosated on Cys residues (Perazzolli et al., 2004; Lindermayr et al., 2005, 2006; Belenghi et al., 2007). Such modifications often result in the inhibition of enzyme activity or alteration in protein function. In analogy to the concept of oxidative stress, an accumulation of nitroso species as a result of either the enhanced production of NO/RNS or the decreased clearance of nitrosated products has been termed nitrosative stress (Ridnour et al., 2004). Although by now it is an established part of NO metabolism in mammalian cells, little is known about the occurrence and consequences of nitrosative stress in plants (Valderrama et al., 2007). The potential of GSNO to transfer NO to protein thiols implies that GSNO biotransformation is a major branch of NO metabolism that could affect many regulatory processes.

It is now recognized that an evolutionarily conserved, GSH-dependent formaldehyde dehydrogenase (FALDH), a type III alcohol dehydrogenase, has activity as a GSNO reductase (GSNOR) (Jensen et al., 1998; Liu et al., 2001). In fact, it has been proposed that the major role of GSNOR/FALDH is in controlling GSNO and SNO levels rather than in detoxifying formaldehyde in living cells. GSNOR metabolizes GSNO to a mixture of products depending on conditions, including GSSG, hydroxylamine, NH_3, and GSH sulfinic acid (Jensen et al., 1998). The overall result is a reduction of GSNO and a decrease in the likelihood of enhanced protein nitrosation reactions.

In plants, there have been limited studies of GSNOR either from the perspective of its formaldehyde-detoxifying activity (Uotila and Koivusalo, 1979; Giese et al., 1994; Martínez et al., 1996; Dixon et al., 1998; Achkor et al., 2003) or from that of its function in GSNO reduction (Sakamoto et al., 2002; Feechan et al., 2005; Rustérucci et al., 2007). In Arabidopsis thaliana, GSNOR is a cytosolic protein that is encoded by a single copy gene (At5g43940) previously named ALCOHOL DEHYDROGENASE2 (Martínez et al., 1996). The gene appears to be expressed throughout the plant, downregulated by wounding and jasmonic acid, and upregulated by salicylic acid (Diaz et al., 2003). Sakamoto et al. (2002) have demonstrated that Arabidopsis GSNOR is capable of reducing GSNO using Escherichia coli extracts expressing recombinant protein.

Information about the phenotypes associated with a loss of GSNOR function is scarce. A T-DNA insertion mutant of the single copy GSNOR gene in Arabidopsis was recently isolated (designated gsnor1-3) (Feechan et al., 2005), and transgenic Arabidopsis plants that overexpress or produce <50% wild-type levels of GSNOR have been generated (Rustérucci et al., 2007). Studies of the disease susceptibility of these plants have yielded contradictory results. Feechan et al. (2005) reported that the gsnor1-3 null mutant was compromised in both R-mediated and basal disease resistance, failing to mount a defense response through the salicylic acid signaling network. By contrast, trans-

genic Arabidopsis plants with reduced GSNOR displayed enhanced resistance to Peronospora parasitica (Rustérucci et al., 2007). Furthermore, systemic acquired resistance and PR1 gene expression were enhanced in antisense plants and impaired in overexpression plants. While the disparity in these results remains to be resolved, there is no doubt that GSNOR plays a role in response to pathogens. No growth or developmental phenotypes were reported associated with the absence or reduction of GSNOR, with the exception of reduced root growth (Espunya et al., 2006), but both groups found an approximate doubling of total cellular SNO species, consistent with the role of GSNOR in SNO metabolism.

We now report that GSNOR activity is necessary for the acclimation of plants to high temperature and for normal development and fertility under optimal growth conditions. Our results demonstrate that GSNOR has an important role in the homeostasis of NO and its metabolites, affecting not only abiotic stress but also plant developmental processes.

RESULTS

The Thermotolerance-Defective Mutant hot5 Encodes GSNOR

We identified an Arabidopsis thermotolerance-defective mutant, hot5-1, in a screen of ethyl methanesulfonate–mutagenized seedlings using a hypocotyl elongation assay that was described previously (Hong and Vierling, 2000). Dark-grown, 2.5-d-old hot5-1 seedlings are completely blocked in hypocotyl elongation after 150 min of 45.8C heat treatment, even following a pretreatment at 38.8C, which allows wild-type seedlings to survive (Figure 1B). The hot5-1 mutant was backcrossed to the wild-type ecotype Columbia (Col) for standard genetic analysis. F2 backcrossed lines showed that the thermotolerance-defective phenotype segregated as a single recessive trait (data not shown). Using established map-based cloning methods (see Methods), the hot5-1 mutation was located toward the bottom of chromosome 5, between BAC clones F6B6 and MLN1. We sequenced all annotated genes in the mapped region using genomic DNA from hot5-1 mutant plants. Sequence analysis revealed a single G-to-A mutation, resulting in a Glu-to-Lys substitution at amino acid 283 in the seventh exon of the GSNOR gene (At5g43940) (Figure 1A). Glu-283 is 100% conserved in GSNOR from plants and other organisms, including bacteria and human (see Supplemental Figure 1 online).

To confirm that GSNOR is indeed the gene responsible for the observed hot5-1 phenotype, we isolated additional alleles of the GSNOR gene. A second missense mutation (hot5-3) was isolated from available Tilling lines (Col erecta background) (Till et al., 2003). The hot5-3 mutation leads to the substitution of a conserved amino acid also in exon 7 (G288R), five amino acids from hot5-1 (Figure 1A; see Supplemental Figure 1 online). Two T-DNA insertion alleles were also obtained, hot5-2 (Col background), which is located in exon 1 and is identical to gsnor1-3 reported by Feechan et al. (2005), and hot5-4 (Wassilewskija [Ws] background) in exon 4 (Figure 1A). The hot5-1, hot5-2, and hot5-3 mutant alleles were backcrossed to the wild-type Col
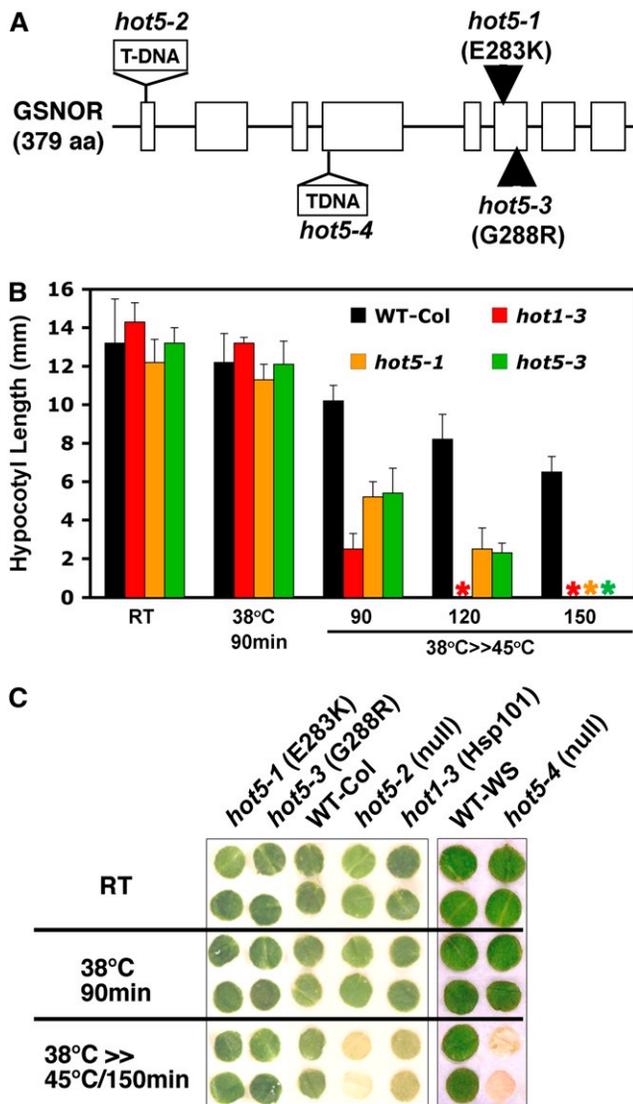
Figure 1. hot5 Mutants Are Defective in the Acquisition of Thermotolerance.

(A) Location of the hot5 missense alleles, hot5-1 and hot5-3, and the T-DNA insertion alleles, hot5-2 and hot5-4, on the GSNOR gene (At5g43940). aa, amino acids.

(B) Ability of wild-type and hot5 mutant seedlings to elongate after the indicated heat treatments in comparison with the wild type and the heat-sensitive Hsp101 null mutant hot1-3. Seedlings were grown on plates in the dark for 2.5 d and treated at 22°C only (room temperature [RT]), at 38°C for 90 min, or at 38°C for 90 min followed by 2 h at 22°C (acclimation treatment) and then by 90, 120, or 150 min at 45°C. Wild-type seedlings continue to elongate after 45°C treatment, but hot5 missense mutations show growth arrest.

(C) Acquired thermotolerance of leaf discs. Leaf disc samples (5 mm in diameter) were punched from rosette leaves of 25-d-old wild-type or mutant plants and then floated on 2 mL of 10 mM MES-KOH buffer, pH 6.8, on 12-well microplates. Heat treatments were performed as described for (B). Leaf discs were returned to 22°C under 12 h of light/12 h of dark and photographed 5 d later.

ecotype, and the hot5-4 allele was backcrossed to the Ws ecotype, two times to remove background mutations.

The hot5 mutants were tested for their ability to acquire heat tolerance in comparison with the null mutant of Heat-Shock Protein101 (Hsp101; hot1-3), which has an established heat-sensitive phenotype (Hong and Vierling, 2001). When tested in the hypocotyl elongation assay for acquired heat tolerance, the phenotype of hot5-3 was equivalent to that of hot5-1 (Figure 1B), and both mutants had a less severe phenotype than hot1-3. However, we were unable to perform the hypocotyl elongation assay on the T-DNA insertion alleles, because although both germinated on plates in the dark, they failed to elongate hypo-cotyls or develop further; we have only been able to grow these homozygous mutants effectively in the light on soil. Therefore, to test the heat acclimation of the hot5 insertion alleles, we developed a new thermotolerance assay, using leaf discs punched from the fourth or fifth leaves of 25-d-old plants (see Methods). The ability of 25-d-old leaf tissue to acquire thermotolerance differed dramatically between the hot5 missense and T-DNA insertion mutants (Figure 1C). The hot5-2 and hot5-4 mutants failed to acquire thermotolerance at this stage; they rapidly lost chlorophyll and turned yellow, exhibiting a phenotype as severe as that of hot1-3. However, in the same assay, hot5-1 and hot5-3 behaved like wild-type plants, remaining green. We conclude that the two missense mutations (hot5-1 and hot5-3) are relatively weak alleles of GSNOR compared with the insertion alleles (hot5-2 and hot5-4). In total, this analysis confirms that mutation of GSNOR prevents the normal development of acquired thermotolerance in plants.

GSNOR Is Not Heat Induced, and HSPs Are Normally Expressed in Mutant Plants

To determine how the hot5 mutant alleles and high temperature affect the abundance of GSNOR protein, protein blot analysis was performed on total proteins extracted from leaf discs as used for the experiment in Figure 1C. Arabidopsis GSNOR antibodies detected an ⊋40-kD band, consistent with the predicted molecular mass of the GSNOR coding sequence (40,697 D). This polypeptide was present at approximately the same abundance in both control and heat-stressed wild-type leaves (Figure 2A). As determined by protein gel blotting of a dilution series of total leaf protein compared with purified recombinant Arabidopsis GSNOR, the HOT5 protein represents ⊋0.02% of total dark-grown wild-type seedling protein (0.01% in leaf protein; see Supplemental Figure 2A online). The hot5-1 missense allele had approximately half the protein amount as the wild type, and the hot5-1 protein appeared to be further destabilized by heat stress, decreasing to about one-third or one-quarter the level seen in wild-type plants (Figure 2A; see Supplemental Figure 2B online). By contrast, the hot5-3 protein accumulated to wild-type levels. Both T-DNA insertion alleles, hot5-2 and hot5-4, had no detectable GSNOR protein, indicating that these are protein null alleles and confirming the specificity of our antibody for the GSNOR protein (Figure 2A).

We further confirmed previous observations of the ubiquitous expression of GSNOR throughout the plant (Martínez et al., 1996; Dolferus et al., 1997) by protein gel blot analysis. Samples were
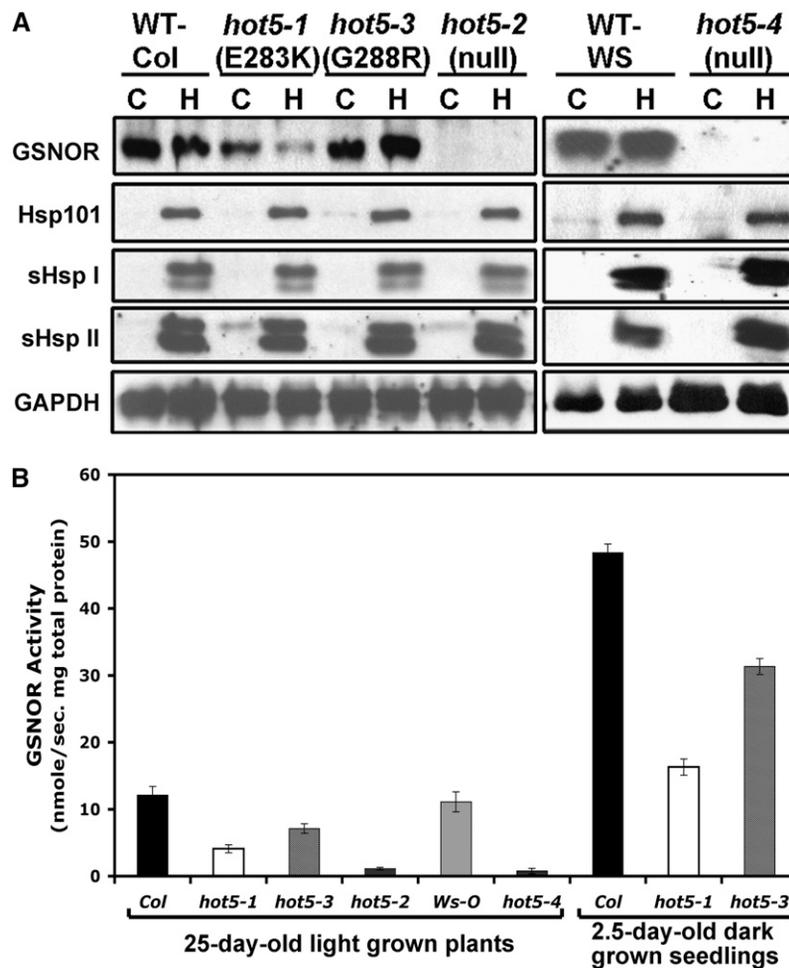
Figure 2. GSNOR Protein Accumulation and Enzyme Activity.

(A) Accumulation of GSNOR and HSPs in wild-type and hot5 mutant plants. Total protein was isolated from control (C; 22⁰C) or heat-stressed (H; 38⁰C for 90 min, followed by 2 h at 22⁰C) 25-d-old leaf discs and analyzed with the indicated Arabidopsis HSP and GSNOR antisera. Equal quantities of total protein (0.5 mg for Hsp101 antibodies, 5 mg for GSNOR and sHSP antibodies) from each of the mutants or the wild type were separated on 7.5% (Hsp101), 10% (GSNOR), or 15% (sHSP) SDS-PAGE gels. Protein blot analysis with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies confirmed the presence of similar protein levels.
(B) GSNOR enzyme activity in wild-type and hot5 mutant plants.

isolated from mature seeds, 2.5-d-old dark-grown hypocotyls, and different organs of mature plants. GSNOR protein was present in all organs tested, including dried seeds (see Supplemental Figure 2C online). These data indicate that the loss of GSNOR activity could affect phenotypes through the plant life cycle.

We next measured the effect of the hot5 mutations on GSNOR enzyme activity in total plant extracts (Figure 2B). In leaves of 25-d-old plants, the GSNO reduction activity of wild-type plants was similar to values reported previously (Feechan et al., 2005), with 12.1 ± 1.3 or 11.1 ± 1.5 nM GSNO-dependent NADH oxidation min^{-1} mg^{-1} total protein seen in wild-type Col and wild-type Ws, respectively. In the missense alleles, activity compared with the wild type was 33.8% in hot5-1 and 58.8% in hot5-3 plants. The hot5-1 protein is likely to have a similar specific activity to the hot5-3 protein, considering that it is of lower

abundance in the mutant plants (Figure 2A). The absence of phenotype in 25-d-old seedlings of the missense mutants suggests that this level of activity is sufficient for wild-type growth. The two null alleles had negligible activity; the low activity detected presumably represents nonspecific GSNO-stimulated oxidation of NADH. These data are consistent with the more severe heat-stress phenotype of light-grown plants carrying the null alleles.

We also measured activity in 2.5-d-old dark-grown seedlings of wild-type Col and the two missense alleles (Figure 2B). Expressed per milligram of total protein, GSNOR activity was actually higher for all seedling samples than in leaves, but this appears to reflect the higher levels of GSNOR protein per milligram of total protein in seedlings versus leaves (see Supplemental Figure 2A online). Surprisingly, the activity in missense mutant, dark-grown seedlings, expressed as a percentage of wild-type

values, was similar to the activity seen in 25-d-old plants (33.7% of wild-type values for hot5-1 and 64.8% of wild-type values for hot5-3). We also measured changes in GSNOR activity after heat stress in the wild type and GSNOR missense mutants. We found no statistically significant change in GSNOR activity under heat-stress conditions in the wild type or mutants, or when protein extraction was performed plus or minus DTT, or when seedlings were grown for 2.5 d in the light instead of the dark (data not shown). The reason that dark-grown seedlings of hot5-1 and hot5-3 have a heat-stress phenotype in the dark, despite having apparently reasonable GSNOR activity at this stage, is not obvious. Possible explanations for this result are that higher GSNOR activity is required in the dark for proper growth after heat stress, that in light-grown seedlings other factors are present that compensate for the reduced GSNOR activity, or that the missense alleles of GSNOR have altered regulation in the dark that is not preserved by our extraction and measuring conditions.

Because HSP expression is known to be an important component of acquired thermotolerance, we also assayed the accumulation of different HSPs by protein blot analysis in the hot5 mutants (Figure 2A). All of the hot5 alleles showed wild-type levels of Hsp101, which is essential for heat tolerance (Hong and Vierling, 2000), as well as cytosolic small HSPs of the class I and II types (Lee et al., 2005). Therefore, we conclude that hot5 mutants are not compromised in signaling mechanisms that lead to the expression of HSPs and that the absence of HSPs is not the cause of the hot5 thermotolerance defect.

hot5 Null Mutants Have Pleiotropic Phenotypes

In addition to their inability to grow following germination on nutrient medium plates in the dark, we also found that the GSNOR null mutants, hot5-2 and hot5-4, had severely reduced seed yields and abnormal growth habits. To investigate GSNOR mutant phenotypes in more detail, we observed the entire life cycle of all hot5 mutant alleles during growth in three different photoperiods, 16 h/8 h, 12 h/12 h, and 8 h/16 h light/dark cycles. The two hot5 missense mutants grew as well as wild-type plants during the whole life cycle under all three light conditions, consistent with our conclusion that these are mild alleles (data not shown). However, the hot5 null mutants showed pleiotropic phenotypes. First, they could not grow in the light on plant growth medium (Haughn and Somerville, 1986) containing 0.5% sucrose. The mutant seed germinated, but growth was arrested right after some root elongation and emergence of small cotyledons, which failed to green, and the seedlings eventually died (Figure 3A). This mutant phenotype was not recovered in the absence of sucrose, on higher sucrose concentrations (1, 2.5, and 5%), by germination directly on water-saturated filter paper, or when ammonium succinate was used to replace all other nitrogen sources in the medium (see Supplemental Figure 4A online). Thus, the basis of this phenotype is unresolved. The hot5 null mutants, however, could be recovered on soil, as shown in Figure 3B, allowing further study of growth phenotypes.

When hot5-2 was grown under long-day conditions (16 h of light), the mutant was less vigorous and had a decreased number of rosette leaves, leaves were pale green and distorted, and bolts were shorter compared with plants grown under 8 or 12 h of light
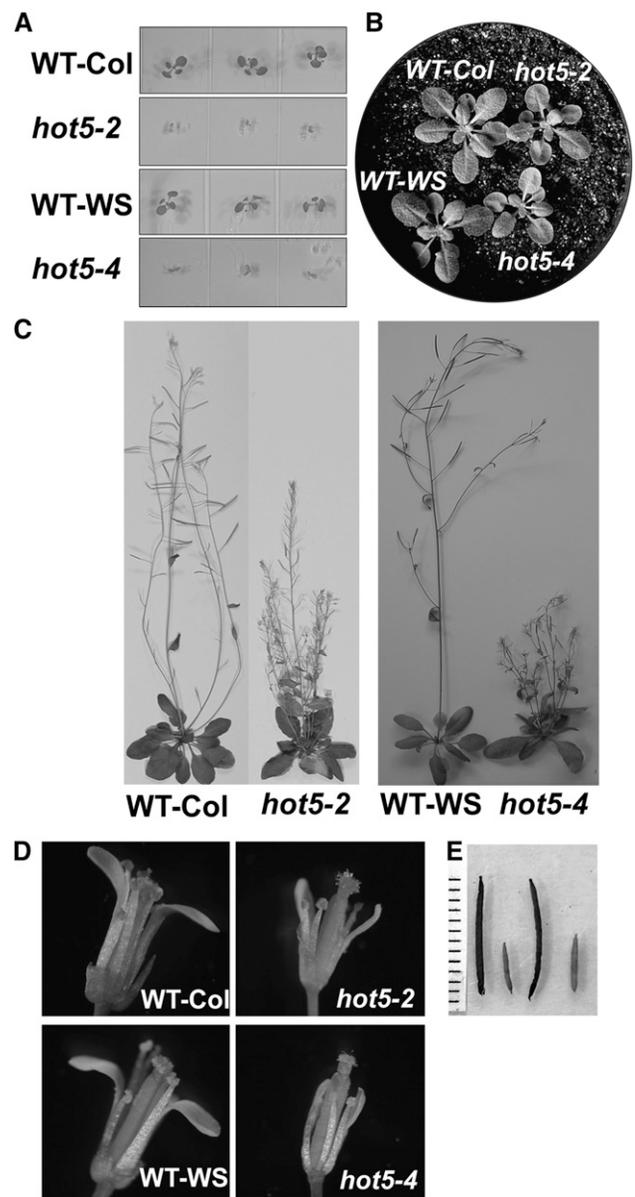

Figure 3. Pleiotropic Phenotypes of hot5 Null Mutants under 12-h/12-h Light/Dark Growth Conditions.

(A) Ten-day-old seedling plants on nutrient plates.
(B) Twenty-five-day-old soil-grown plants.
(C) Forty-five-day-old mature, soil-grown plants.
(D) Flower phenotype. For these photographs, one sepal, petal, and stamen were detached.
(E) Full-grown silique. From left to right: Col wild type, hot5-2, Ws wild type, and hot5-4. The ruler at left shows millimeters.

(data not shown). Indeed, under long days, the chlorophyll content of hot5-2 was only 62% of that of wild-type plants (see Supplemental Figure 3A online). After bolting, the hot5 null mutants were highly branched and semidwarf under all light conditions (Figure 3C). The roots of hot5 -2 were also reduced in

length compared with those of wild-type plants (see Supplemental Figure 3B online), but this difference is consistent with the reduced growth of the rosette, and its relationship to the reduced root length reported previously for GSNOR antisense plants is not clear (Espunya et al., 2006). The plants were also long-lived, continuing to produce leaves for as long as 25 d after wild-type plants had senesced.

The most dramatic phenotype of the hot5 null mutants was reduced fertility. Leaf numbers before bolting were not altered in hot5-2 compared with the wild type under either long or short days. Under 12 h of light, the hot5 homozygous null mutants produced many flowers and siliques but set very few seeds per plant. The mutants showed normal floral organ formation, with a wild-type number of sepals and petals and normal pistil formation. However, petals of mutant flowers were somewhat shriveled and smaller than wild-type petals, and stamens did not elongate normally, although pollen was produced at wild-type levels (Figure 3D). Most flowers in the hot5 null mutants could not produce seeds; consequently, the siliques did not elongate normally (Figure 3E).

There is no doubt that the failure of the hot5-2 and hot5-4 stamens to elongate properly contributes to the severely reduced fertility of these mutants. To determine whether the pollen and stigma of hot5-2 function normally for fertilization, we performed manual self-pollination and reciprocal test crosses between hot5-2 and wild-type plants (Table 1). Self-pollination of hot5-2 produced only 17.4 ± 6.2 (SD) seeds/silique, in contrast with 73.2 ± 5.3 seeds/silique for the wild type. In the reciprocal crosses, 23.1 ± 8.2 seeds/silique were generated using hot5-2 as the female with wild-type pollen, and 42.4 ± 7.7 seeds/silique were generated with hot5-2 pollen and wild-type females. These data indicate that in addition to reduced anther length, loss of HOT5 function compromises both the male and female functions required for fertilization and/or seed development.

GSNOR Affects Intracellular NO/Nitrosation Levels

By metabolizing GSNO, a cytoplasmic reservoir of NO and a nitrosating species, GSNOR potentially modulates cellular NO status. To determine whether the absence of GSNOR indeed affects NO/nitroso levels, and how this is further affected by elevated temperature, we examined endogenous NO production using the NO-sensitive fluorescent dye 4-amino-5-methylamino-2',7'-difluorescein diacetate (DAF-FM DA) (Arnaud et al., 2006). For staining, protoplasts were prepared from leaves of 25-d-old wild-type and hot5-2 mutant plants either before or after heat

stress. NO-dependent fluorescence signals were dramatically higher in the cytosol and chloroplasts of hot5-2 protoplasts compared with wild-type protoplasts from untreated leaves; in fact, no significant DAF-FM DA staining was observed in wild-type plants (Figure 4A). The same high levels of DAF-FM DA staining were also observed in protoplasts of hot5-4 (data not shown). Despite the severe effect of heat on the viability of hot5-2 and hot5-4 leaf tissues, heat treatment led to only a minor increase in NO-related fluorescence in the wild type, and no apparent change was seen in the mutant when heat stress was performed prior to protoplast isolation. We were unable to visualize intact cells when protoplasts were heat stressed after isolation and stained, so we could not test for rapid or transient heat-induced changes in DAF-FM DA staining in protoplasts.

Feechan et al. (2005) reported that the hot5-2 mutant (named gsnor1-3 by this group) has increased SNO species compared with the wild type, and increased SNO levels were also reported for plants in which GSNOR was reduced using an antisense strategy (Rustérucci et al., 2007). To confirm this observation and to determine the effects of heat stress, we quantified total nitroso species in leaves from wild-type and hot5 null plants using gas-phase chemiluminescence (Feelisch et al., 2002) (Figure 4B). The hot5 null mutants were found to have approximately double the amount of nitroso species compared with the wild type, consistent with previous reports. Heat stress did not significantly change nitroso species levels in either the mutant or the wild type. Unexpectedly, nitrate levels were also markedly higher in the hot5 null mutants than in the wild type (Figure 4C), despite the fact that all plants had been grown at the same time on the same soil. The increase in nitrate content appears to be correlated with the increase in nitroso species concentration, suggesting a link between protein nitrosation and the nitrate assimilation pathway. Collectively, these results suggest that hot5 null mutants have an increased basal NO tone, which translates into a higher level of nitrosative stress. They further indicate that GSNOR is likely required to prevent excessive nitrosation of intracellular targets and that the effects of heat stress are minor compared with the effects of GSNOR mutation.

Endogenous NO Status Affects Heat Tolerance

The high levels of NO and nitroso products in the hot5 null mutants suggest that this phenotype is causally linked to the acquired thermotolerance defects. To test this hypothesis, leaf discs of wild-type and hot5-2 mutant plants were floated on MES-KOH buffer containing either of two different NO donors, sodium nitroprusside (SNP) or DETA/NO, or the NO scavenger CPTIO, and then treated at 45°C for 2 h following pretreatment at 38°C (see Methods) (Figure 5A). Under heat stress, SNP led to severe yellowing and cell death in the wild type and further enhanced the hot5-2 phenotype. In comparison, when leaf discs were floated on KCN, an analog of SNP that does not release NO, disc yellowing was not observed. Treatment with 10 mM of the other NO donor, DETA/NO (which releases only NO [Hrabie et al., 1993]), also increased leaf yellowing in heat-stressed wild-type plants. Consistent with the involvement of NO/nitroso products in the heat-sensitive phenotype, 100 mM CPTIO treatment not only partially restored the appearance of hot5-2 leaf discs

Table 1. Seed Production in Test Crosses with hot5-2

F1, Female Stigma ✕ Male Pollen	Seed No./Silique
Col ✕ Col	73.2 ± 5.3
hot5-2 ✕ hot5-2	17.4 ± 6.2
Col ✕ hot5-2	23.1 ± 8.2
hot5-2 ✕ Col	42.4 ± 7.7

Five or six siliques were counted for each F1 hybrid. The data shown are average values with standard error from the means ± SE indicated.
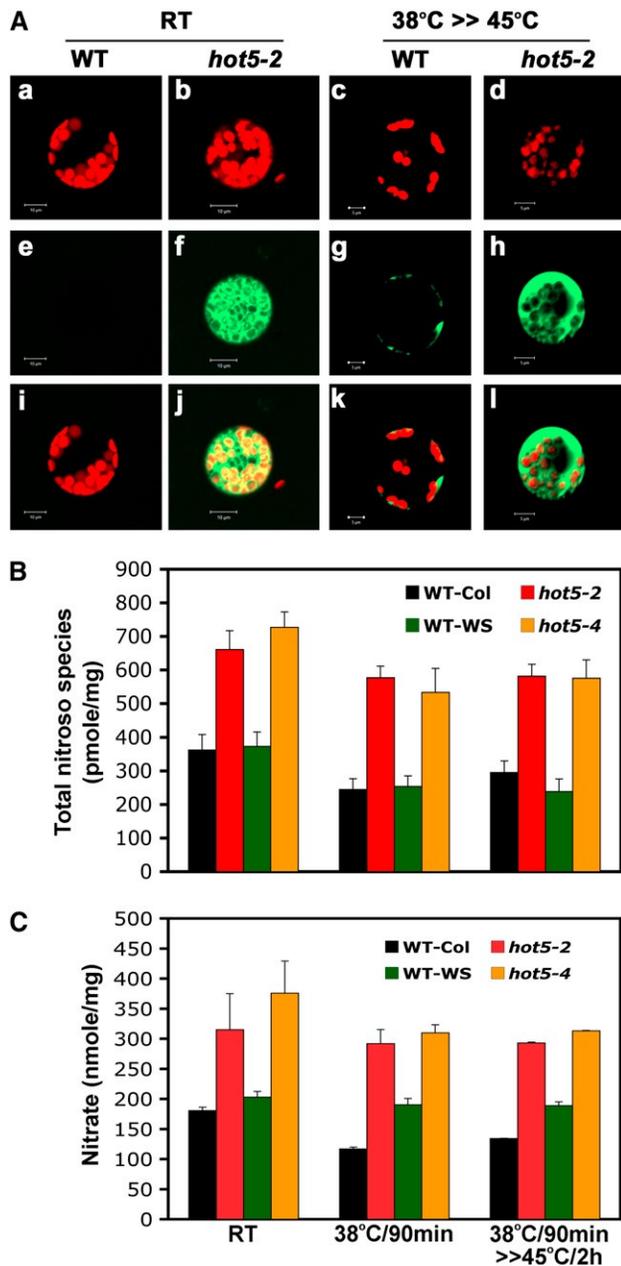
Figure 4. Endogenous NO Status, Total Nitroso Species, and Nitrate Levels in hot5 Null Mutants.

(A) DAF-FM DA staining for NO and its metabolites. Staining was performed in the Col wild type and the hot5-2 null mutant from leaves that were maintained at room temperature or heat-treated before protoplast isolation. NO production and the associated potential for nitrosation were visualized in protoplasts stained with DAF-FM DA by confocal microscopy. Chlorophyll autofluorescence ([a] to [d]), DAF-FM DA staining ([e] to [h]), and merged images ([i] to [l]) are shown. Bars ¼ 10 mm. RT, room temperature.

(B) and (C) Total nitroso species (B) and nitrate (C) from wild-type and hot5 null mutant plants. Values were normalized against total protein amounts. Data are means of three independent experiments (n ¼ 3 to 4).

to that of the wild type but also was able to block the effect of SNP (Figure 5A).

Results of SNP and CPTIO treatments were also quantified by the measurement of chlorophyll content over time after heat stress in leaf discs from 25-d-old plants (Figure 5B). Four days after heat treatment, buffer-treated hot5-2 retained only ⸳30% of chlorophyll and SNP-treated hot5-2 was fully bleached. By contrast, wild-type leaf discs retained high levels of chlorophyll in buffer alone, and when treated with SNP they retained ⸳60% of their chlorophyll after 4 d. Treatment with CPTIO dramatically rescued the hot5-2 chlorophyll loss, with 75% of initial chlorophyll content remaining at 4 d after heat treatment. To show that the effect of CPTIO was specific to the hot5 mutant and not just a general effect of NO scavenging, we also tested the ability of CPTIO to rescue the heat sensitivity of the Hsp101 null mutant, hot1-3. In contrast with hot5-2, the thermotolerance defect of hot1-3 was not rescued by the NO scavenger, indicating that the heat-sensitive defect of hot5 is unique and distinct from the defect in the hot1-3 mutant (Figure 5B).

We next determined whether the phenotypes observed for the wild type and hot5-2 in the presence of the exogenous NO scavenger or NO donor correlated with cellular NO status. Protoplasts were isolated at 2 h after heat treatment from leaf discs exposed to CPTIO or SNP. Treatment with CPTIO dramatically decreased the level of DAF-FM DA fluorescence in hot5-2 (Figure 5C) compared with buffer alone (Figure 4A). In addition, the DAF-FM DA fluorescence in the wild type was significantly increased by SNP treatment compared with buffer alone (Figure 4A). Thus, the heat-sensitive phenotype and NO/nitrosation levels are correlated.

To confirm that excess NO or metabolites could also explain the thermotolerance defect of the weak hot5 missense mutations, we examined the effect of treatment with the NO donors and scavenger on the hypocotyl elongation of heat-treated, dark-grown hot5-1 seedlings (Figure 5D). Treatment of seedlings with these agents just before heat stress produced quantitative differences in subsequent elongation in the dark, consistent with the results with hot5-2 leaf discs. CPTIO very clearly enhanced the thermotolerance of hot5-1 seedlings, while SNP, but not KCN, increased the heat sensitivity of wild-type and hot5-1 seedlings, and addition of CPTIO with SNP reversed this effect. DETA/NO treatment also impaired the heat tolerance of both the wild type and hot5-1, although in addition it reduced hypocotyl growth at room temperature. In total, these data demonstrate the involvement of excess NO and/or nitrosative stress in the heat-sensitive phenotype of the missense mutations, confirming that the control of endogenous NO status is critical for survival of heat stress.

NO Status in the hot5 Missense Mutations Correlates with Heat Sensitivity

The fact that the missense hot5 mutations showed a heat-sensitive phenotype only as dark-grown seedlings prompted us to compare the NO status of dark-grown seedlings and 25-d-old plants of the missense mutants. We first visualized DAF-FM DA fluorescence in hot5-1 and hot5-3 root tips of seedlings grown and loaded with dye in complete darkness (Figure 6A; hot5-3 data not shown). Compared with the wild type, both missense
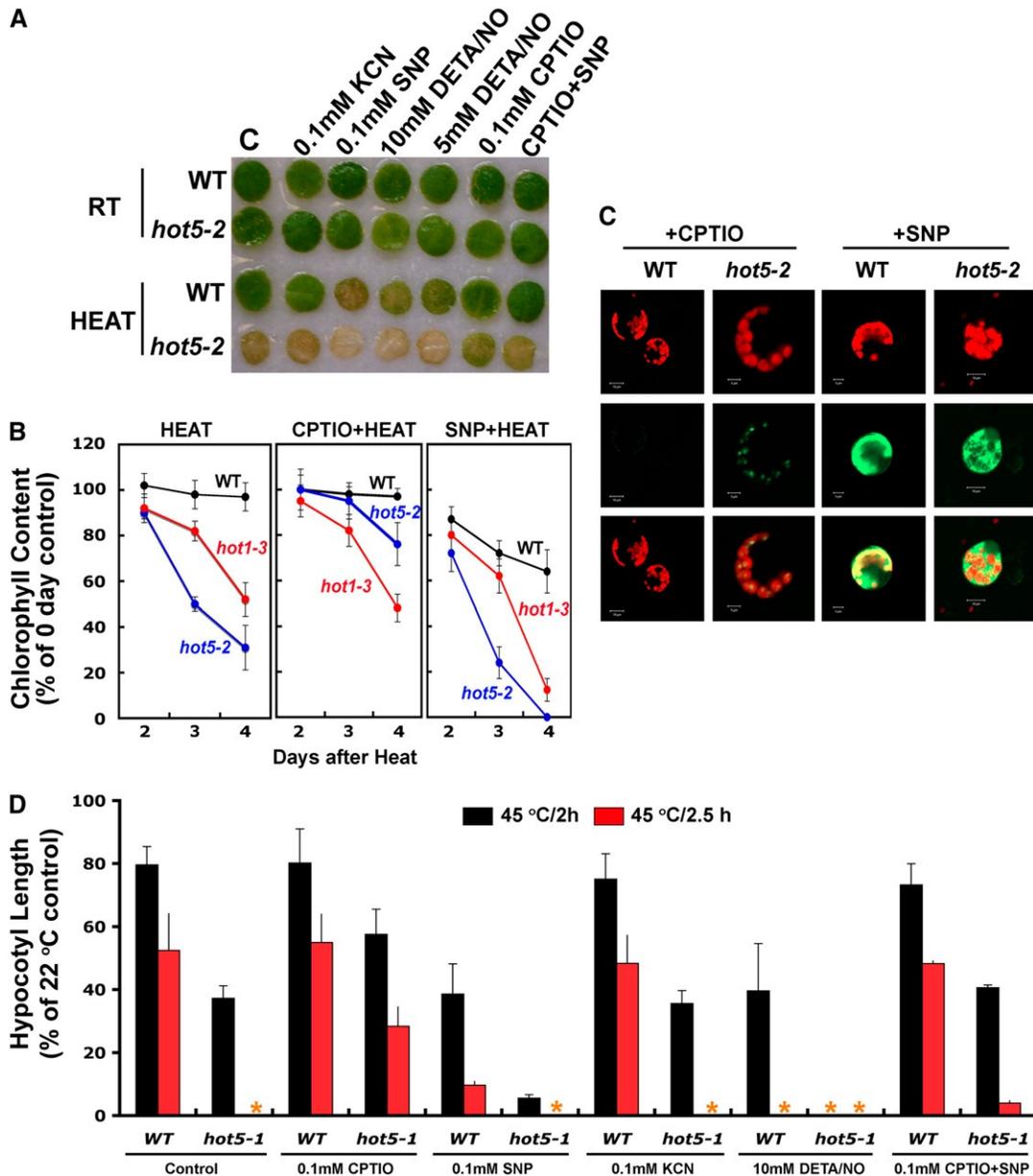
Figure 5. The Relationship of NO Status and Thermotolerance.

(A) As in Figure 1C, leaf discs from the wild type or the hot5-2 mutant were floated on the indicated compounds and either kept at room temperature (RT) or heat-stressed. The photograph was taken at 5 d after heat stress.

(B) Decline in total chlorophyll in leaf discs of the indicated genotypes following heat treatment with no addition or the addition of CPTIO or SNP as discussed in the text. At least six leaf discs from separate plants at each sampling time were used.

(C) NO-related fluorescence of protoplasts isolated from leaves treated with CPTIO or SNP.

(D) Intracellular NO status affects the hypocotyl elongation of the hot5-1 mutant grown in the dark after heat treatment. After growth for 2.5 d in the dark, seedlings were treated with the agents indicated and then heat-stressed for the times shown (after pretreatment at 38⁰C). After an additional 2.5 d in the dark, hypocotyl lengths were measured and expressed as a percentage of the unheated sample.

mutants showed much higher levels of NO-related fluorescence after growth in the dark. The DAF-FM DA fluorescence in hot5-1 was also eliminated by pretreatment of seedlings with CPTIO, as expected for fluorescence generated from NO (Figure 6A). Furthermore, protoplasts from light-grown, 25-d-old hot5-1 plants

had wild-type, basal fluorescence levels, correlated with the wild-type heat tolerance phenotype of the missense mutants at this growth stage (Figure 6B). Light-grown, 2.5-d-old hot5-1 and hot5-3 seedlings also showed wild-type levels of DAF staining. Thus, the endogenous NO status of the hot5 missense alleles,
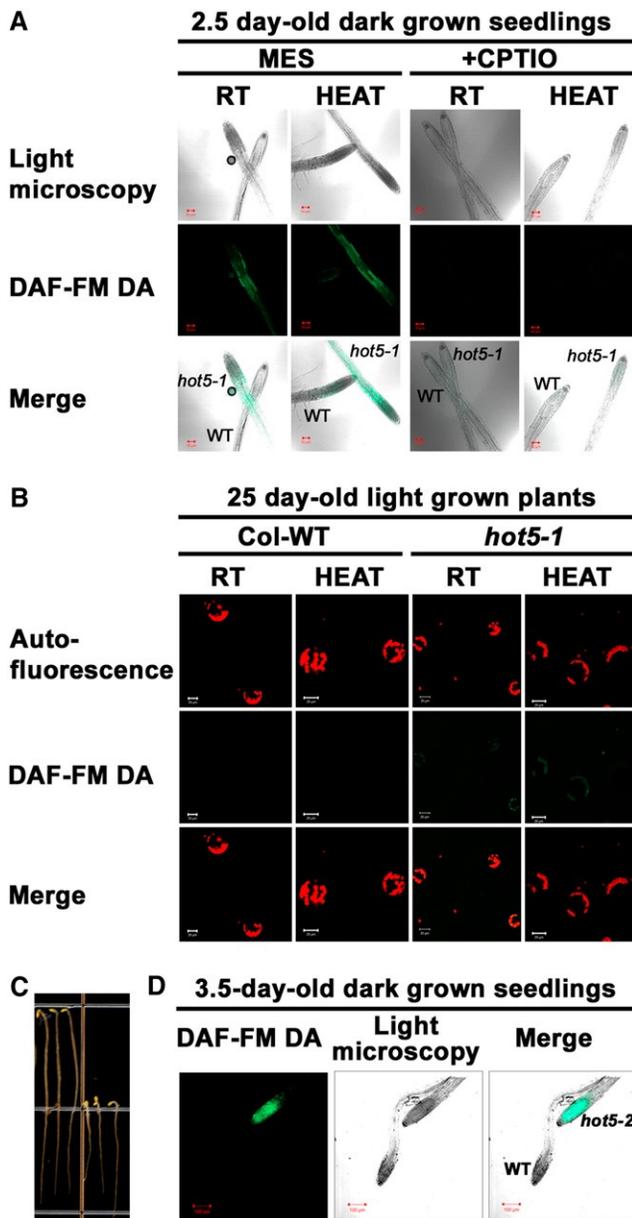
Figure 6. hot5-1 Shows Increased DAF-FM DA Staining Only as Dark-Grown Seedlings.

(A) NO-related fluorescence in roots of dark-grown hot5-1 seedlings with or without treatment with CPTIO. RT, room temperature; MES, buffer only.
(B) Protoplasts from control or heat-stressed leaves of 25-d-old hot5-1 plants exhibit wild-type, basal levels of NO-related fluorescence.
(C) hot5-2 growth phenotype on nutrient medium in the dark. Seedlings of hot5-2 were grown in the dark for 3.5 d before staining with DAF-FM DA in the dark. The first three seedlings are wild type and second three are hot5-2. The distance between lines is 13 cm.
(D) hot5-2 also has elevated DAF-FM DA staining in roots after growth in the dark compared with the wild type.

in both the light and dark, correlates with the heat-sensitive phenotype.

Although we were unable to determine the heat sensitivity of the hot5-2 and hot5-4 null alleles as dark-grown seedlings, to determine whether they had the same high DAF-FM DA staining phenotype as the missense alleles when grown in the dark, null mutant seeds were grown in the dark to generate root material (Figure 6C). When stained with DAF-FM DA, these null mutant roots also showed very high levels of fluorescence (Figure 6D). Light-grown seedlings of the same age also had high levels of DAF staining (data not shown). Thus, the missense and null alleles of hot5 share the inability to regulate NO status with dark-grown seedlings, further confirming that this phenotype results from the hot5 mutations.

The NO-Overproducing nox1 Mutant Shows a Thermotolerance Defect Correlated with NO Status

The observation that endogenous NO status affects acquired thermotolerance predicts that mutants that overaccumulate NO would be heat-sensitive. This possibility was tested using the NO-overproducing mutant nox1 (also known as cue1) (He et al., 2004), grown both in the dark and in the light, compared with hot5-1 and hot1-3 as references. When tested for hypocotyl elongation in the dark, nox1 does not show any defect even after 150 min of 45°C heat treatment, although nox1 has a short hypocotyl under normal conditions compared with wild-type and hot5-1 plants (Figure 7A). Consistent with the absence of a heat phenotype, dark-grown nox1 seedlings also did not stain with DAF-FM DA (Figure 7B). In contrast with this dark-grown phenotype, 10-d-old light-grown seedlings of nox1 were defective in acquired thermotolerance (Figure 7C). Like the hot5 null mutants (Figure 4A), protoplasts from light-grown nox1 also showed increased DAF-FM DA levels in the absence or presence of heat treatment, correlated with the thermotolerance defect (Figure 7D). These data further support the connection between excess NO-related nitrosation and plant heat sensitivity.

We also tested thermotolerance in the noa1 mutant (formerly nos1), which produces less endogenous NO (Crawford et al., 2006), and a nitrate reductase-deficient mutant, nia1/nia, which exhibits minimal nitrate reduction and must be grown on an alternative nitrogen source (Wang et al., 2004). Both 2.5-d-old dark-grown seedlings and 10-d-old light-grown seedlings were indistinguishable from wild-type seedlings in their heat tolerance (see Supplemental Figure 4 online).

DISCUSSION

By analyzing both missense and null mutations of the gene encoding GSNOR, we have uncovered an important role for this enzyme in modulating cellular NO levels and nitrosation status in plants. Specifically, we demonstrated that GSNOR function is required for acclimation to high temperature and for normal plant growth and fertility. Previous studies supported the conclusion that GSNOR, a type III alcohol dehydrogenase originally associated with the detoxification of formaldehyde (Uotila and Koivusalo, 1979; Giese et al., 1994; Martínez et al., 1996; Dixon et al., 1998; Achkor et al., 2003), acts in plants as well as other
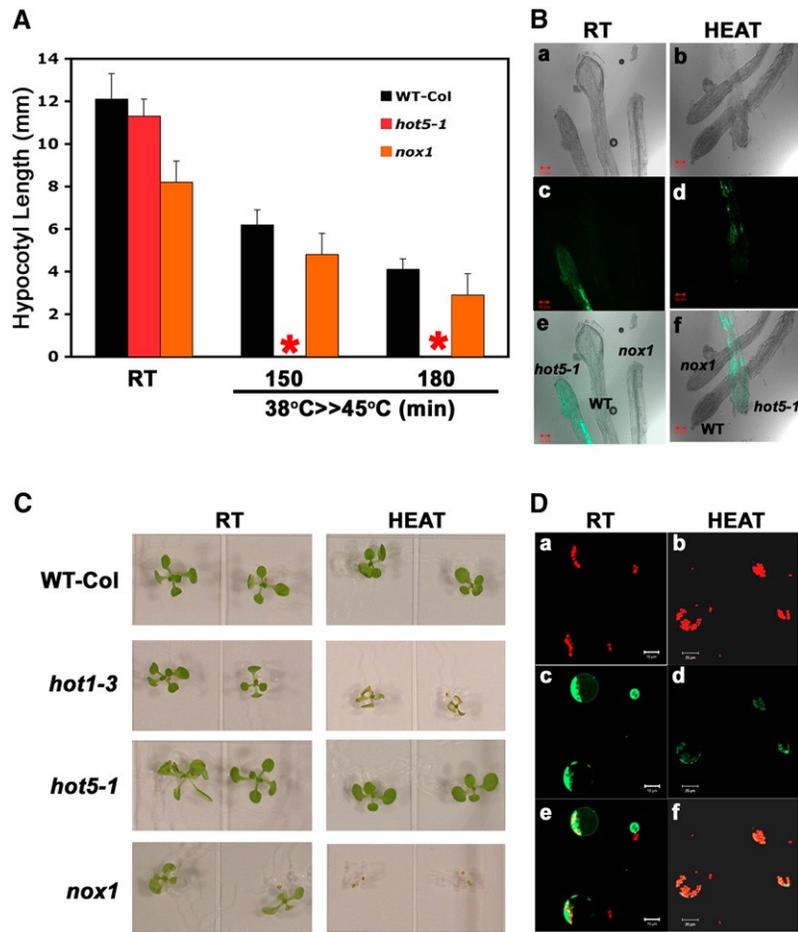
Figure 7. Thermotolerance and NO Status Phenotypes of the nox1 Mutant.

(A) The nox1 mutant exhibits wild-type thermotolerance as 2.5-d-old dark-grown seedlings. The asterisks indicate no growth after heat stress. RT, room temperature.

(B) nox1 shows wild-type levels of NO-related fluorescence in roots in the dark. Light microscopy ([a] and [b]), DAF-FM DA staining ([c] and [d]), and merged images ([e] and [f]) are shown.

(C) The nox1 mutant is defective in acquired thermotolerance as 10-d-old seedlings grown in the light.

(D) NO-related fluorescence in nox1 is high in protoplasts isolated from light-grown plants. Chlorophyll autofluorescence ([a] and [b]), DAF-FM DA staining ([c] and [d]), and merged images ([e] and [f]) are shown.

organisms to metabolize GSNO (Sakamoto et al., 2002; Feechan et al., 2005; Rustérucci et al., 2007). GSNOR is a potentially significant player in the modulation of cellular NO status because it effectively removes GSNO, a compound with NO-generating and thiol-nitrosating (NO^b-transferring) potential, from the cellular pool. GSNOR will also act to regulate the availability of GSNO for glutathiolation reactions, in which it acts by modifying other cellular thiols, including those on proteins, to form mixed disulfides (R-SSG). This reaction has the potential to affect the redox status and activity of proteins; in addition, it gives rise to the formation of nitroxyl (NO^-), a redox cousin of NO with a biological action profile distinct from that of NO (Fukuto et al., 2005). Thus, the effects we describe on thermotolerance, plant growth, and fertility may be mediated by several different pathways or by multiple mediators acting in concert. Although GSNOR does not directly act on S-nitrosated protein substrates, GSNOR knock-

out mice, Arabidopsis, and yeast cells all showed increased SNO levels (Liu et al., 2001, 2004; Feechan et al., 2005; Rustérucci et al., 2007). Our studies confirm and extend these results, indicating that GSNO modulates cellular nitrosation status. Consistent with this notion, the nitroso content of leaves from the hot5 mutants was about twice that of wild-type leaves, and the fluorescence signal obtained with the NO probe, DAF-FM DA, was clearly higher in the mutants compared with the wild type. This family of fluorescence probes senses NO utilizing nitrosation chemistry following the oxygen-dependent conversion of NO into RNS and the chemical conversion of the weakly fluorescent precursor into a more highly fluorescent molecule (Rodriguez et al., 2005). Thus, a higher fluorescence signal is not necessarily indicative of the presence of free NO but is an integrated readout of cellular nitrosation chemistry (Rodriguez et al., 2005). In aggregate, GSNOR activity and regulation must be recognized

as affecting processes controlled by NO-related pathways in plants.

The direct cause of the heat sensitivity of the hot5 mutants is not known. Assessment of the levels of major HSPs indicated that GSNOR mutants were not defective in the production of these protective proteins. The connection of heat sensitivity to excess nitrosation, however, is demonstrated by several observations. First, intense NO-related fluorescence staining was observed in dark-grown seedlings of the HOT5 missense mutants (hot5-1 and hot5-3), which is where the heat-sensitive phenotype is exhibited, and not in light-grown seedlings, which are not heat-sensitive. Second, decreasing NO levels with the NO-scavenger CPTIO partially rescued the heat-sensitive phenotype of both dark-grown hot5-1 and hot5-3 and light-grown hot5 null mutants. Conversely, increasing NO with the NO donors SNP and DETA-NO increased the heat sensitivity of wild-type seedlings and leaves. Finally, the NO-overproducing nox1/cue1 mutant showed NO-correlated thermotolerance defects. These observations support the hypothesis that elevated levels of GSNO enhance heat sensitivity due to the perturbation of pathways sensitive to reactive oxygen species/RNS, which are likely already under strain due to heat stress.

Although both the hot5-1 and hot5-3 missense mutants had reduced GSNOR activity compared with the wild type, it is very interesting that we did not see a significant difference in GSNOR activity in the missense mutants when comparing dark-grown seedlings and 25-d-old plants. The missense mutants accumulated DAF-FM DA–staining species and showed the thermotolerance defect only as dark-grown seedlings, suggesting that GSNOR activity might be lower in the dark than in the light in these mutants. The fact that DAF-FM DA staining was also seen in dark-grown hot5-2 and hot5-4 null mutants further supports the idea that this phenotype results from reduced GSNOR activity. We suggest several possible reasons for the apparent discrepancy between the significant GSNOR activity detected in total extracts of dark-grown missense mutants and their DAF-FM DA staining. First, it is possible that there are overall higher levels of GSNO production/flux in dark-grown seedlings and, therefore, higher GSNOR activity is required in the dark for the removal of these species to enable proper growth after heat stress. It is also possible that in light-grown seedlings other components are present that compensate for the reduced GSNOR activity in the mutants and limit the accumulation of excess nitroso species. Another hypothesis is that the hot5-1 and hot5-3 proteins have altered regulatory properties compared with the wild-type protein, being inactive in the dark, and that our extraction conditions relieve this inactivation (e.g., dissociation of an inhibitor or removal of a labile inhibitory modification). This interesting phenotype of the missense mutants no doubt reflects the complexity of the tissue- and environment-specific regulation of nitroso species in plants.

Our data do not suggest that GSNOR is a regulatory player in thermotolerance; we have no evidence that NO is involved in heat stress signaling. Although there is one previous report that NO levels increased during heat stress (Gould et al., 2003), we did not observe a major heat-dependent increase in NO-related fluorescence staining in isolated protoplasts. However, we applied heat stress before protoplast isolation and, therefore,

cannot rule out a transient NO increase in response to heat stress. We were unable to maintain intact protoplasts when cells were heat stressed after isolation and stained. Some increase in DAF-FM DA staining following heat stress could be observed in roots of dark-grown seedlings, consistent with the previous report (Gould et al., 2003), but staining was transient and significantly lower than in the mutants in the absence of stress (see Supplemental Figure 5 online).

The general importance of GSNOR in plants is emphasized by its ubiquitous presence throughout the plant. While we have documented the expression of GSNOR by protein gel blot analysis in all organs examined, others have visualized its presence using immunocytochemistry (Barroso et al., 2006; Espunya et al., 2006). Based on protein blot analysis compared against a standard of purified GSNOR protein, we estimate that the GSNOR content of leaves accounts for $\sim$0.01% of total protein (see Supplemental Figure 2 online), and we saw no evidence for a heat-induced protein increase. Constitutive expression of GSNOR during development, as well as a range of stresses, is supported by public microarray data (www.genevestigator.ethz.ch), and the absence of mRNA induction during heat stress has been confirmed by our own microarray studies (Larkindale and Vierling, 2007). The lack of evidence for a significant regulation of GSNOR at the transcriptional level or the level of protein abundance suggests that GSNOR is regulated primarily at the posttranscriptional level of enzyme activity. We currently hypothesize that some manner of redox regulation through Cys modification is one mechanism likely to control GSNOR activity. Notably, GSNOR has a structural zinc atom coordinated by four fully conserved Cys residues (see Supplemental Figure 1 online), a redox regulatory feature of other proteins. In addition, we note that plant GSNOR has two conserved Cys residues absent in GSNOR from E. coli and human, Cys-370 and Cys-284, the latter directly adjacent to the hot5-1 missense mutation. There are also three other Cys residues outside of the active site that are common to eukaryotic GSNOR and that might serve a redox-regulatory role.

Despite the significant role that GSNOR may play in the regulation of nitrosative stress, studies devoted to understanding the role of this enzyme in plants are quite limited. The focus of studies to date has been on the role of GSNOR in pathogen defense pathways and formaldehyde metabolism (Martínez et al., 1996; Dixon et al., 1998; Sakamoto et al., 2002; Achkor et al., 2003; Feechan et al., 2005; Rustérucci et al., 2007). Although Feechan et al. (2005) worked with the identical null mutation we used in this study, hot5-2 (gsnor1-3 in their report), they did not report any morphological or developmental phenotypes of the mutant. Our observations indicate that balanced GSNO metabolism and cellular NO/nitrosative status is critical not only for thermotolerance but also for normal growth and development under optimal growth conditions. The most dramatic phenotype we observed was reduced fertility. In fact, it was most effective to maintain the null alleles as heterozygotes, and for all experiments individual progeny of the heterozygotes were genotyped to identify homozygous plants for physiological experiments. We found all pleiotropic phenotypes in the backcrossed hot5-2 null mutant as well as in a second null mutation, hot5-4, confirming that the phenotypes can be ascribed to the absence of functional HOT5 protein.

The hot5 null mutants have more than one defect that leads to reduced fertility. The stamens do not elongate properly, such that anthers remain below the stigma surface at the time of anthesis (Figure 2D). Furthermore, results of self-pollination and reciprocal crosses to the wild type indicate poor function of both the male and female gametophytes of hot5 null mutants (Table 1). Interestingly, release of NO has been proposed as one signal involved in pollen tube repulsion from the ovule after fertilization (Johnson and Lord, 2006). This repulsion prevents penetration of the ovule by more than one pollen tube and is a critical step in the fertilization process. McInnis et al. (2006) also recently reported significant levels of NO in pollen and suggested that pollen-derived NO is important in the pollen–stigma interaction. We surmise that increased NO and its metabolites in the pollen and/ or ovule lead to this defect, perhaps by interfering with pollen tube guidance to the ovule.

Consistent with its constitutive expression throughout the plant, HOT5 appears to be required during the entire life of the plant. In addition to the fertility defect, hot5 null mutants had an increased number of flowering stalks, multiple short shoots, and were long-lived plants that continued to produce leaves even after wild-type plants had senesced. These phenotypes may be linked in some way to the reduced fertility. Furthermore, while hot5 null mutant seeds could germinate on plant growth medium plates, all further growth was arrested, although seeds could be germinated in soil to produce mature plants. We were unable to rescue the germination phenotype of the hot5-2 null mutant on plant growth medium, including the NO scavenger CPTIO (100 mM to 1 mM) (data not shown). We also observed that hot5-2 was significantly less vigorous and had reduced chlorophyll when grown under long days (16 h light) (growth conditions used by Feechan et al. [2005] in studying pathogen resistance). NO is reported to accumulate in chloroplasts and to stimulate photosynthetic electron transport (Zhang et al., 2006). Thus, NO accumulation could be affected by differences in photoperiod and might alter chloroplast development and chlorophyll biosynthesis. Previous studies indicate that NO broadly participates in the plant life cycle, from germination to seedling and mature plant growth (Beligni and Lamattina, 2000; Bethke et al., 2006; Zhang et al., 2006), and then decreases in senesced leaves (Corpas et al., 2006). Thus, GSNOR activity can be expected to have an effect on all of these processes.

NO is also reported to delay flowering through effects on both photoperiod and autonomous flowering time determinants (He et al., 2004). The nox1/cue1 mutation disrupts a chloroplast phosphoenolpyruvate/phosphate translocator. The mutant overproduces NO apparently due to high accumulation of L-Arg, a substrate for NO production (He et al., 2004). The NO-overproducing nox1/cue1 mutant had a delayed-flowering phenotype. By contrast, plants carrying a mutation in NOA1, which produce less endogenous NO, flowered earlier than wild-type plants. This evidence suggests that flowering time is altered by endogenous NO levels (He et al., 2004). However, we did not find evidence for the alteration of flowering time, despite the obvious alteration in NO status in GSNOR null plants. Leaf numbers before bolting were not altered in hot5-2 compared with wild-type plants under either long or short days. This result may be due to differences in the NO-derived species present in the

GSNOR mutants compared with the previously studied mutants with altered NO status.

In addition to increased total NO adducts and dramatically enhanced NO-related fluorescence staining, an unexpected finding was that the leaves of hot5 mutants have roughly twice the concentration of nitrate compared with wild-type leaves. Thus, the pattern of nitrate content under basal conditions mirrors that of levels of nitroso species, suggesting a link between protein nitrosation and nitrate assimilation. Since the plants used in the biochemical studies were all grown on the same substrate (soil), the higher levels of nitrate in the hot5 null mutants cannot be attributed to differences in nutrient availability. Rather, they must result from differences in uptake and transport or in nitrate consumption along the nitrate assimilation pathway. However, given the complexity of nitrogen metabolism, its multilayered regulation and connection to other metabolic pathways (Stitt et al., 2002; Lamattina et al., 2003), how cellular nitrosation status may be coupled to nitrate assimilation requires further investigation.

Although the major role for GSNOR is now proposed to be in GSNO metabolism, the enzyme is nevertheless capable of acting in formaldehyde detoxification. In plants, one-carbon (C1) metabolism can generate formaldehyde, which can react spontaneously with GSH to form S-hydroxymethylglutathione. GSNOR acting as a FALDH oxidizes S-hydroxymethylglutathione to S-formylglutathione. It is clear that GSNOR exhibits FALDH activity in vivo in plants and other organisms. The FALDH enzyme in the aerobic soil bacterium Paracoccus denitrificans is critical for methyltrophic growth (Ras et al., 1995). A yeast FALDH deletion mutant (sfa1) showed impaired growth in the presence of formaldehyde (Fernandez et al., 1999; Achkor et al., 2003), and in Arabidopsis, overexpression of the FALDH gene (GSNOR) conferred a high resistance to formaldehyde (Achkor et al., 2003). However, we do not think that the absence of formaldehyde detoxification activity leads to the heat-tolerance defect of GSNOR mutants, based on the following observations. S-Formylglutathione produced by GSNOR must subsequently be hydrolyzed to GSH and formic acid by S-formylglutathione hydrolase (SFGH) (Jensen et al., 1998). We analyzed a T-DNA knockout mutant (SALK_002548) of the At SFGH gene (At2g41530) (Kordic et al., 2002). As the SFGH knockout mutation is blocked in the formaldehyde detoxification pathway, this mutant should be heat-sensitive if formaldehyde detoxification is the problem with the GSNOR mutant. However, the SFGH knockout mutant showed similar phenotypes to wild-type plants in all growth conditions (data not shown). Furthermore, when different concentrations (0.2 mM to 0.2 mM) of formaldehyde were directly applied to hot5-1 seedling plants in the dark or to hot5-2 leaf discs in the light, no additional phenotypic defects were observed (data not shown). These results suggest that the heat-sensitive phenotypes of hot5 are not caused by defects in formaldehyde metabolism.

In addition to the heat-stress phenotype of hot5 null mutants, photoperiod-dependent phenotypes suggest that these mutants may be sensitive to other abiotic stresses. However, when either hot5-1 dark-grown hypocotyls or hot5-2 light-grown leaf discs were treated in salt, cold, or high osmotic conditions, the hot5 mutants were not more sensitive than wild-type plants (data not shown). We cannot rule out the possibility that GSNOR regulation

of NO status may be important under stress conditions not yet tested.

In summary, GSNOR regulates cellular nitrosation levels by metabolizing GSNO, which is a mobile reservoir of NO in plant cells. Therefore, understanding the mechanisms that regulate the activity of GSNOR is a critical aspect of the study of the overall regulation of NO-related signaling and nitrosative stress in plants.

METHODS

Plant Materials and Thermotolerance Assays

Arabidopsis thaliana seeds of the indicated genetic backgrounds and genotypes were surface-sterilized, planted on nutrient medium plates (Haughn and Somerville, 1986) containing 0.5% (w/v) sucrose, and kept at 4°C for a minimum of 3 d. Plates were prepared with exactly 10 mL of medium on a leveling table to ensure even heat treatment and were sealed after planting with Parafilm to prevent desiccation. Plants were grown in illuminated growth chambers ($\sim$100 mmol m^{-2} s^{-1}) on a 22/18°C, 12- or 8-h day/night cycle for analysis of growth phenotypes. To obtain mature hot5-2 and hot5-4 plants, heterozygous seeds were sown directly on soil, and all plants were genotyped by PCR to identify the homozygotes. Note that because of the reduced vigor of null mutants under long days, material for all stress and other physiological assays of these mutants was obtained from plants grown under 12 h of light. For thermotolerance assays, 2.5-d-old dark-grown and 10-d-old light-grown seedlings were treated as described (Hong and Vierling, 2000). For the leaf disc assay, discs (5 mm in diameter) were punched from third to fifth fully expanded rosette leaves of 25-d-old soil-grown plants and then floated on 2 mL of 10 mM MES-KOH buffer, pH 6.8, on 12- or 24-well microplates. Plates were incubated at 22°C only, at 38°C for 90 min, or at 38°C for 90 min, followed by 2 h at 22°C and 2.5 h at 45°C. Leaf discs were returned to 22°C under 12 h of light/12 h of dark and photographed 5 d later.

We also tested thermotolerance in the noa1 mutant (formerly nos1), which produces less endogenous NO (Crawford et al., 2006), and a nitrate reductase–deficient mutant, nia1/nia, which exhibits minimal nitrate reduction and must be grown on an alternative nitrogen source (Wang et al., 2004). Both 2.5-d-old dark-grown and 10-d-old light-grown mutant seedlings were indistinguishable from the wild type in their heat tolerance (see Supplemental Figure 4 online). The nox1 (He et al., 2004) and noa1 (Crawford et al., 2006) mutants were obtained from Z.-M. Pei (Duke University) and N.M. Crawford (University of California at San Diego), respectively. The nia1-1/nia2-5 seeds (Col background) were obtained from the ABRC (CS6512).

NO-Related Chemical Treatments

The DETA/NO (DETA NONOate; diethylenetriamine nitric oxide adduct; half life of NO release ¼ 56 h at 22°C and 20 h at 37°C) was synthesized by Katrina Miranda as described previously (Hrabie et al., 1993).

The NO donor DETA/NO as well as the NO donor SNP and the NO scavenger CPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidadazoline-1-oxy-3-oxide] were dissolved in 10 mM PBS, pH 7.4, and used to treat seedlings on plates at final concentrations from 1 mM to 10 mM. KCN (100 mM) and 100 mM SNP plus 100 mM CPTIO were used as negative controls.

For the hypocotyl elongation test, seeds were sterilized and plated in rows on 2-mL nutrient medium plates containing 0.5% (w/v) sucrose on 3.5-cm circular plates, which were wrapped in foil. Plates were incubated at 4°C for a minimum of 3 d and then placed in a vertical position at 22°C for 3 d. One hour before heat treatment (38°C for 90 min followed by 2 h at 22°C and then 2.5 h at 45°C), plates were briefly opened under dim green light and treated with the NO-related chemicals. Two milliliters of solution was added on plates, which were placed in a horizontal position for 1 h at 22°C. For heat treatment, the remaining solutions were poured out and the plates were rewrapped in foil. Hypocotyl lengths were measured after an additional 2.5 d in the dark.

Identification of hot5 Mutant Alleles

The hot5-1 mutant was originally isolated from an ethyl methanesulfonate mutant pool in a screen for thermotolerance-defective mutants (Hong and Vierling, 2000). For genetic mapping of the hot5-1 mutation, 1024 plants showing the hot5-1 mutant phenotype were selected. For fine mapping, different markers on the bottom of chromosome 5 were developed for simple sequence polymorphism, cleaved amplified polymorphic sequence, and single nucleotide polymorphism analyses.

Single knockout mutants of the HOT5 gene were obtained from the GABI (German Plant Genomics Program; 315D11; hot5-2 in the Col background) and FLAG (Versailles Genomic Resource Center; FLAG_298F11; hot5-4 in the Ws background) T-DNA collections using the accession number of HOT5 (At5g43940) in the database (http://signal.salk.edu/). Homozygous mutants were identified by PCR analysis using the recommended primers from each T-DNA collection. While some seeds could be recovered from the null mutants, routine experiments were performed by identifying the homozygous mutants by PCR from among the progeny of the heterozygotes.

Tilling analysis (in the Col ecotype, carrying the erecta mutation) was performed on the HOT5 gene, encompassing approximately amino acid residues 96 to 379 (Arabidopsis Tilling Resource; http://tilling.fhcrc.org:9366). The hot5-3 mutant was recovered as a hypocotyl thermotolerance-defective mutant from a total of eight missense mutations analyzed.

Both the homozygous hot5-1 and hot5-3 missense mutant alleles were backcrossed to Col wild-type plants, and one homozygous F3 line for each mutation was used for phenotypic analyses. For the T-DNA null mutant alleles, heterozygous hot5-2 or hot5-4 plants were backcrossed to Col or Ws wild-type plants, respectively, and then genotyped to identify wild-type, heterozygous mutant, and homozygous mutant plants. All homozygous plants were finally obtained after two backcrosses.

Purification of the HOT5 Protein

A HOT5 cDNA was cloned to the pJC20 expression vector and transformed to BL21(DE3) Escherichia coli cells. HOT5 was overexpressed with 0.05 mM isopropylthio-b-galactoside in 0.05 mM ZnCl–containing Luria-Bertani medium. Cells were harvested after overnight induction at 30°C and then extracted in 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.05 mM ZnSO$_4$, 50 mg/mL DNase I, 2 mM MgCl$_2$, 100 mg/mL lysozyme, 1% (v/v) Triton X-100, and 20 mM Tris, pH 8.0. Cell extracts were stirred at 4°C for 30 min and then sonicated. The protein was loaded onto a HiPrep 16/10 DEAE FF column and eluted with a gradient of NaCl (0 to 200 mM) in 20 mM Tris buffer, pH 8.0. Following concentration, the HOT5 protein fractions were loaded onto a HiPrep Sephacryl S-100 column equilibrated with 100 mM NaCl containing 100 mM Tris buffer. Eluted HOT5 fractions were subjected to a final concentration step for antibody production. Polyclonal antibodies were generated in rabbits by Cocalico Biologicals.

SDS-PAGE and Protein Blot Analysis

Dark-grown seedlings (2.5 d old) were treated at 38°C for 90 min, and total protein was extracted thereafter in SDS sample buffer. Equal quantities of total protein (0.5 mg for Hsp101 antibody and 5 mg for small HSP, GSNOR, and GAPDH antibodies) were separated by SDS-PAGE on 7.5% (Hsp101), 10% (GSNOR and GAPDH), or 15% (small HSP) acrylamide gels and processed for protein gel blot analysis. Protein amounts were

measured using a Coomassie Brilliant Blue dye binding assay (Hong et al., 2003) with BSA as a standard. Protein blots were probed with rabbit antiserum against HOT5, Hsp101, or the small HSPs Hsp17.6C-I and -II (Hong and Vierling, 2001) at a dilution of 1:1000. As a loading control, blots were probed for cytosolic GAPDH using a GAPC antibody (a gift of Ming-Che Shih, University of Iowa) as described (Chan et al., 2002). Blots were incubated with goat anti-rabbit horseradish peroxidase, and bands were visualized with the enhanced chemiluminescence protein gel-blotting detection reagent (Amersham International) and BioMax film (Kodak).

Measurement of Nitrate, Nitroso Species, and Chlorophyll Content

Leaf extracts were prepared by homogenization of 150 mg of material in 0.5 mL of PBS containing 10 mM N-ethylmaleimide and 2.5 mM EDTA and then either immediately centrifuged for 5 min or snap-frozen and stored in liquid nitrogen for later analysis. The concentration of nitrate in these leaf extracts was determined by ion chromatography using a dedicated HPLC system for the simultaneous detection of nitrite and nitrate (ENO-20; Eicom) following methanol precipitation (1:1, v/v). The content of nitroso species (comprising SNO and N-nitroso products) in extracts from hot5-2, hot5-4, and wild-type plants was quantified by reductive denitrosation following injection of leaf extracts into a mixture of iodine/iodide in glacial acetic acid with subsequent detection of the released NO by gas-phase chemiluminescence reaction with ozone, as described (Samouilov and Zweier, 1998; Feelisch et al., 2002; Wang et al., 2006a). Molar concentrations of nitrate and total nitroso species were normalized for protein content.

Chlorophyll was extracted from individual leaf discs by boiling in 95% methanol. Chlorophyll concentration was normalized to the fresh weight of the leaf discs and calculated as described (Lichtenthaler, 1998).

Imaging of NO Status in Arabidopsis Protoplasts

The NO status of seedling roots or protoplasts was visualized by staining with DAF-FM DA (Arnaud et al., 2006) and confocal microscopy. The third to fifth fully expanded rosette leaves of 25-d-old Arabidopsis plants were used for the preparation of protoplasts according to an established method (Sheen, 1995), with minor modifications (Lee et al., 2007).

Protoplasts were resuspended in 150 mL of 25 mM DAF-FM DA, 0.4 M mannitol, 15 mM MgCl$_2$, and 4 mM MES/KOH, pH 5.7, and allowed to incubate for 15 min at 22°C in the dark. DAF-FM DA treatment of hypocotyls was performed under complete darkness or green dim light to minimize light exposure. Plants were treated at 22°C only, at 38°C for 90 min, or at 38°C for 90 min followed by 2 h at 22°C and then 2 h at 45°C. All samples for NO visualization were isolated at 2 h after heat treatments and compared with room temperature treatments.

Confocal Microscopy

Leaf tissues and protoplasts were visualized by confocal laser scanning microscopy using an LSM model 510 META microscope (Zeiss) equipped with a Plan-Apo 63⨯ 1.4 lens (numerical aperture). NO-related fluorescence after DAF-FM DA loading was captured following excitation at 488 nm and detection at 505 to 570 nm (BP505-570 infrared filter). Auto-fluorescence of chlorophyll was detected at 645 nm (LP 615 filter) (Lee et al., 2007). The Zeiss LSM Image Browser 3.2 program was employed for image acquisition, and Photoshop 6.0 (Adobe Systems) was used for image processing.

Measurement of HOT5 Enzyme Activity

GSNOR activity was measured by monitoring the decomposition of NADH (Jensen et al., 1998; Sakamoto et al., 2002). Oxidation of NADH, dependent on the presence of the substrate GSNO, was determined spectrophotometrically at 340 nm. Crude leaf or seedling extracts (25 mg) were prepared in 100 mL of 0.05 M HEPES buffer (20% glycerol, 10 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, and 1 mM e-aminocaproic acid, pH 8.0), centrifuged to remove insoluble material, and then clarified with a desalting column (Zeba desalting column; Pierce). Enzyme activity was determined at 25°C by incubating the desalted fraction (10 mL) in 180 mL of 0.1 M phosphate buffer containing 10 mL of 6 mM NADH as cofactor and 10 mL of 6 mM GSNO as substrate. GSNOR activity was monitored for 1 min after the addition of NADH using an Agilent 8453 UV spectrophotometer. The rates were corrected for background NADH decomposition of each extract containing no GSNO. Rates were averaged over selected intervals during which the absorbance decline was linear. Final NADH decomposition values were normalized against total protein amount. Data are means of three independent experiments.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative and GenBank/EMBL data libraries under accession numbers At5g43940 and AAB06322 (GSNOR gene).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Amino Acid Sequence Alignment of GSNOR from Arabidopsis (Accession Number AAB06322), Rice (Accession Number BAD21999), Maize (Accession Number CAA71913), E. coli (Accession Number NP_414890), and Human (Accession Number NP_000662).

Supplemental Figure 2. Accumulation of GSNOR Protein in the Wild Type and hot5 Missense Mutants.

Supplemental Figure 3. Chlorophyll Content Depends on Photoperiod in the Wild Type and hot5-2, and Root Growth Phenotype of hot5 Null Mutants.

Supplemental Figure 4. Thermotolerance Assay of noa1 and nia/nia2 Mutants.

Supplemental Figure 5. NO-Related Fluorescence after Heat Stress.

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