Supplementary Information

Supplementary Figures and Figure Legends

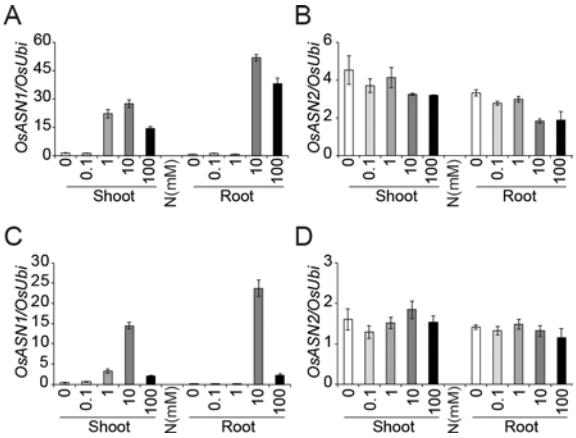

Fig. S1. Effect of nitrogen (N) availability on the expression of two rice asparagine synthetase (*OsASN*) genes. (A, B) Effect of varying amounts of ammonium (NH_4^+) ions, as the N source, on the expression of *OsASN1* (A) and *OsASN2* (B) in shoots and roots. Seedlings were grown for 4 days on the modified 1/ 2MS medium containing 0, 0.1, 1, 10, or 100 mM NH_4^+ as the only N source. (C, D) The expression of *OsASN1* (C) and *OsASN2* (D) in shoots and roots grown on the modified half-strength MS medium for 10 days. Data represent mean ± SE of three replicates.

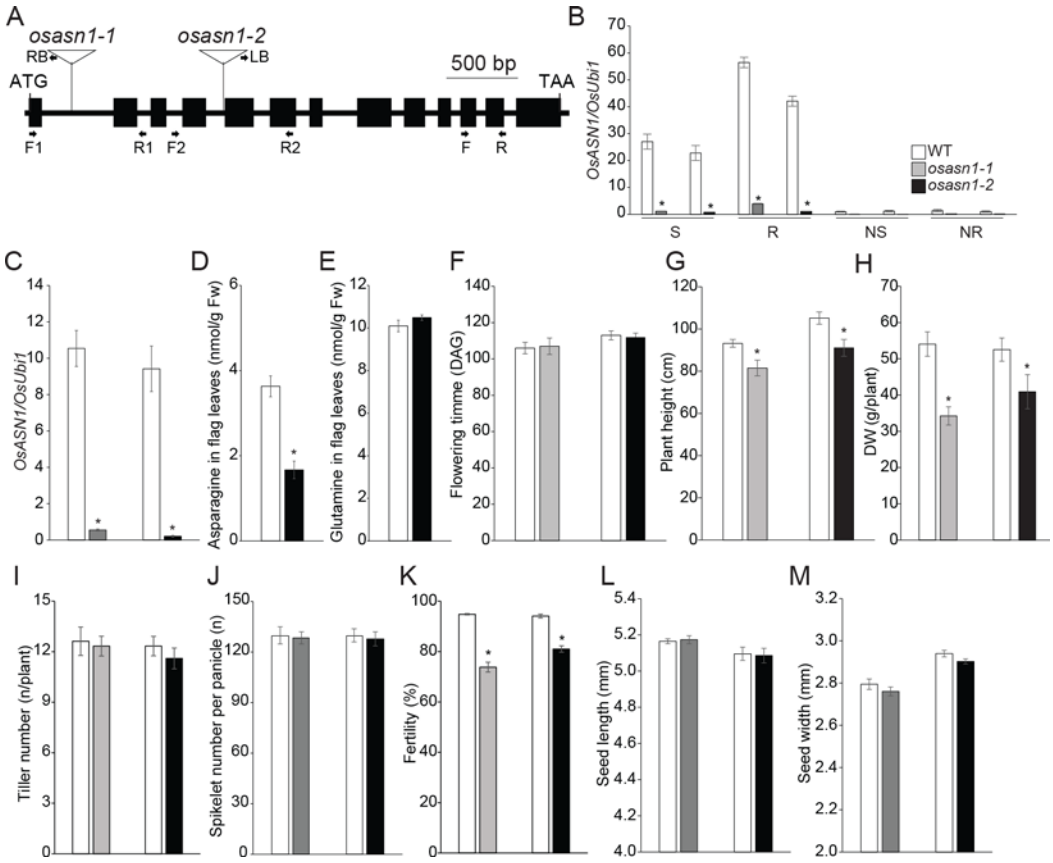

Fig. S2. Isolation and characterization of *osasn1* knockout mutants. (A) Schematic diagram showing the insertion positions of T-DNA in *OsASN1*. Boxes and lines indicate exons and introns, respectively. In *osasn1-1* (line 3D-02739) and *osasn1-2* (line 3A-05359), T-DNA was inserted in the 1st and 4th introns, respectively. Horizontal arrows indicate gene-specific primers (F1, R1, F2, and R2) and T-DNA-specific primers (LB and RB) used for genotyping the T2 progeny. (B) Quantitative RT-PCR (qRT-PCR) analysis of *OsASN1* expression in mutant and wild-type (WT) plants. Total RNA was isolated from 7-day-old seedling shoots and roots of *osasn1-1*, *osasn1-2*, and segregated WT plants grown on N-sufficient (10 mM NH_4^+) or N-limited (0.1 mM NH_4^+) media, and subjected to qRT-PCR analysis using gene-specific primers (F and R). S, shoots of plants grown in N-sufficient medium; NS, shoots of plants grown in N-limited medium; R, roots of plants grown in N-sufficient medium; NR, roots of plants grown in N-limited medium. Rice *Ubiquitin* (*OsUbi*) gene was used as an internal control. (C) Expression of *OsASN1* in flag leaves of WT and mutant plants at the flowering stage. Data represent mean ± SE of three biological replicates. Asparagine (D) and glutamine (E) concentrations in flag leaves of WT and *osasn1* plants. (F) Flowering time of WT and *osasn1* mutants grown in the paddy fields. DAG, days after germination. Measurements of plant height (G), dry weight (DW) of aboveground biomass per plant (H), tiller number per plant (I), spikelet number per panicle (J) and fertility (K) of rice plants

grown in paddy fields. Data represent mean ± SE of five biological replicates. Significant differences between mutant and WT plants are indicated with an asterisk (*$P \leq 0.05$; Student's *t*-test). Measurements of the length (L) and width (M) of WT and *osasn1* mutant seeds ($n = 20$).

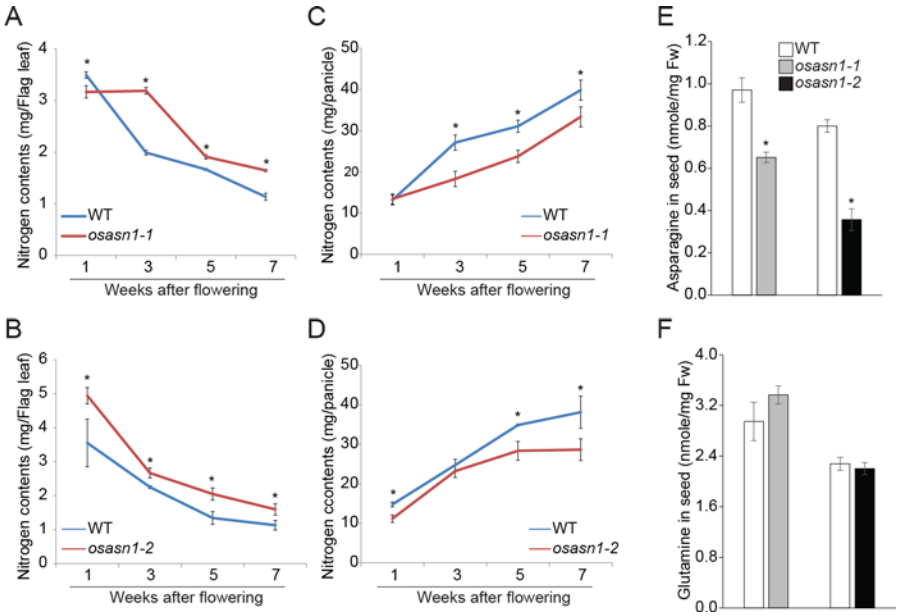

Fig. S3. Total N content of wild-type (WT) and *osasn1* mutant plants at the grain filling stage. Plants were grown in paddy fields under natural field conditions. (A, B) Total N content of flag leaves of WT and knockout mutants. (C, D) Total N content of panicles from the main culm of WT and knockout mutants. Asparagine (E) and glutamine (F) concentrations in mature seeds of WT and *osasn1* plants. Data represent mean ± SE of five biological replicates. Significant differences between mutant and WT plants are indicated with an asterisk (*$P \leq 0.05$; Student's *t*-test).

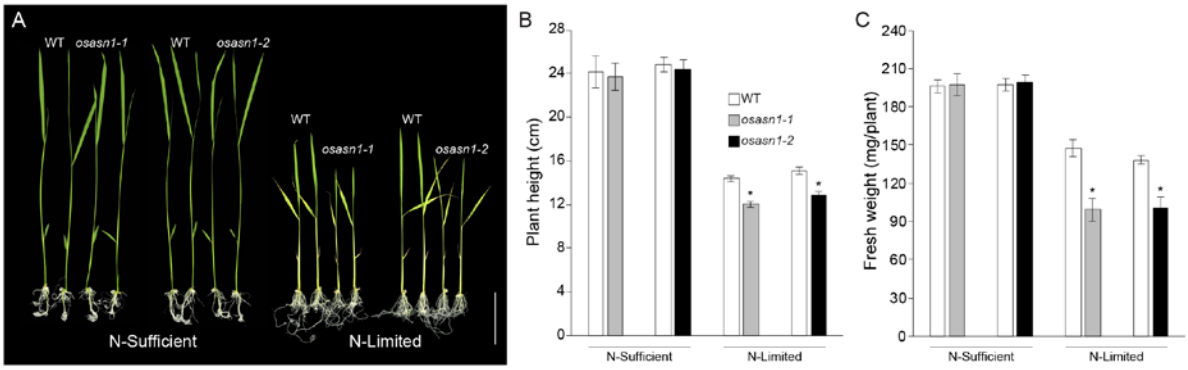

Fig. S4. The *osasn1* mutants showed enhanced sensitivity to N-limitation at the 14-day old seedling stage. (A) Phenotypes of WT and knockout mutant plants. Plants in were grown in N-sufficient (10 mM NH_4^+) or N-limited (0.1 mM NH_4^+) media for 14 days. Scale bars = 5 cm. (B, C) Measurement of plant height (B) and fresh weight (C) of WT and *osasn1* seedlings. (D) Data represent mean ± SE (n=10). Significant differences between mutant and WT plants were determined using Student's *t*-tests, and indicated with an asterisk (*P < 0.05).

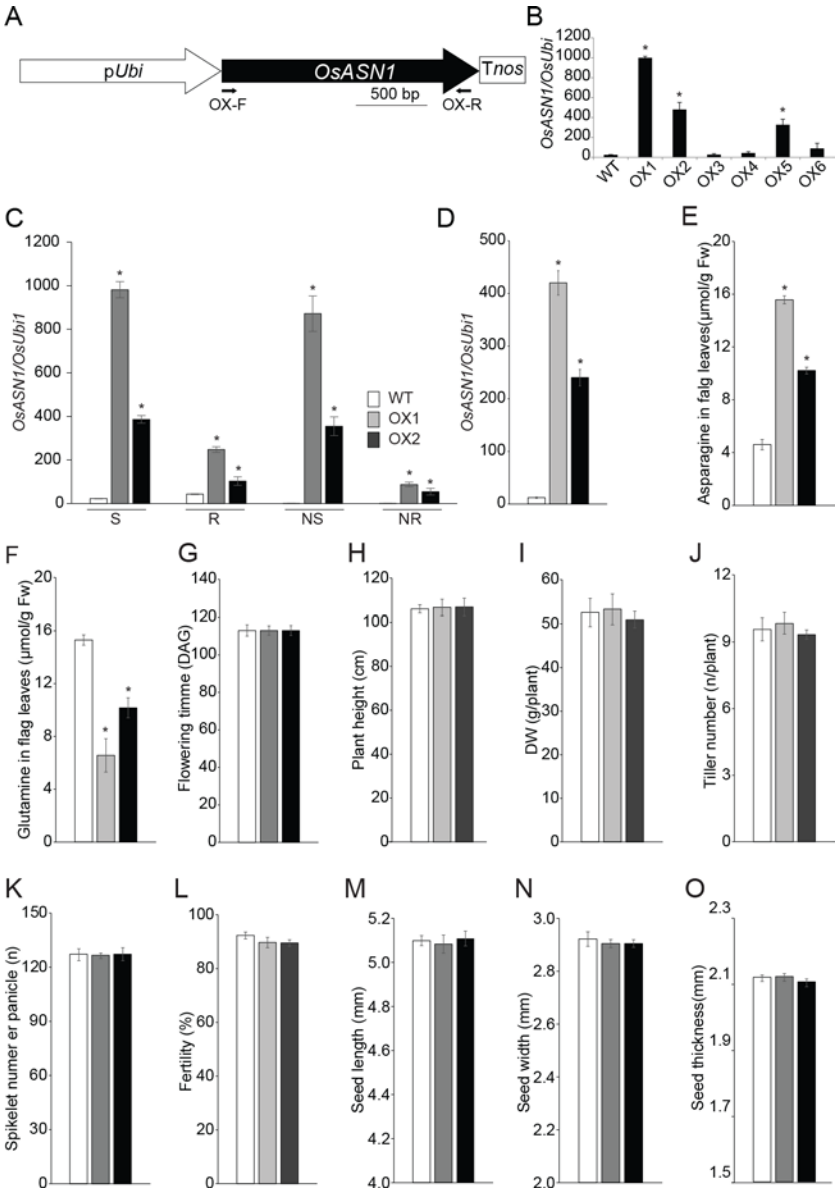

Fig. S5. Generation and characterization of *OsASN1* overexpressing (OX) transgenic lines. (A) Schematic representation of the vector expressing *OsASN1* under the control of maize *ubiquitin* promoter (p*Ubi*) and *nopaline synthase* terminator (T*nos*). *OsASN1*-specific primers (OxF and OxR) were used to amplify the full-length cDNA. (B) qRT-PCR analysis of *OsASN1* in WT and overexpressor (OX) plants using RNA isolated from shoots of seedlings grown in a modified 1/2 MS media containing 10 mM NH_4^+. Data represent mean ± SE of three biological replicates. (C) qRT-PCR analysis of *OsASN1* expression in OX1, OX2, and WT seedlings grown on N-sufficient (10 mM NH_4^+) or N-limited (0.1 mM NH_4^+) media. S, shoots of plants grown in N-sufficient media; NS, shoots of plants grown in N-limited media; R, roots of plants grown in N-sufficient media; NR, roots of plants grown in N-limited media. (D) Expression of *OsASN1* in flag leaves of OX and WT plants at the flowering stage. (E) Asparagine (E) and glutamine (F) concentrations in flag leaves of OX and WT plants. (G-L)

Measurements of the flowering time (G), plant height (H), dry weight (DW) of aboveground biomass per plant (I), tiller number per plant (J), spikelet number per panicle (K), and fertility (L) of OX and WT plants grown in paddy fields. Data represent mean ± SE of five biological replicates. Significant difference between WT and OX plants are indicated with an asterisk (*$P \leq 0.05$; Student's *t*-test). (M-O) Measurements of grain length (M), width (N), and thickness (O) of seeds of WT and OX plants ($n = 20$).

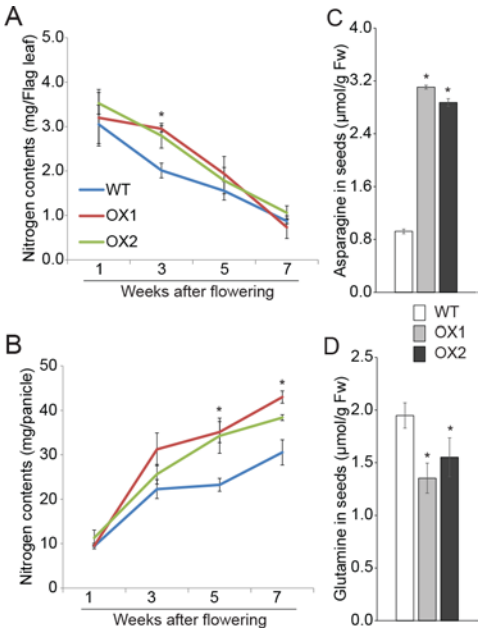

Fig. S6. Total N contents of WT and OX plants at the grain filling stage. (A) Total N content of flag leaves. (B) Total N content of panicles from the main culm. Plants were grown in paddy fields under natural conditions. Asparagine (C) and glutamine (D) concentrations in mature seeds of OX and WT plants. Data represent mean ± SE of four biological replicates. Significant differences between WT and OX plants are indicated with an asterisk (*$P \leq 0.05$; Student's *t*-test).

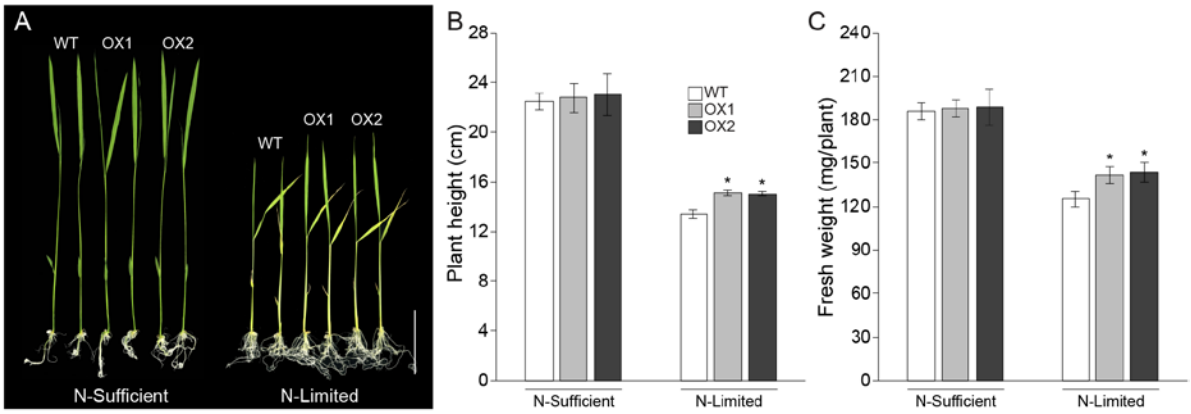

Fig. S7. Transgenic *OsASN1* OX lines showed enhanced tolerance to N limitation at the 14-day old seedling stage. (A) Phenotypes of OX1, OX2, and WT plants grown. Plants were grown in N-sufficient (10 mM NH_4^+) or N-limited (0.1 mM NH_4^+) media for 14 days. Scale bars = 5 cm. (B, C) Measurement of the height (B) and fresh weight (C) of WT and OX seedlings.

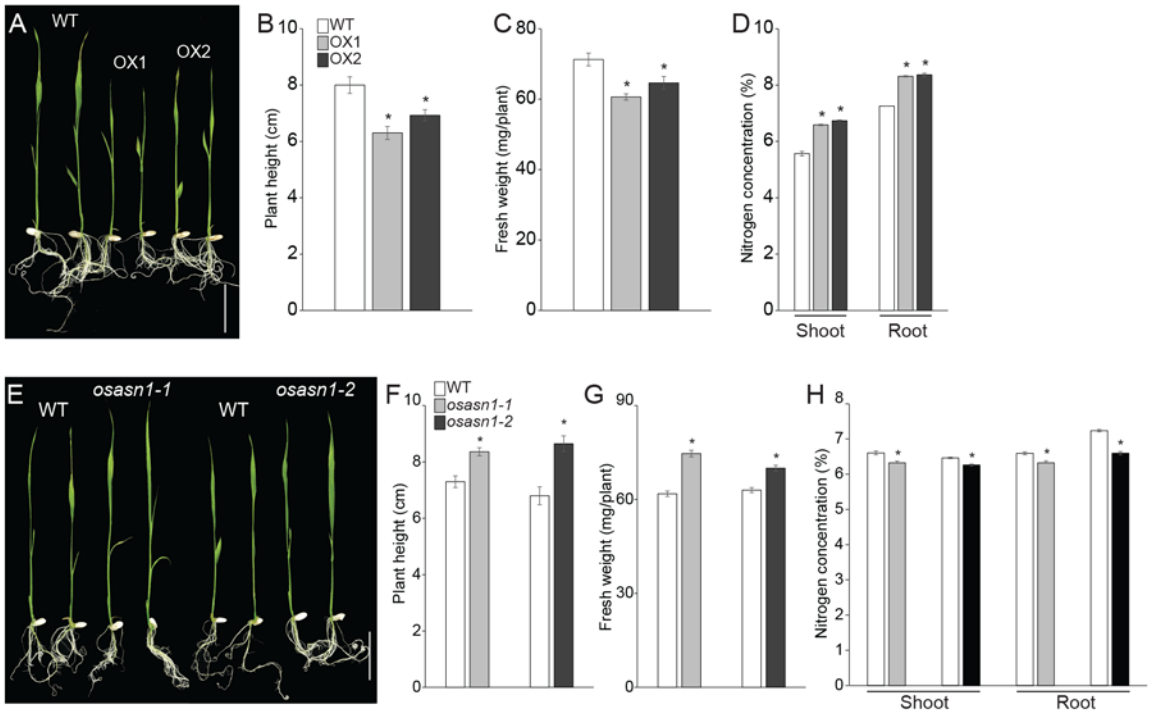

Fig. S8. High N tolerance test at the seedling stage. Seeds were germinated, and plants were grown for 8 days on a 1/2MS solid medium containing 100 mM NH_4^+. (A) Phenotypes of WT and OX plants. Scale bars = 2.5 cm. Measurement of the height (B), fresh weight (C), and N concentrations in shoots and roots (D) of WT and OX plants. (E) Phenotypes of WT and *osasn1* mutants. Measurement of the height (F), fresh weight (G), and N concentrations in shoots and roots (H) of WT and *osasn1* mutant plants. Data represent mean ± SE of 10 randomly selected plants. Significant differences between OX and WT plants and between *osasn1* and WT plants are indicated with an asterisk (*$P < 0.05$).

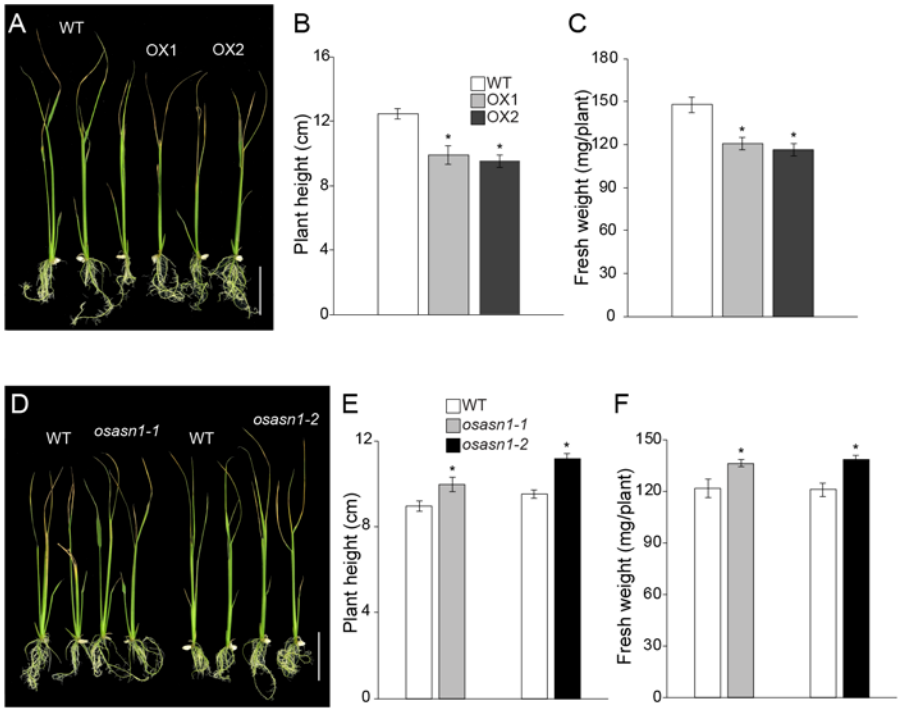

Fig. S9. Growth test of WT, OX plants and *osasn1* mutants grown under high-N (100 mM NH_4^+ NH_4^+) condition at the seedling stage for 14 days. Plants were grown for 14 days on a 1/2MS solid medium containing 100 mM NH_4^+. (A) Phenotypes of WT and OX plants. Scale bars = 2.5 cm. Measurement of the height (B), fresh weight (C) of WT and OX plants. (D) Phenotypes of WT and *osasn1* mutants. Measurement of the plant height (E) and fresh weight (F) of WT and *osasn1* mutant plants. Data represent mean ± SE of 10 randomly selected plants. Significant differences between OX and WT plants, and between *osasn1* and WT plants are indicated with an asterisk (*$P < 0.05$).

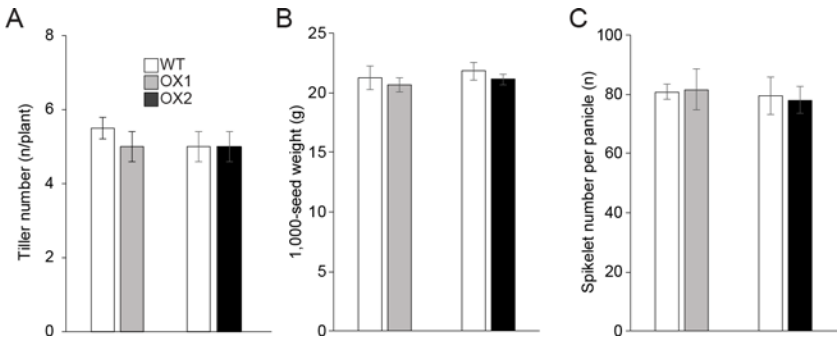

Fig. S10. Characterization of *OsASN1* overexpressing transgenic plants to N limitation at the reproductive stage. Tiller number per plant (A), 1,000-grain weight (B) and spikelet number per panicle (C) of WT and OX plants. Data represent mean ± SE of five replicates. Significant differences between OX and WT plants are indicated with an asterisk (*$P < 0.05$).

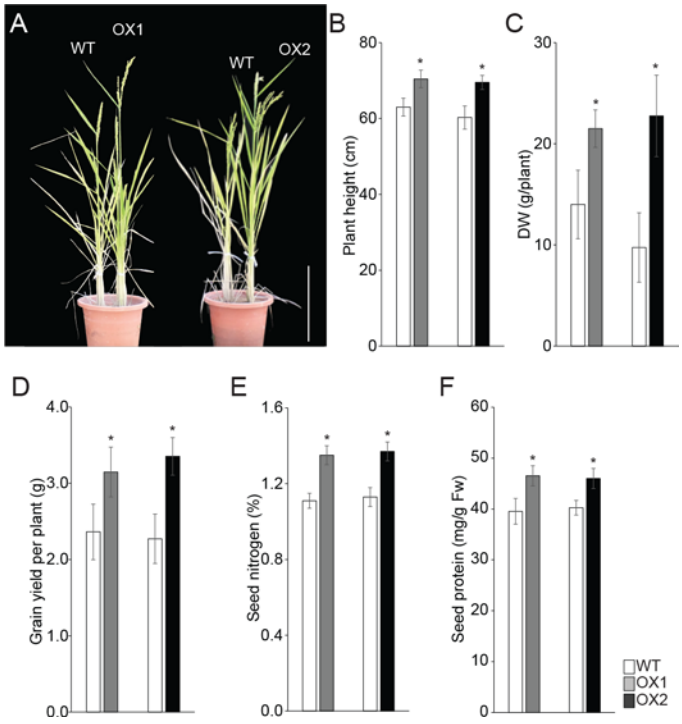

Fig. S11. Improved tolerance of *OsASN1* OX lines to N-limiting conditions at the reproductive stage in 2017. (A) Morphological comparison of OX1, OX2, and WT plants grown outdoors under N-limiting condition until harvest in pots. Representative photographs were taken at 5 weeks after flowering. Scale bars = 20 cm. Measurement of plant height (B), dry weight (DW) of aboveground biomass (C), total grain yield per plant (D) of WT and OX plants. N (E) and protein (F) concentrations in mature seeds of WT and OX plants grown in conditions described in (A). Data represent mean ± SE of five replicates. Significant differences between OX and WT plants are indicated with an asterisk (*$P < 0.05$).

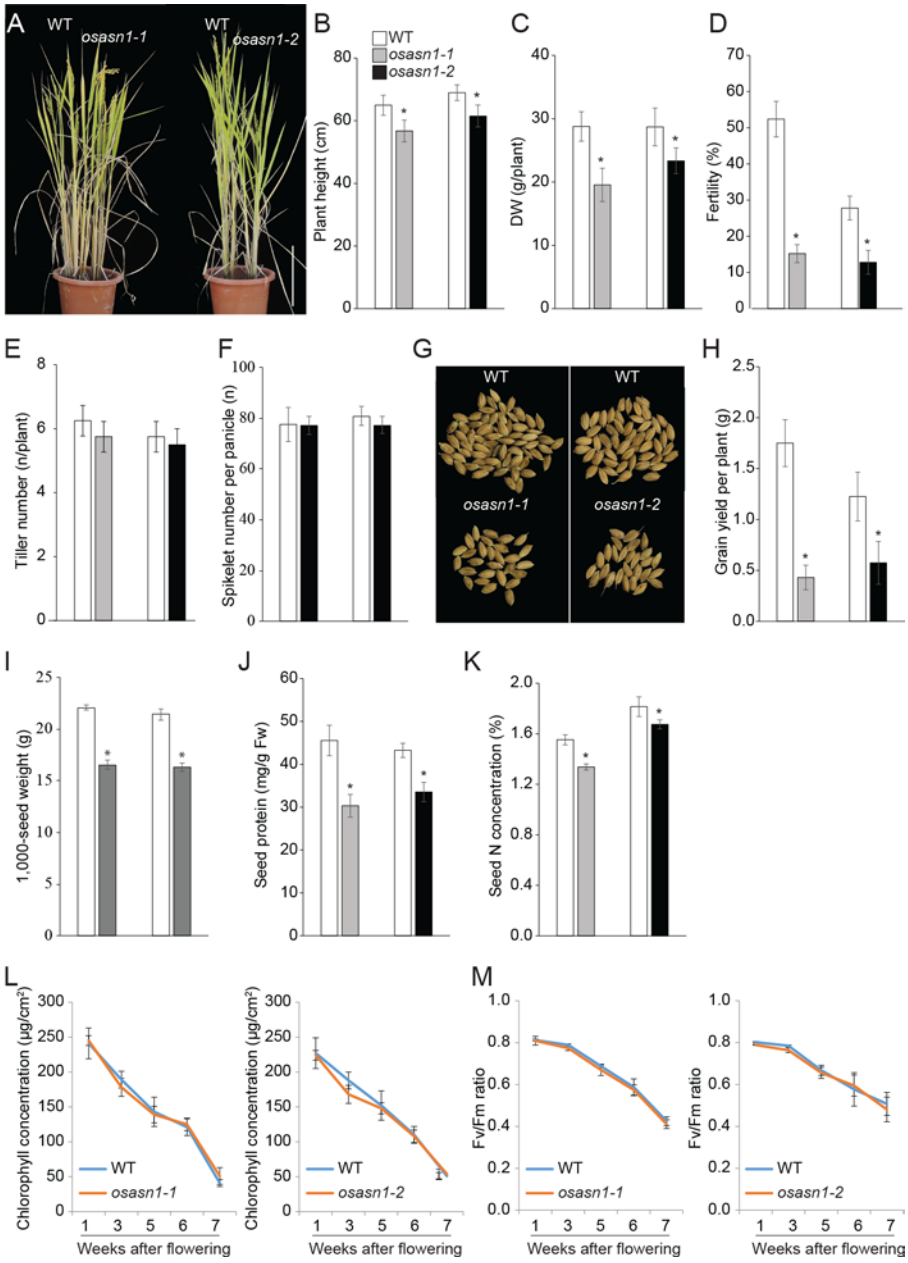

Fig. S12. Phenotypic analysis of *osasn1* plants under N-limiting conditions at the reproductive stage in 2016. (A) Phenotype of WT and *osasn1* mutant plants. Plants were grown in an outdoor field condition until harvest in pots containing 1/16th of the level of N in regular N-enriched nursery soil. Representative photographs were taken at 5 weeks after flowering. Scale bars = 20 cm. Comparison of plant height (B), dry weight (DW) per plant (C), grain filling rate (D), tiller number per plant (E), spikelet number per panicle (F), and total seed weight per plant (G, H) of WT and *osasn1* mutant plants. 1,000-seed weight (I), N (J) and protein (K) concentrations in mature seeds of plants grown in conditions described in (A). (L, M) Measurement of total chlorophyll concentrations (L) and photochemical efficiency (M) of WT and *osasn1* mutant plants. Data represent mean ± SE of five replicates.

Significant differences between *osasn1* and WT plants are indicated with an asterisk (*$P <$ 0.05).

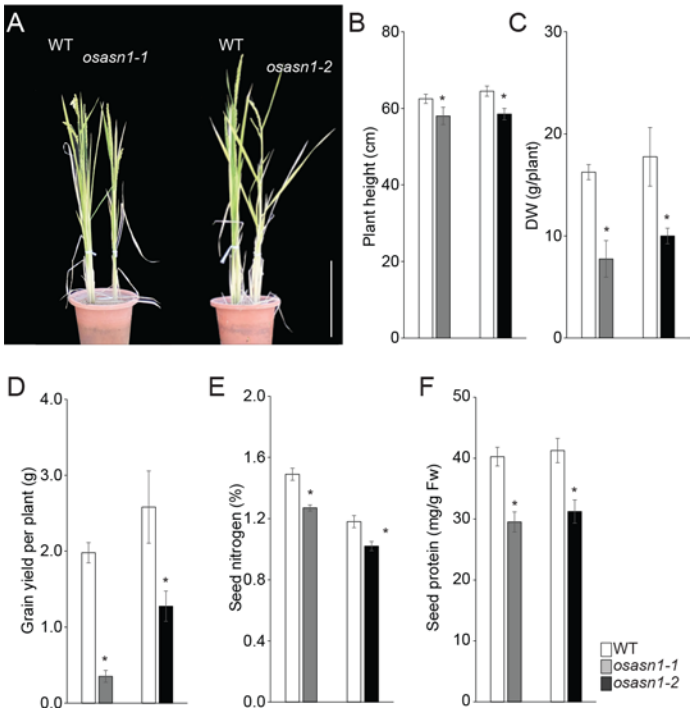

Fig. S13. Phenotypic analysis of *osasn1* mutant plants under N-limiting conditions at the reproductive stage in 2017. (A) Phenotype of WT and *osasn1* mutant plants grown outdoors under N-limiting condition until harvest. Representative photographs were taken at 5 weeks after flowering. Scale bars = 20 cm. Comparison of plant height (B), dry weight (DW) per plant (C), and total seed yield per plant (D) between WT and *osasn1* plants. N (H) and protein (I) concentrations in mature seeds of WT and *osasn1* plants grown in conditions described in (A). Data represent mean ± SE of five replicates. Significant differences between *Osasn1* and WT plants are indicated with an asterisk (*$P < 0.05$).

Table S1. List of primers used in this study.

Primer name[a]	Primer sequence (5′→3′)
OsASN1-F	TACAGGCAGAAAGAGCAGTTCA
OsASN1-R	CAGCGTTCTTCATCATTTCATC
OsASN2-F	TGGTTTGAAAGGTTCTCCTGAT
OsASN2-R	GGTAAATGACTTCCTCCAGTGC
OsUbi-F	CTGCTGCTGTTCTAGGGTTCAC
OsUbi-R	CAAAACGTTTCAGACACCATCA
OX-F	AAAGCTTATCCATCCACCGTCTCAATC
OX-R	AAGGTACCAGCTTCGAGCGTTATGCTTC
F1	AATCCATCGTCAGTTCGTCAC
R1	ACCATCACGCTACCGTACAAG
F2	ATCCATCCACCGTCTCAATCT
R2	ATCTCTCCATTGGCCTTCAAT
LB (T-DNA)	TAAGAGGATAATTGATTTGCTTAC
RB (T-DNA)	CTGATCATTCCAATCCTAGTACAAT
OsAMT1;1-F	GATCTCGATCGTCACGTCATAA
OsAMT1;1-R	GCATGCCTGTACACAAGAGAAG
OsAMT1;2-F	ATCATATCGATCGTGTCCGTTT
OsAMT1;2-R	CATACAGGTGCAAATGCAGAGA
OsAMT1;3-F	CCGGAGGAGCTGAGCTAGTAGT
OsAMT1;3-R	CCGGAGGAGCTGAGCTAGTAGT
OsGS1;1-F	GTCCAACATGGACCCTTACATC
OsGS1;1-R	AACCAAATCGAAATGGAATGAG
OsGOGAT1-F	CAAGCAAGAGAGCAGCTGATAA
OsGOGAT1-R	ACAAGATCAGCCTCAATGGTCT

[a]F and R indicate forward and reverse primers, respectively.